

**DEVELOPMENT OF JUVENILE CULTURE TECHNIQUES AND
TESTING OF POTENTIAL BIOMARKERS OF
ENVIRONMENTAL STRESS IN FRESHWATER MUSSELS
(BIVALVIA: UNIONIDÆ)**

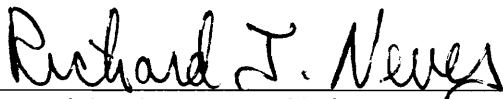
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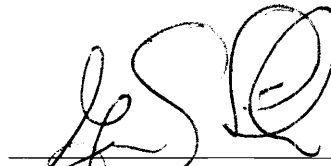
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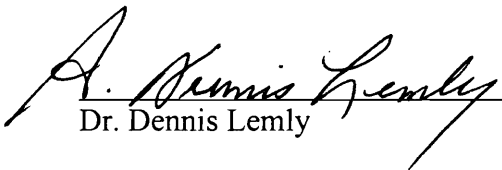
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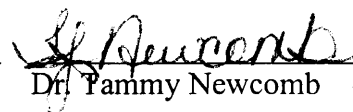
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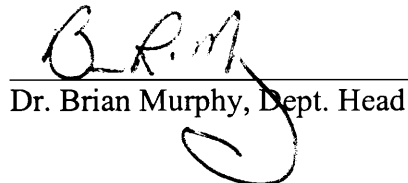

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ABSTRACT

The freshwater mussel fauna of the Clinch River in Southwest Virginia has declined in recent decades, principally due to habitat degradation from poor land-use patterns and pollutants. A study was undertaken to determine the feasibility of using river water in a flow-through culture system to rear juvenile freshwater mussels. The culture method placed juvenile mussels, confined in small dishes, into oval troughs supplied with untreated river water. Two of three years produced acceptable survival rates of 27% and 19% to an age of 90 days or greater. The third year yielded very low survival rates of less than 3%, demonstrating that failures in culture production can occur. Growth rates of juveniles in the culture system using river water were almost double those in laboratory culture systems, provided that juveniles were placed in the oval troughs during June. Otherwise, growth was comparable to that attained in laboratory culture systems.

Several factors were investigated to assess their effect on the growth and survival of juvenile mussels in culture systems. Substrate size, silt ($<120\ \mu\text{m}$) or sand ($120 < x < 600\ \mu\text{m}$) had no effect ($p < 0.05$) on the survival and growth of juvenile mussels. Temperature had a strong influence on the growth of juveniles ($p < 0.01$), with higher temperatures leading to greater growth rates. Growth in laboratory culture systems was modelled well by a linear regression based on the number of degree-days above 15°C as: $L(\text{mm}) = 0.81 \cdot 10^{-3} \cdot \text{degree-days} + 0.347$ with $R^2 = 0.89$. Flow velocity of the overlying water affected survival rates in the oval troughs supplied with river water and one of the laboratory experiments ($p < 0.05$). Flow velocities in the range of 5-10 cm/sec produced the best survival rates, as high as 50% in some dishes. Juvenile densities in the range of 100/56 cm^2 to 100/144 cm^2 did not effect survival or growth.

Physiological parameters were measured for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*), held in cages at 8 locations in the Clinch River basin, to determine their usefulness as biomarkers and the suitability of the habitats. Parameters measured were: glycogen, glucose, cellulolytic enzyme activity, acetylcholinesterase (AChE) activity, phosphofructokinase (PFK) activity, fructose-1,6-bisphosphatase

(FBPase) activity, and RNA:DNA. Experiments were repeated during three seasons to determine what effect time of year had on the biomarkers. The sites chosen were a mixture of habitats with good mussel populations and depauperate mussel populations due to anthropogenic impacts. None of the potential biomarkers clearly indicated that a given site was unsuitable during each period tested. However, several parameters suggested that depauperate sites were poor during some season(s). The potential biomarkers that correlated best with the mussel populations at sites were cellulolytic enzyme activity and acetylcholinesterase activity. While other assays did provide evidence of habitat degradation during individual seasons, they were inconsistent in identifying impacted sites. A composite of all the assays did accurately identify the sites with extirpated mussel populations during at least one season.

Seasonal variability was pronounced for many of the biomarkers, rendering their use in field applications dependent on the prior determination of seasonal patterns. The seasonal variations indicated that mussels and clams do experience significant changes in energy dynamics throughout the year. FBPase activity was greatest in the fall in both species ($p < 0.05$), up to 8 times greater than spring values in mussels. PFK activity was much greater in the summer (72-118 mUnits/mg tissue) than other seasons (5-25 mUnits/mg tissue) in mussels, but did not differ in clams ($p < 0.05$). The activity of these two enzymes was also markedly different for the two species. Mussels had FBPase activities from 99-172 mUnits/mg tissue and PFK activities from 23-118 mUnits/mg tissue during the spring and summer, while clams had activities of 1250-2450 and 0.4-5.0 mUnits/mg tissue for FBPase and PFK, respectively.

Additionally, many of the biomarkers exhibited different patterns among sites for Asian clams and mussels ($p < 0.05$), such that Asian clams cannot be used as surrogates for freshwater mussels in the identification of suitable habitats without verification of similar responses under the particular application.

ACKNOWLEDGEMENTS

We all have a whole gang of folks that have helped us get where we are going in ways we often don't even realize. So for all of those I do not mention in this brief paragraph, thanks for the influence you have had on me. I would like to give my sincerest appreciation to my advisor, colleague, and friend, Dr. Dick Neves, for all his help in my research and, more importantly, my education. His advice, insight, and questions have helped strengthen my scientific and professional development. I would also like to thank my committee members: Dr. Dennis Lemly, Dr. George Libey, Dr. Alan Heath, Dr. Lou Helfrich, and Dr. Tammy Newcomb. Each and every one was always willing to help in any way they could and played a major role in improving this project and my education. I also want to extend my thanks to the faculty of the Department of Fisheries and Wildlife Sciences at Virginia Tech for taking a chance on me and an interest in me. The teaching quality, both inside and outside the classroom, was first rate, and the lunchroom discussions were often just as enlightening. Many thanks have to go to the broad array of mussel-heads that have help in so many ways. These folks include Jess Jones, Bill Henley, Lora Zimmerman, Susan Rogers, Brian Watson, Dave Neils, Michelle Steg, Catherine Gatenby, Jon Burrell, Marty O'Connell, Mike Latham, and all our summer technicians. In addition, the fisheries and wildlife graduate students were such an inspiration and stimulation to interact with, and I hope that I will continue to be able to keep in touch. I want to express my deep gratitude and love for my parents who raised me to be inquisitive, confident, persistent, and a bit stubborn. They gave me wonderful opportunities to grow emotionally, physically, and intellectually, for which I am grateful. Thanks y'all for all the support. Finally, I want to express my deepest love and thanks to my wife, Glenda, and son, Jared, for putting up with the years of hard work. Without their commitment to me and my education, none of this would have been possible. Their love is what keeps me going, and provides me a base to come home to. Jared is always there to remind me why we are in this game, for the next generation and the one after that. Glenda is always there to remind me that life is worth living to the fullest, and that I should strive to always test myself. Thank you very much, one and all.

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CHAPTER 1

REARING OF JUVENILE MUSSELS IN CULTURE SYSTEMS SUPPLIED WITH NATURAL RIVER WATER

INTRODUCTION

North America has the largest diversity of unionid mussels, with 297 species and subspecies (Turgeon et al., 1998). Many of these are now on endangered or threatened species lists at both the state and federal levels. Historically, there were approximately 45 species of unionids in the Clinch River in southwest Virginia (Sheehan et al., 1989). However, due to two toxic spills, the section of river below the Clinch River Steam Plant in Carbo, Russell Co., VIRGINIA is depauperate of mussels (Stansbery et al., 1986). Despite a timespan of 28 years, the unionid fauna has failed to recover significantly in this reach of river. Mussel populations in other aquatic systems also have been depleted over the last few decades (Starrett, 1971; Coon et al., 1977; Bates, 1982; and Williams and Schuster, 1989). Additionally, the introduction of the exotic zebra mussel (*Dreissena polymorpha*) has negatively impacted native unionid populations. Zebra mussel population explosions in the Illinois and Ohio rivers have caused significant mortalities among the unionid fauna (Lidyanskiy et al., 1993). The nature of freshwater mussel reproduction, with the obligatory parasitic glochidial stage, also subjects them to a high risk of impact from habitat perturbations due to effects on host fish. For these reasons, research is needed to assist the recovery and well being of freshwater mussel populations.

In many cases, populations have been so depleted that recovery or maintenance can only be effected through some type of stocking program. Therefore, the culture of juvenile mussels is a vital part of restoration efforts for the imperiled mussel fauna in the United States. This has been identified as one of the priorities for research and development related to the conservation of unionids (Neves, 1997). The development of

techniques for successfully transforming and rearing juvenile mussels is necessary for the widespread restoration of mussel communities. In order to accomplish restoration efforts on the scale that is needed, large numbers of juveniles are required. Transforming and rearing juvenile mussels is an attractive way to influence the comeback of mussels since many of the populations in danger are declining for lack of ample recruitment (Neves et al., 1997). One study estimated that the mortality rates for the various stages of young pearl mussels (*Margaritifera margaritifera*) are 95% or higher for each stage (Young and Williams, 1984). This study estimated that, in a viable population of mussels, 99.9996% of glochidia did not successfully find a host fish and died, 95% of the glochidia that did find a host fish died before metamorphosis, and 95% of metamorphosed juveniles died before reaching maturity. While the numbers may not be consistent among diverse species of mussels, the extremely high losses during the glochidial and early juvenile stages of life seem to be realized in all freshwater mussels. Therefore, strategies which reduce the mortality rates during these critical times can exert significant impacts on the long-term viability of mussel populations.

Captive culture is one approach to this activity. This is a strategy employed by the marine shellfish industry to increase yields of such bivalves as mussels, clams, and oysters (Burrell, Jr, 1985; Manzi, 1985; Lutz, 1985). The most common methods of culturing oysters, marine mussels, and marine clams utilize natural waters to supply nutritional and chemical requirements and protective mechanisms to decrease losses due to predation and physical disruption (Huner and Brown, 1985). Such operations take “seed” from captive broodstock or wild drift, and grow the animals in a protected

environment in which factors such as temperature and food supply are maintained to encourage rapid growth and high survival. Extensive research has been performed with oysters to develop the techniques to hold and spawn broodstock, provide for larvae, collect spat, and culture the oysters to market size (Walne, 1979; Burrell, Jr., 1985; Quayle, 1988). Oyster bottom culture operations place vulnerable juveniles in relatively natural habitats where they can be protected from physical harm, such as that caused by high flow events or wave action, and many predators (Quayle, 1988). The requirements for the successful culture of oysters are a suitable substrate for the young animals to attach, plenty of food in the water column, enough flow to deliver the food, and protection from predators (Burrell, Jr., 1985).

One approach to the culture of juvenile freshwater mussels involves using an artificial stream system which emulates the riverine habitat in which recruitment occurs. The development of such an approach would yield several advantages over most other, more artificial, means of rearing young mussels. By using river sediments and ambient river water, juvenile mussels will benefit from exposure to more natural conditions while still being protected from many dangers such as predation by a wide range of benthic animals, flood scour, and periodic sedimentation. Rearing juvenile mussels in such a system will also provide them with a natural food supply. Exposure to the phytoplankton, bacteria, and organic matter that is present in river water may provide nutritional benefits which exceed those of less complex diets in aquaculture settings and ease the transition from artificial rearing facilities to the release stream. Overall, if this method of rearing mussels can be developed, it will likely prove to be the most successful

method of obtaining mussels for stocking.

Evidence in the literature suggests that culture systems that employ existing surface waters have the potential to be effective for rearing juvenile mussels. Howard (1914) showed that rearing juvenile *Lampsilis radiata luteola* was more effective in river water than in aquaria. Howard (1916) was then able to continue rearing these animals in the cages until they reached maturity and the females became gravid. However, another study using juvenile *Lampsilis radiata luteola* produced disappointing survival rates (Corwin, 1920). Results from these, and other early studies of the propagation of juvenile freshwater mussels, suggested that the best methods were those using natural waters (Howard, 1923). Few studies were conducted from this time until the early 1980's, when attempts were made to produce transformed juveniles without the use of fish hosts (Isom and Hudson, 1982; Hudson and Isom, 1984). Further support for the efficacy of rearing juvenile unionids in stream water was provided by Buddensiek (1995). He stated that juveniles held in river water had high survival rates (up to 20% after 12 months) and good growth. These studies suggested that a culture system supplied with natural river water would have a high probability of success.

The objective of this study was to investigate the feasibility of using a culture system supplied with river water to rear juvenile freshwater mussels. The culture system investigated was simple, low maintenance, and small enough that it could be used by state hatcheries to assist in mussel recovery efforts. Physical factors that might affect the growth and survival of juvenile mussels were evaluated. The realized growth and survival rates of juveniles were compared to those of laboratory culture methods.

MATERIALS AND METHODS

Experiments to investigate factors influencing the survival and growth of juvenile mussels were conducted using three different culture systems. The first was a series of flow-through oval troughs with a constant supply of untreated river water. The second consisted of a recirculating trough with chiller units, which allowed the control of water temperature. The third system, which was also recirculating, was designed with an inlet header that attempted to establish an approximate laminar flow in the tank. Survival of juveniles was determined by separating the juveniles from the substrate, either with sieves or other means, and counting all live animals. Survival was computed as the number of live juveniles recovered, divided by the total number of juveniles present at the start of the time period. Juvenile size was measured with a dissecting microscope and ocular micrometer at a magnification of 65-400X.

Flow-Through System with Natural River Water

This study was conducted in a flow-through culture system using ambient river water. The location of this culture system was on the property of the Clinch River Steam Plant (CRSP) at Carbo, Virginia. This facility was located in a bend of the Clinch River in Russell County, Virginia at river mile 266.1. The water for the culture system was taken from the Clinch River immediately upstream of the steam plant. Mussel populations in this upstream reach of the river appeared to be relatively healthy with young adults present, indicating that water quality was suitable for rearing juvenile mussels.

The system was designed to allow simulation of ambient river conditions, in an environment that could be protected from floods, predators, and sedimentation. The feed water was introduced into a U-shaped channel (Figure 1), which served as the flow delivery system for the oval troughs in which the juvenile mussels were reared. It also served as a settling chamber to remove much of the sediment introduced into the system. This channel had eleven holes drilled through each side at the same height. Each of the 22 holes was fitted with a short tube to guide the flow of water out of this channel and into the oval troughs in which the mussels were held. Therefore, the channel served as both a flow regulator and a sedimentation chamber.

The flow from the U-channel was directed into oval troughs, which were capable of holding approximately 75 L of water. Each of these troughs was equipped with a raised center that extended above the water level and a standpipe drain to regulate water level. The flow of water into these troughs was directed along the long axis of the oval on one side of center to encourage the establishment of a continuous current in a circular fashion around them. In addition, each of the oval troughs was fitted with a motorized paddle wheel to maintain a unidirectional current. This flow simulated the continual current experienced by mussels in a natural riverine setting.

Within these troughs, newly transformed juvenile mussels were placed in 7.5 x 5.0 x 5.0 cm rectangular glass containers (during the first year, 1993) or 7.5 x 7.5 x 3.3 cm plastic containers (during the next two years, 1994 and 1995). Each of these containers was initially set up with one of two substrate types (particle size <130 μ m or

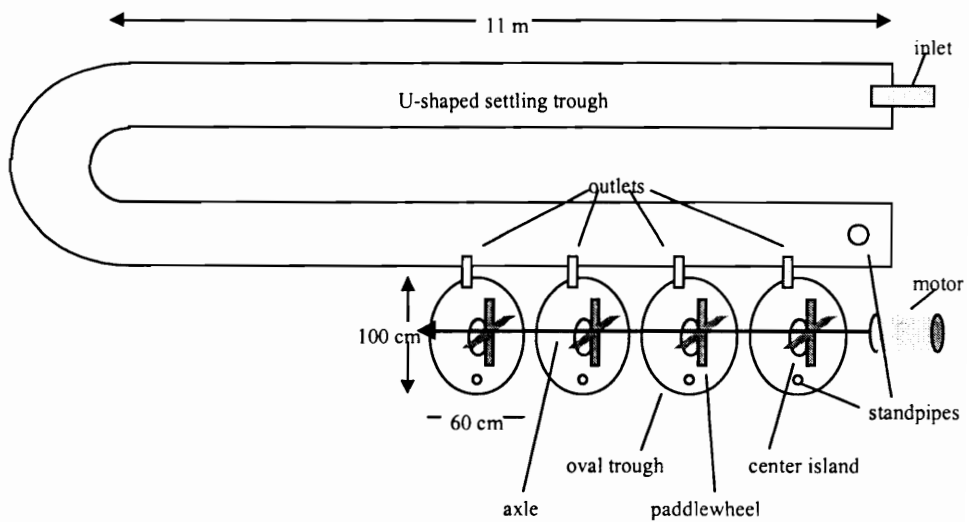


Figure 1. Diagram of the culture system used in the culture of juvenile mussels at the Clinch River Steam Plant. There were a total of 11 oval troughs on each leg of the U-shaped trough.

120-600 μm) being tested for its suitability to rearing juvenile rainbow mussels (*Villosa iris*). In addition, two depths of each substrate were tested, 5 mm and 20 mm. Once the juveniles were settled in the substrate, the maintenance of a continuous current ensured that the mussels were exposed to a constant supply of food. These oval troughs served as a place where presumably favorable conditions for survival and growth could be maintained to encourage successful rearing of juvenile mussels.

Gravid female *V. iris* were collected from Copper Creek at Nickelsville and transported to the Virginia Tech Aquaculture Center in Blacksburg, Virginia. When the glochidia were mature, they were taken by flushing water from a 3 or 5 cc syringe with a 3.8 cm 21 gauge needle through the marsupia of the mussels. Rock bass (*Ambloplites rupestris*), a known fish host for this mussel species, were subsequently infested with these glochidia. The fish were infested by placing them in a small container or bucket with just enough water to cover their backs and two airstones to keep the water agitated. The collected glochidia were then added to the container. The fish were held in this water for approximately 45 min to allow the glochidia to contact the gills and become attached. These fish were then held in 37 L aquaria until the glochidia transformed into juveniles, which took 2-3 wk. At this point, they dropped from the fish gills and settled to the bottom of the aquaria. Using light siphoning pressure, the transformed juveniles were collected from the bottom of the aquaria and stored until transport to the culture system at the CRSP. This transport occurred within one week after juvenile transformation.

Once transported to the rearing facility at the CRSP, the juveniles were introduced into the oval troughs, confined in the glass or plastic containers. One hundred mussels

were placed in each container in 1993 and 1994, while 50 mussels per container were used in 1995. Each container was partially filled with one of two sieved substratum fractions; smaller than 130 μm and 130-600 μm . The source of the substratum was the Clinch River above the CRSP in 1993 and 1995, and the Clinch River at Nash Ford in 1994. In 1995, a subsample of eight containers from each sediment treatment was taken monthly to determine growth and mortality among the juveniles. The total number of containers used for each substrate treatment was equal and depended on the number of metamorphosed juveniles obtained each year, ranging from 4-20 per treatment. Total mortality rates were determined at the end of 3 to 5 mo, when the system had to be dismantled for the winter. Differences in growth and mortality between the two substrate types and depths were determined using t-tests and ANOVA analyses.

During the 1994 and 1995 experiments, the containers were placed in the oval troughs in such a way as to yield four blocks (quadrants) within each trough. The first block was the section immediately to the right of the inlet flow and closest to the settling trough, the second was the section between the paddlewheel and the standpipe, the third quadrant was on the left side of the standpipe, and the fourth was on the left side of the inlet flow. This placement allowed testing of whether the position in the trough affected the growth or survival of juveniles. Each quadrant contained one container of each of the four treatments; shallow, small substrate; deep, small substrate; shallow, large substrate; and deep, large substrate. Five oval troughs were used in this manner. Each trough could then be used as a replicate with four quadrants.

Finally, to determine whether confinement adversely affected growth and survival

of juvenile mussels, an experiment was conducted in which the juveniles were not confined in containers. The bottom of each of two oval troughs was initially filled to a depth of approximately 10 mm with substrate less than 120 μm in size. Each trough received approximately 500 juveniles at the start of the experiment, and the system was allowed to run for 3 mo. At the end of this period, the growth and survival of juveniles was evaluated.

Two other factors were evaluated for their influence on the growth and survival of juvenile mussels. Data from the automatic data recorders at the water intake of the CRSP were used to determine the temperature regime to which the juveniles were exposed. The data chart temperatures were adjusted based on measurements taken in the culture system. This information was used to determine whether water temperature had an effect on the growth or survival of juvenile mussels in the culture system. At the end of the 1995 experiment, the flow velocities (m/sec) at each of the four quadrants of all oval troughs were measured with a digital Marsh-McBirney flow meter (Flo-Mate model 2000, Fredrick, Md.). These measurements were taken as close to the depth of the juvenile container edges as possible. Two measurements were made for each quadrant, one at the location of the outside pair of containers and one at the location of the inside pair of containers. The flow measurements were averages of 10 sec integrations over the course of 1 min. Regression analysis was used to determine the effect of this parameter on growth and survival of the juvenile mussels.

Temperature Experiment

This experiment was conducted in environmental chambers in Cheatham Hall,

Virginia Tech, Blacksburg, Virginia, using 570 L Living Streams fitted with chiller units (Frigid Units, Inc., Toledo, Ohio) which generated a unidirectional flow, aerated the water, and controlled the temperature (Figure 2). Heaters were placed in one stream to maintain a constant 25°C. The bottom of each stream was lined with washed pea-sized gravel to provide a substratum for natural algal and bacterial growth to occur. The streams were filled with Clinch River water, taken from Nash Ford in Russell County approximately 2 wk before the initiation of the experiment to allow colonization of natural flora. The three streams were maintained at 12°C, 18°C, and 25°C, respectively. These temperatures simulated late fall/early spring, mid-spring, and summer river temperatures, respectively. Juveniles were placed in these streams in 7.5 x 7.5 cm plastic containers filled to a depth of 10 mm with fine sediment (<120µm). The containers were placed in an array of six rows along the length of the Living Stream by three columns across its width. Subsamples were taken at 30 and 60 days after the initiation of the experiment to determine growth and survival of the juveniles at each temperature. Water that evaporated was replaced with distilled deionized water. The experiment was terminated after 60 days.

Stocking Density Experiment

A system was constructed to simulate the Living Stream, generating a unidirectional current by pumping water from underneath a false bottom (Figure 3). In this system, a false bottom was fitted into a 90 L tank with approximately 2 cm between

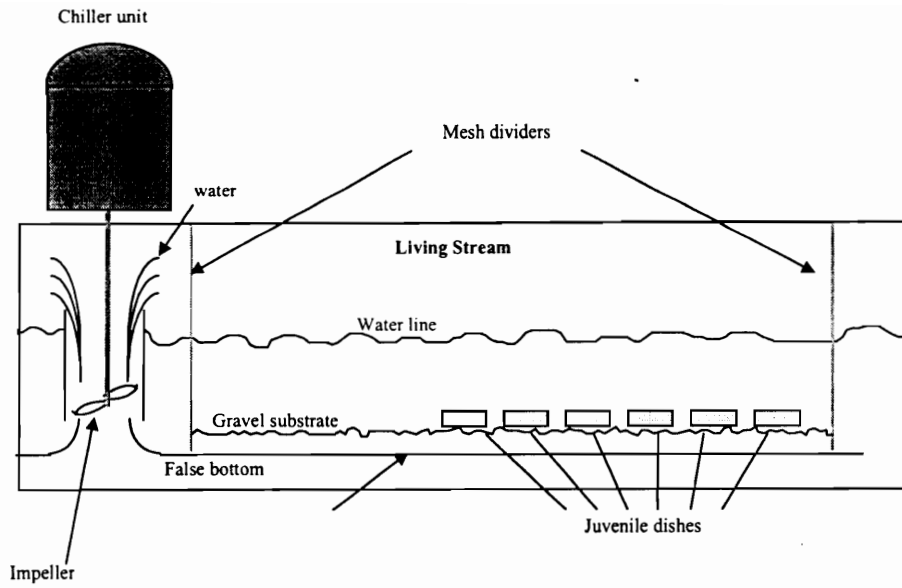


Figure 2. Diagram of the Living Stream system used during the temperature experiment in the environmental chamber.

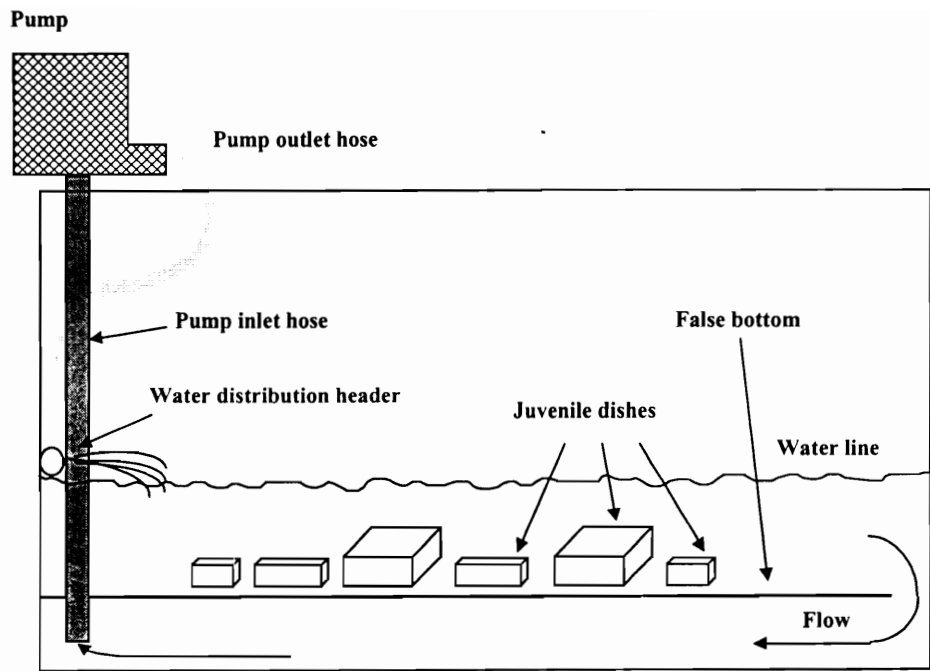


Figure 3. Diagram of recirculating system used for density experiment.

the downstream end of the false bottom and the end of the tank. A small external water pump was placed on the wall of the tank to circulate the water. Recirculated water was dispersed at the surface of the tank by means of a distribution header, consisting of a tank-wide, ½ inch diameter PVC pipe in which holes were drilled. Acid-washed rectangular plastic containers were filled with fine sediment to a depth of 10-15 mm. Three sizes of containers were used, 75 x 75 mm, 130 x 75 mm, and 130 x 130 mm. Photoperiod was 14 hr light and 10 hr dark. At the start of the experiment, 6 replicates of each size dish, with 100 juveniles in each, were placed in the culture tank. The tank was filled with an equal mix of conditioned town water and well water to obtain a hardness of approximately 200 mg/L of CaCO₃. The juveniles fed on the natural flora and detritus that developed in the sediment since no regular supplements of algae were made to the system. The system was maintained on a 12 hr light/dark cycle, except when filamentous algae became problematic; at that time, the duration of the light cycle was shortened. Water temperature in the tank ranged from 22°C to 27°C and was allowed to follow ambient room temperature since it remained suitable for juvenile mussel culture. The experiment was allowed to run for 90 days, the containers were removed from the system, the juveniles were sieved from the sediment, and growth and survival were determined. After the juvenile dishes were removed from the system, flow velocities were measured at various points within the culture tank with a digital Marsh-McBirney flow meter (Flo-Mate model 2000, Fredrick, Md.). Flow measurements were averages of 2 sec integrations over the course of 1 min. Regression analysis was used to determine the influences of flow on juvenile growth and survival.

RESULTS

Natural River Water Artificial Streams

The pilot study utilized two substrate particle size fractions to compare growth and survival in each size fraction, one smaller than ($<130\mu\text{m}$) and the other of similar size to the newly transformed juveniles ($130\text{-}600\ \mu\text{m}$). The results of this comparison indicated that there was no significant difference in survival between these two treatments, based on the two-sample t-test ($p<0.05$). After a period of 115 days, the average survival rates per container were 27.7% and 27.3% for the fine and coarse substrate treatments, respectively (Figure 4). The range of survival rates was 3-37% for the fine substrate treatment, and 1-50% for the coarse substrate treatment. Growth, measured as total length of the shell, revealed that juveniles reared in fine sediment had a higher growth rate ($p<0.10$) than those in the coarse sediment. The mean lengths of juveniles in the fine and coarse sediment fractions were 2.22 mm and 1.97 mm, respectively. When compared on the basis of the area of an ellipse, with length and width measurements corresponding to those of the juvenile's shell, size was not statistically different between the two treatments, using both the two-sample t-test ($p=0.13$) and the Wilcoxon Signed-Rank test ($p=0.12$). However, the p-values for these comparisons were low enough to suggest that there may well be a difference in the growth performance between these two treatments. Compared in terms of shell area, mussels in the fine sediment attained an average size of $2.64\ \text{mm}^2$, whereas those in the coarse sediment attained an average size of $2.16\ \text{mm}^2$ (Figure 5). In further analysis, an empirical distribution function (EDF) plot of the two treatments showed that the growth in the fine substrate not only was greater on average, but also demonstrated less variance than that in

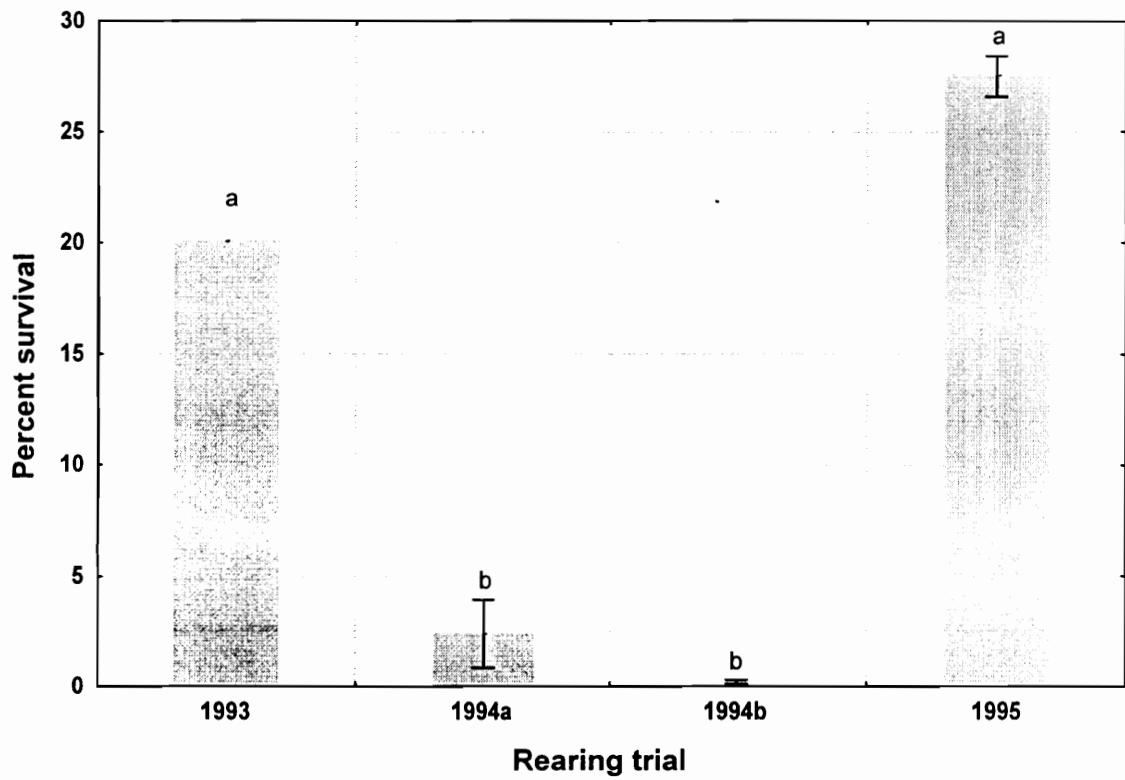


Figure 4. Mean percent survival per container of juvenile mussels for rearing trials in oval troughs. Bars with different letters are statistically different at the $\alpha = 0.05$ level.

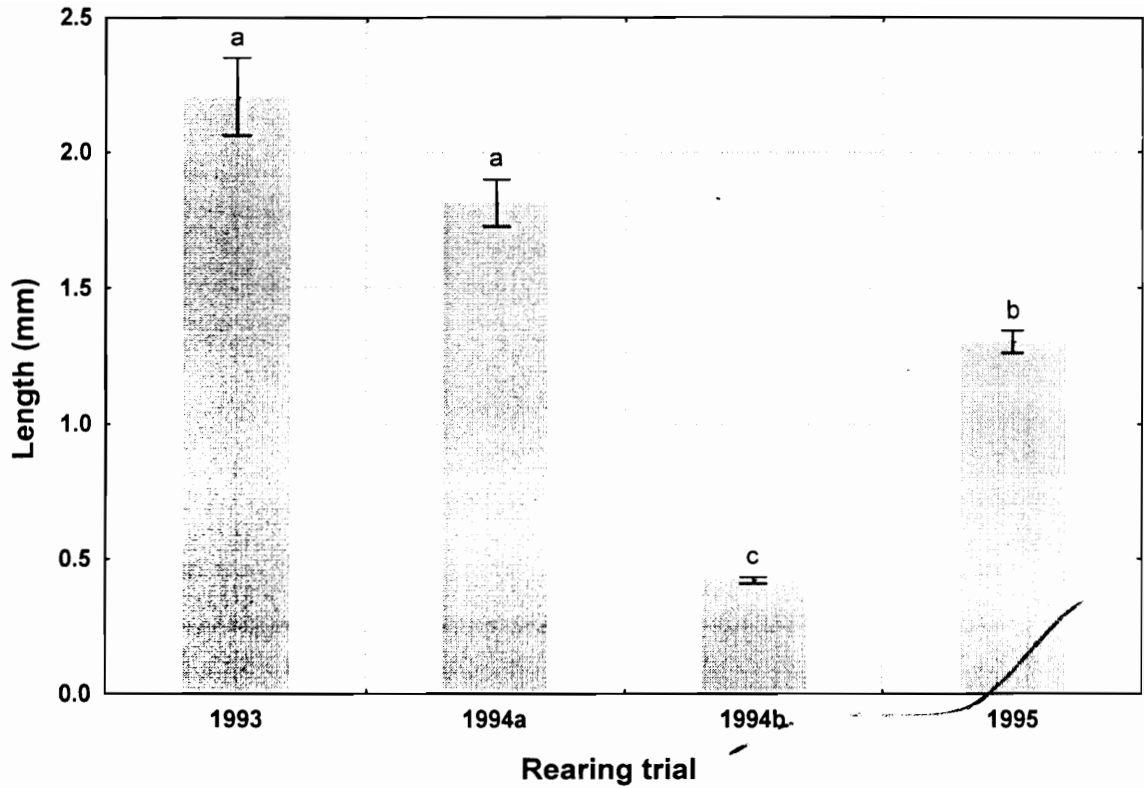


Figure 5. Mean shell length of juveniles at the end of each rearing trial. All trials were conducted in the same culture system, but differed in years and time of initiation. Bars with different letters are statistically different at the $\alpha = 0.05$ level.

the coarse substrate. These results indicated that this approach to rearing juvenile mussels was promising. The pilot study showed reasonable levels of survival and substantial growth over the course of 4.5 mo.

Another factor possibly affecting results was that the number of Asian clams (*Corbicula fluminea*) present in the glass containers was statistically different between treatments ($\alpha = 0.05$). The coarse substrate containers had significantly more Asian clams in them than the containers with the fine substrate. This could be due to the fact that pediveligers or small juveniles did not get sieved out of the larger substrate fraction but were effectively removed from the smaller substrate size. Another possible explanation is that these animals were brought in with the river water and survived better in the coarse substrate than in the fine substrate. The mean number of Asian clams in each container was 1.7 for the fine substrate treatment and 7.8 for the coarse substrate treatment.

TABLE 1. Mean (range) of shell growth from pilot rearing experiment. Size attained by juveniles after 4.5 months in the culture system at the Clinch River Steam Plant in Carbo, Virginia.

Substrate Size	Shell length (mm)	Shell area (mm²)
fine*	2.22 (0.95 - 3.25)	2.64 (0.83 - 5.53)
coarse*	1.97 (0.90 - 3.25)	2.16 (0.53 - 5.58)

* fine substrate is <120 μ m and coarse substrate is between 120 and 600 μ m.

Following the pilot study in 1993, the experimental design was revised in 1994 to include two substrate depths (5 mm and 20 mm), the same two substrate sizes, and many more replicates. Attempts to produce transformed juvenile mussels were begun in April using wild-caught rock bass from the New River drainage. The first successful production of transformed juvenile mussels occurred in June, and mussels were placed in the culture system on June 22, 1994. This batch consisted of 11 containers of 100 animals each (1100 animals in total). Round, plastic containers, approximately 10 cm in diameter, were used for this trial. These animals were held in the culture system for 112 days until October 12, 1994. The survival rate per container ranged from 0% to 17% (Figure 4). The mean length reached by the living juveniles was 1.81 mm, which compares to 2.10 mm for the juveniles held about 20 days longer in the previous year (Figure 5).

The next batch of transformed juveniles was placed in the culture system on September 2 and September 9. The batch was delivered to the culture system on two dates because transformed juveniles excysted from the host fish over the course of 10 days. This batch consisted of 80 containers of 100 juvenile mussels each (8000 juvenile mussels in total). These juveniles were left in the culture system until December 11, a total of 100 and 93 days, respectively. Survival of these juveniles was low, with no more than 3 live mussels found in any container of 100 (Figure 2). They also did not grow well during this time (Figure 3). None of the containers showed growth of more than 50% of the initial size. The valves left by dead mussels were readily found and allowed me to account for approximately half the animals (range of 33 - 173 valves or 16-86% of the

animals). The presence of so many shells indicates that predation was not the cause of death for most of the juveniles. These results suggest that the juvenile mussels did not grow appreciably after early September in the culture system.

One other item of interest was noted during the 1994 experiment. While a substantial number of Asian clams was found in the experimental containers in the 1993 season, none were found in the containers held in the fall of 1994. This could be due to the later date at which the experiment was initiated or to some other unknown event or condition which prevented Asian clams from successfully settling in containers during that year.

In the summer and fall of 1995, the experiment was repeated in the culture system. During this trial, a subset of the juvenile containers was removed at days 30, 74, and 94+ and searched for live juveniles and empty shells. Survival was better during this trial than during the 1994 trial (Figure 2). Some growth also occurred, but the growth rates were much lower than those observed during the 1993 experiment (Figure 3), only reaching a mean length of 1.38 mm and area of 1.30 mm² after 95 days.

Survival rates were computed as the number of live juveniles in the substrate at the sampling time divided by the initial number of juveniles placed in the container at the beginning of the experiment. At day 30, the survival rate averaged 40.8% for juveniles held in the fine sediment (<120 μm). Coarse sediment containers were not sampled, as no method of recovering juvenile mussels from substrata of a similar size as the animals had been developed. Mean survival to 74 days for the two fine sediment treatments combined was 17.3%, corresponding to a 42.4% rate of survival from day 30 to day 74.

Survival to day 94-98 was 19.1%, indicating that virtually no mortality occurred between days 74 and 94. This final survival rate was determined by combining the rates for all four sediment treatments. A method for effectively recovering juvenile mussels from the coarse substrate had been developed by this sampling time. The substrate size and substrate depth had no significant effect on the survival rate of juvenile mussels ($p < 0.10$). Water velocity at the site of the container in the oval troughs did have a slight effect on the survival rates ($p < 0.05$) when survival rate was regressed on water velocity. The strength of this relationship was low with R^2 values of 0.38 and 0.27 for the day 74 and day 94+ samples, respectively.

The wide disparity among the three years of trials can be explained in part by differences in the temperature regimes in the streams (Figure 6). During the 1993 experiment, juvenile mussels were in the streams a total of 119 days with a mean temperature greater than 15°C (Table 2). In comparison, the early 1994 batch had 106 days greater than 15°C , the late 1994 batch had 41, and the 1995 batch had 79. The growth of the juveniles was correlated with the number of days averaging 15°C or greater ($p < 0.05$).

Juvenile Culture Under Controlled Temperatures

During the lab experiment conducted to determine the effects of temperature on the growth and survival of newly transformed juvenile mussels, definite trends in survival and growth were found among treatments. At day 30, mean survival was 79.3%, 71%, and 49.7% in the 12°C , 18°C , and 25°C streams, respectively. Mean length was 0.40 mm, 0.45 mm, and 0.50 mm in the 12°C , 18°C , and 25°C streams, respectively. No juveniles survived to 60 days in the 25°C treatment. Mean survival to day 60 was 76% and 33%

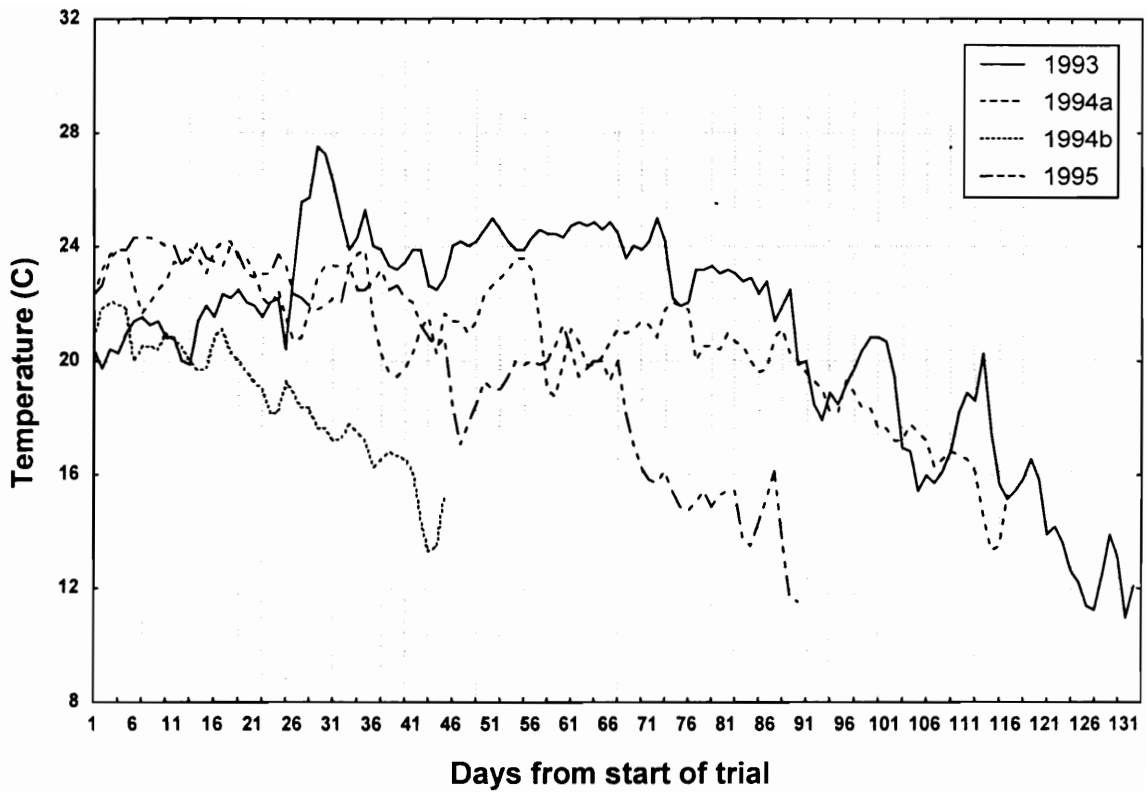


Figure 6. Water temperature profiles during the juvenile culture experiments in the culture system at the Clinch River Steam Plant, Carbo, Virginia.

for the 12°C and 18°C streams, respectively. The survival rates were different ($\alpha = 0.05$) for each treatment, using Tukey's multiple comparison. Mean length at day 60 was 0.36 mm and 0.44 mm for the 12°C and 18°C treatments, respectively, and were also statistically different ($\alpha = 0.05$). The lengths reached by the juveniles in the 12°C stream were indistinguishable from the shell lengths at the start of the experiment, as no growth occurred at the low temperature.

Table 2. Effects of daily mean temperature on the growth and survival of juvenile mussels held in the culture systems at the Clinch River Power Plant in Carbo, Virginia.

Year	Number of days with mean temperature above 15°C	Mean length (mm)	Mean area (mm ²)	Percent survival
1993	119	2.10	2.40	27.5
1994a	106	1.81	1.87	2.2
1994b	41	0.36	0.10	0.0
1995	79	1.38	1.30	19.1

Effect of Density on Growth and Survival

Juvenile mussels had high growth and survival rates, regardless of density in the containers. While this experiment did not shown any detectable difference in growth or survival among the three densities, very different growth results were obtained from the above experiment. At 60 days, the juveniles ranged from 1.5-2.0 mm in length.

However, the temperature ranged between 23°C and 27°C, substantially higher than the temperatures of the Living Streams in which good survival was attained.

The discrepancy between the growth of juvenile mussels in the Living Streams (temperature experiment) and the density experiment led to an evaluation of differences in the two systems. The most prominent differences were that the density experiment had a higher total hardness and lower pH. The density experiment had a total hardness of 280 mg/L, while the hardness levels of the Living Streams in the environmental chamber ranged from 87 to 146 mg/L. The pH in the environmental chamber streams ranged from 8.0 to 8.6 and that of the density experiment ranged from 6.9 to 7.2. Flow velocities in both systems were low, and no disturbance of the surface of the substrate was observed.

Combined Analysis

In order to compare the growth results of these experiments, both within this study and to other studies, a method of normalization was employed. The growth achieved during each experiment was calculated on the basis of the number of degree-days above 15°C experienced by the juveniles. Since it was clear that temperature significantly affected the growth rate of juvenile mussels, this was determined to be a suitable method for comparing results. Once the data from each experiment were normalized on this degree-day basis, a regression was performed to estimate the rate of growth of juvenile rainbow mussels. The regression equation, calculated with the larger juveniles from the 1993 experiment and first batch in 1994 excluded, was:

$$\text{Length(mm)} = 0.809 \cdot 10^{-3} \cdot \text{Degree-days} + 0.347.$$

Data from 1993 and the first batch in 1994 were omitted because they were determined to

be outliers relative to the other trials based on residuals analysis. The resulting regression had an R^2 value of 0.88 and $p < 0.001$, indicating that this regression accounted for most of the difference in the growth among the experiments. Growth during the 1993 experiment was more than double the predicted amount, a length of 2.22 mm vs. a predicted length of 1.01 mm. The first batch in 1994 produced similar results, with an actual mean length of 1.81 mm and a predicted length of 0.82 mm.

Comparisons of growth rates among experiments in my study and with other studies (Gatenby, 1994) revealed that growth of rainbow mussels was similar under different laboratory culture conditions (Table 3, Figure 7). The regression equation obtained using the data from my laboratory culture experiments accounted for most of the variation of growth when the data were normalized on the basis of degree-days greater than 15°C. The regression was used to predict the growth of juvenile *V. iris* reported by Gatenby et al. (1997) and Gatenby (1994), and the results were consistent. The predicted shell length after 60 days using my regression was 0.74 mm while the actual lengths they reported ranged from 0.64-0.74 mm. When the same equation was applied to the 100 day lengths reported by Gatenby et al. (1997) and Gatenby (1994), the results were a predicted value of 0.99 mm versus actual lengths of 0.83-1.35 mm.

Table 3. Relationship of degree-days above 15°C to predicted and measured mean length (mm) for juvenile *Villosa iris*.

Degree-days above 15°C	Mean length (mm)	Predicted length (mm)	Source (if other than this study)
90	0.450	0.420	this study
169	0.421	0.484	this study
180	0.440	0.493	this study
180	0.413	0.493	Gatenby, 1994
240	0.658	0.541	Gatenby, 1994
258	0.585	0.556	this study
300	0.500	0.590	this study
400	0.807	0.671	Gatenby, 1994
465	0.760	0.723	this study
468	0.767	0.726	this study
480	0.690	0.735	Gatenby, 1994
660	1.070	0.881	Gatenby, 1994
800	1.009	0.994	Gatenby, 1994
819	2.17	1.010	this study
1088	2.968	1.227	Gatenby, 1994
1120	1.234	1.253	Gatenby, 1994

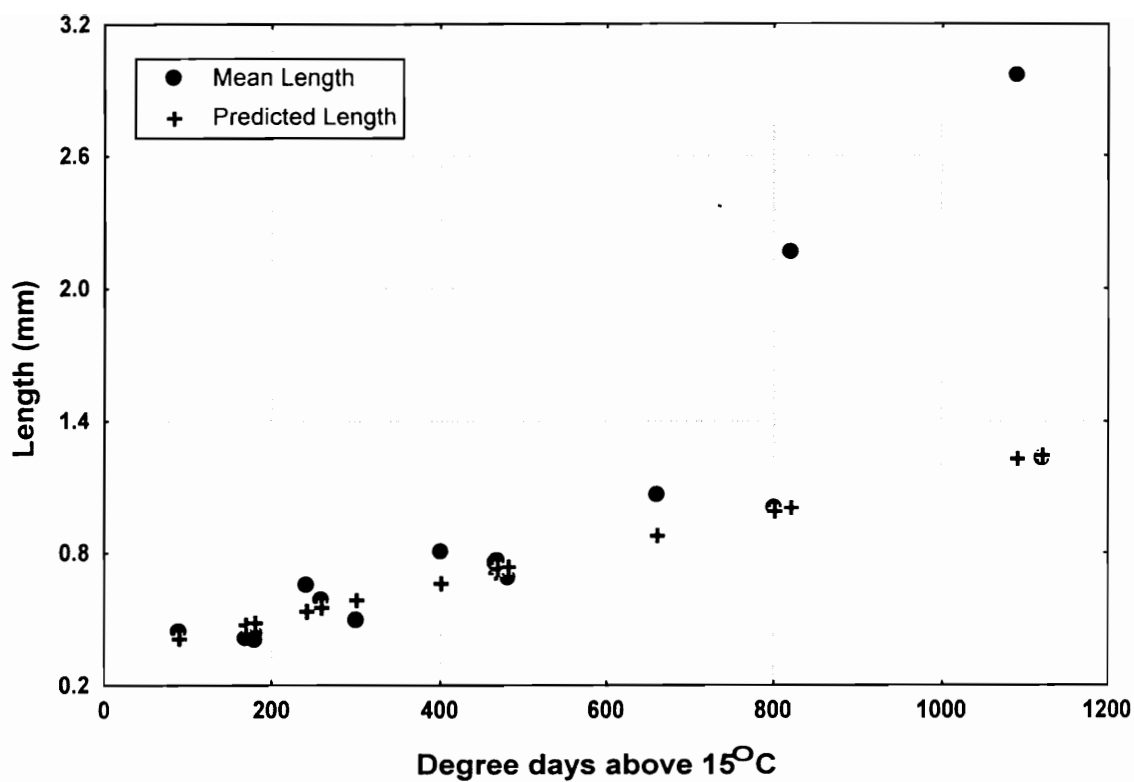


Figure 7. Plot of mean measured length and predicted length of juvenile *V. iris* after culture at the given number of degree-days above 15°C. The regression equation used to calculate the predicted lengths was $L(\text{mm}) = 0.809 \times 10^{-3} \times \text{degree-days} + 0.347$.

DISCUSSION

Results of culture trials showed that rearing juveniles in protected oval troughs supplied with suitable river water is a viable method to culture juvenile mussels for conservation purposes. While the success of the technique varied from year to year, the potential for excellent growth and acceptable survival was demonstrated. The growth achieved when juveniles were delivered to the artificial stream in early summer was more than twice that achieved in captive laboratory settings. In addition, survival rates of approximately 20% after 3 months far exceed the estimated survival of wild metamorphosed juveniles (Young and Williams, 1984). Use of this type of an artificial stream system could be an effective addition to a restoration program by producing substantial numbers of relatively large juveniles, likely to have greater chances of survival than laboratory-reared juveniles. My study also revealed that several factors were important to the success of this culture technique, including temperature regime, flow condition, and season.

The presence of a suitable substrate was believed to be necessary to obtain high growth rates and survival. Earlier studies provided evidence that the presence of silt in the system improved the survival and growth rates of cultured juvenile freshwater mussels (Hudson and Isom, 1984; Yeager et al., 1994; Gatenby et al., 1996). The reason that substrate improves the performance of culture systems is not clear, but there is evidence to suggest that one of the benefits is an increase in available food. Yeager et al. (1994) found that newly transformed juvenile *V. iris* obtained food through the use of

pedal feeding. In this process, the juvenile would extend its foot out from the gaped valves and rapidly sweep the foot to draw particles toward the open mantle cavity. This process also was reported by Gatenby et al. (1996) for *V. iris*. In their study, juveniles cultured with fine sediment had twice as much material in their gut than juveniles fed an algal diet in the absence of fine sediment. Given this line of evidence, they hypothesized that rearing juveniles in sand rather than fine sediment, or silt, would yield slower growth and lower survival. The use of larger sizes of substrate was also hypothesized to inhibit growth and survival by reducing juvenile mobility and efficiency of pedal-feeding.

The lack of differences in juvenile mussel growth and survival in the two substrate sizes may be due to the introduction of additional fine sediment and organic matter from the supply water. Many of the dishes were covered with a 1-5 mm thick layer of this fine sediment within 30 days. This additional particulate matter may have provided the same benefits that Hudson and Isom (1984), Yeager et al. (1994), and Gatenby et al. (1996) reported in their studies. More than 95% of the juveniles recovered during the 30 day sampling were in this loose, flocculent layer of fine sediment which had settled on the original substrate. This result agreed with that of Yeager et al. (1994), in which juveniles allowed to burrow 4 hr or less were all found in the top 1 cm of substrate. The reason for the majority of mussels occurring in this layer of substrate is not known. Water chemistry parameters such as dissolved oxygen, pH, conductivity, etc. can influence the suitability of microhabitats for juvenile mussels (Buddensiek et al., 1993). The juveniles in my study may have avoided the substrates provided because they compacted, inhibiting interstitial water exchange and physical movement. Later

sampling during my study indicated that larger juveniles could use the more compacted substrate. In subsequent samplings on days 74, 94, and 98, higher percentages of the juveniles were located in the original substrate. Since the younger juveniles depend on pedal-feeding for nutrition, the newly settled, loose layer may provide a suitable food resource, while the older animals can supplement their diet through filter feeding. This would explain how the larger juveniles are able to exploit the more stable, packed substrate, unused by the younger juveniles.

In the wild, the suitability of sandy substrates may be contingent on the delivery of ample food supplies to the microhabitat. Since it is believed that sediment provides nutritional benefits to young juvenile mussels (Gatenby et al., 1996), some other food source would be required to support juveniles in sand substrate of a stream. Further evidence for the beneficial influence of some amount of sediment was provided by Buddensiek (1995), who found that juveniles (*Margaritifera margaritifera*) held in cages had higher survival rates when a moderate amount of sediment accumulated in the cages. Cage cells with no sediment and cells which were more than two-thirds filled with sediment exhibited lower survival rates. Juvenile mussels in a stream have been reported to occur most often in eddies behind boulders in riffles and runs, habitats which would accumulate small to moderate amounts of fine particulates (Neves and Widlak, 1987). The evidence seems clear that the presence of fine sediment provides benefits to young juvenile mussels through processes which are not well understood.

Flow velocity of the overlying water also influenced the success of rearing juvenile mussels. Juveniles had higher survival rates in areas of the artificial streams

which had lower flow velocities and less physical disturbance, similar to depositional zones in streams. Neves and Widlak (1987) found that juvenile mussels were most common in depositional zones in a headwater stream of the Upper Tennessee River Basin. Localized flow velocities in the artificial streams ranged from 0 to 0.20 m/sec at a depth similar to the top lip of the dishes. Dishes held in locations with flow velocities above approximately 8 cm/sec exhibited significantly disturbed substrates. In contrast, Buddensiek et al. (1993) determined that a high rate of exchange between the overlying water and the interstitial spaces was beneficial to juvenile eastern pearlshells (*M. margaritifera*). Data from my stocking density experiment indicated that juveniles in dishes placed in areas of low flow velocities exhibited lower survival rates than those in locations with moderate flow velocities. However, none of the flow velocities in this system disturbed the surface of the substrate in the dishes. Hudson and Isom (1984) also reported that juveniles of *Anodonta* (= *Utterbackia*) *imbecillis* exhibited better growth in systems with higher flow velocities. This line of evidence suggests that some moderate flow is necessary for good growth and survival of juvenile mussels, but flows high enough to physically disrupt the surface of the substrate are detrimental to them.

Temperature significantly affected the growth and survival of juvenile mussels. Juvenile *V. iris* held in controlled-temperature Living Streams at 12°C did not grow over the course of 60 days in natural river water, juveniles held at 18°C exhibited a small amount of growth, and juveniles held at 25°C had relatively high growth rates. Mortality was positively correlated to growth; very low at 12°C, moderate at 18°C, and high at 25°C. A similar study with juvenile *M. margaritifera* also found that temperature was

positively correlated with growth and mortality, yielding correlation coefficients (R^2) ranging from 0.332 to 0.809 (Buddensiek, 1995). A wild population of *M. margaritifera* also has been shown to have higher individual growth rates as temperature increases (Hruska, 1992).

Further evidence for the effect of temperature on the growth of juvenile mussels was obtained from the artificial stream system experiments at Carbo, Virginia. The growth of the juveniles held in the oval troughs was markedly different in the two years in which adequate survival was achieved. Growth rates during the 1993 experiment were very high, while they were much lower during the 1995 experiment. One of the major differences between these two trials was the date at which juveniles were delivered to the troughs. In 1993, newly transformed juvenile *V. iris* were placed in the oval troughs in late June. Initiation of the experiment at this time allowed the juveniles to begin growth in the river water during the expected spring peak in algae density (Fogg and Thake, 1987; Biggs, 1996). The 1995 experiment was not initiated until the first week of August, almost 1.5 mo later in the growing season. Analysis of the temperature and growth data suggests that the juveniles did not grow at temperatures below 15°C. Under conditions of declining water temperatures, the juveniles would be expected to grow less when they are placed in the artificial stream later in the growing season.

V. iris growth rates were very similar among all laboratory trials. The greatest percent difference between the regression predicted values and the measured lengths in the range of laboratory studies available was 35%. These similar growth rates were attained in several different culture systems. However, juvenile rainbow mussels placed

in oval troughs with river water during early summer grew at twice the rate of the predicted growth based on the laboratory experiments, suggesting that natural growth rates are much higher than those attained in the laboratory culture systems. If the juveniles are not placed in artificial streams until later in the summer, the potential growth rate becomes comparable to that of the laboratory culture systems. How these growth rates compare to those of wild, newly transformed juveniles is unknown.

Density effects were brought into question during the 1994 trial in the artificial stream system, during which almost all the juveniles perished. One of the possible causes for the very poor survival was that the juveniles were held at a density in the containers which was too high for the food supply, water exchange rate, or behavioral needs of the juveniles. The controlled laboratory experiment, in which the density of the juveniles in containers was manipulated, showed that survival and growth of juveniles at densities ranging from 100 juveniles/56 cm² (twice the density of juveniles in the 1994 artificial stream experiment) to 100 juveniles/144 cm² were not different. Gatenby et al. (1997) successfully reared juvenile *V. iris* for up to 100 days at densities up to 100 juveniles/25 cm², indicating that these high densities are not detrimental to young juvenile mussels. However, during this experiment the juveniles were consistently fed with algae. Further experiments in the laboratory with larger individuals have suggested that densities of 100 juveniles/177 cm² may cause the animals to attempt to disperse. Kat (1982) found that small *Elliptio complanata* commonly moved into and out of defined plots, and that this movement was related to the density of the juveniles in the plot. Therefore, newly transformed juvenile mussels can be cultured at densities of 100/56 cm² or higher, but

they may need more space as they grow to prevent reductions in growth due to overcrowding. Density limits in the culture of marine mussels have been discussed and modelled in the literature (Manzi, 1985; Hickman, 1992), but the limits are based on available seston (Lutz, 1985). Available food was not believed to be a limiting factor in our culture system because similar survival rates were attained in the highest and lowest density dishes without the addition of food.

Water hardness was also recognized as a potentially important factor in the culture of juvenile freshwater mussels during my study. The controlled-temperature experiment and the stocking density experiment were both initiated with the same batch of freshly transformed juveniles. Therefore, the juveniles in both experiments were of the same 'quality'. The fact that the juveniles in the stocking density experiment survived and grew much better than those in the controlled-temperature experiment indicated that something about the system used in the stocking density experiment favored the success of culturing juveniles. It was determined that the major difference between the two systems was that the density experiment system was filled with a mixture of well water and conditioned city water, yielding a hardness of 200-250 mg/L as CaCO₃. The other system had hardness levels of 90-150 mg/L CaCO₃. Both systems were allowed to establish algal flora, which provided the food resource for the juveniles. A subsequent study found that hardness levels influence survival and growth in newly transformed juvenile *L. fasciola* (Steg, 1998). Since it has been reported that juvenile survival is positively correlated with size in wild populations (Young and Williams, 1984; Buddensiek, 1995), increased growth may be a factor in improving survival in higher

hardness water. Based on this and other studies, it seems clear that water hardness in the 200-250 mg/L range increases the growth and survival rates of juvenile *V. iris* and *L. fasciola*.

Evaluation of my method of rearing juveniles compared to others in the literature showed mixed results. While juvenile survival to 90+ days during two years was acceptable, one culture trial yielded almost no survival. Based on the size of the valves left behind and the number of growth lines on the shells, the juveniles in this trial died within 3 wk after transformation, indicating that the juveniles either starved (Hudson and Isom, 1984) or died in response to some temporary water quality problem that went undetected. The fact that this trial was not initiated until the first of September may favor the possibility of starvation. The total abundance of phytoplankton during September can be lower than that in June and July (Biggs, 1996). The composition of the phytoplankton can also shift in relation to seasons of the year (South and Whittick, 1987; Biggs, 1996), providing different qualities of food among seasons. The different algal communities to which the trials were likely exposed probably provided food stuffs of various qualities, especially in relation to lipids and fatty acids which are important to early juvenile growth (Gatenby et al., 1997). The survival rates obtained in my study were not as good as the highest rates reported for *V. iris* by Gatenby et al. (1997). Buddensiek (1995) also reported higher survival rates for *M. margaritifera* juveniles held in mesh-sealed cages in rivers. This may reflect the exposure of the juveniles in my study to more environmental perils than the controlled laboratory study of Gatenby et al. (1997). However, growth was high for juveniles placed in the culture system early in the growing season. The

mean size (2.1 mm) attained during the 1993 trial after 131 days was over 50 % greater than that reported by Gatenby et al. (1997) after 100 days (1.35 mm). The mean size of juveniles in my 1993 and early 1994 trials were also larger than those of *L. fasciola* juveniles in a laboratory culture experiment with regular supplements of algae feed (Steg, 1998). The juveniles from the early batch in my 1994 trial, placed in the artificial streams on June 22, also exceeded the growth rate of other culture attempts. However, juveniles that were not placed in the culture streams until late in the season (August or September) did not grow as much as those of Gatenby et al. (1997), or Steg (1998). This underscores the importance of initiating the culture process early in the year. The culture method used in my study yielded growth and survival rates comparable to or greater than those of other successful culture methods, provided that the juveniles are placed in the artificial streams early enough in the growing season to utilize spring and early summer conditions.

Summary

Culture systems supplied with river water have the potential to be very effective systems for the culture of juvenile mussels. The growth possible with such a system exceeds that of any known laboratory culture system to date. Survival rates are also comparable to the laboratory culture systems, as long as the juvenile mussels have an adequate growing season. Increases in the growth rate of juveniles could be due to the presence of some resource(s) that provide nutritional, metabolic, or physical habitat benefits to the mussels that are not available in current laboratory culture systems. The timing of culture initiation in the more 'natural' river-fed streams is crucial. My study

showed that when juveniles are placed in these artificial streams at a date that provides too few growing days, growth is decreased and survival may be affected. My culture system is easy to set up, easy to maintain, and modest in cost. However, the system does not protect juveniles from all possible environmental perils, including pollutants, temperature extremes, and some predators.

Culture systems supplied with natural river water are a feasible method of culturing juvenile mussels for reintroductions and augmentation of existing populations. The river water provides the juveniles with appropriate food resources and chemical conditions. However, the fact that the growing season is limited by water temperature, and possibly sunlight and primary productivity, makes the timing of culture initiation crucial for best results. Juveniles placed in the culture system after late spring/early summer will have significantly reduced growth. This problem may be solved by heating the incoming river water, but the food resource issues may confound the results.

Since the system used in my study is so simple to set up and maintain, American Electric Power at Carbo, Virginia should consider using it as part of an overall mussel propagation program. The costs of monitoring the culture system and rearing the juveniles would be minimal, and would require very little of the existing environmental staff time (less than 2 hrs/wk) except at set-up and 'harvest' times. Design improvements that should be considered include developing a reliable way of heating incoming water during the winter months so that culture can continue throughout the year and developing a better way of maintaining flow in the oval troughs, as the paddlewheels generate enough turbulence to disturb the substrata.

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CHAPTER 2

INVESTIGATION OF POTENTIAL BIOMARKERS OF ENVIRONMENTAL STRESS IN FRESHWATER MUSSELS

INTRODUCTION

There is an urgent need for the development of reliable and practical methods for assessing the health of mussels in river systems. Few of these methods exist at present. One way in which this can be pursued is through the use of bioindicators of stress, or biomarkers. Sufficient development of biomarkers would allow the identification and diagnosis of environmental problems before they become catastrophic for the resident fauna. My study was undertaken to address whether several physiological and biochemical parameters may be useful as biomarkers to identify impacted habitats for mussels.

The Clinch River is one of the major tributaries of the Upper Tennessee River basin, in southwestern Virginia and northeastern Tennessee. The majority of the Clinch River watershed is in the Valley and Ridge Physiographic Region, with a topography characterized by long steep ridges and narrow valleys. The Clinch River has been regarded as having one of the most diverse mussel and fish faunas of any comparable sized stream in North America (Neves, 1991; Jenkins and Burkhead, 1994). In addition, the upper Clinch River is one of the few remaining free-flowing tributaries in the Tennessee River basin, providing critical habitat for the aquatic fauna that has evolved in the region (EPA, 1996). Prior to the construction of Watts Bar and Norris dams by the TVA, and subsequent impounding of the river, 60 species of unionids were recorded from the Clinch River (Ortmann, 1918). Ahlstedt (1984) reported finding 43 species of mussels in the Clinch River upstream of Norris Reservoir. The upper Clinch River currently harbors 17 federally endangered species of mussels.

Anthropogenic Impacts in the Clinch River Watershed

Human activities are negatively impacting the watershed, causing declines in native unionid populations. The range of impacts includes mining effluents and run-off, inadequate sewage treatment facilities, agricultural run-off, urban run-off, sedimentation from poor land-use practices and logging, and accidental spills (Helfrich et al., 1986). Impoundment of rivers has also destroyed unionid habitat in many southeastern rivers (Bogan, 1993; Williams et al., 1993), but there are no impoundments on the Clinch River in Virginia. The sum of the impacts from these stressors seems to be driving the native mussel fauna toward extinction.

Coal mining and its associated activities have had a detrimental effect on the Clinch River. Various processes associated with the extraction, separation, and transportation of coal in the central and southern Appalachian Mountains have diminished aquatic resources. The exposure of mine tailings and debris to air allows the leaching of metals to occur (Nelson et al., 1991). As wastewaters and drainage water from mines becomes more acidic, the problem of metals leaching becomes even more severe (Helfrich et al., 1986; Kelly, 1988; Nelson et al., 1991). The Clinch River is highly buffered and alkaline, so the acidity is generally not a direct problem for the biota. However, exposure to heavy metals, such as cadmium, chromium, copper and zinc, can be highly detrimental to freshwater mussels (Naimo, 1995).

The Clinch River Steam Plant, operated by American Electric Power, in Carbo, Virginia has historically impacted the Clinch River. Industrial spills have accounted for acutely lethal pollution events, impacting the river as far downstream as the Tennessee

border (Cairns et al., 1971). Both zinc and copper have been measured at levels great enough to harm at least some fractions of the biota (Clements et al., 1992). Copper appears to be especially toxic to the juvenile life stage of unionids, negatively affecting mussels at concentrations of 24 $\mu\text{g/L}$ (Jacobson et al., 1993). Adult mussels have LC_{50} 's to copper exposure of 39-137 $\mu\text{g/L}$ (Cherry et al., 1991). However, since 1994, a new wastewater treatment facility at the Clinch River Steam Plant has reduced the copper concentrations of cooling tower and other waste water discharges to levels less than ambient concentrations (EPA, 1996). The reductions in effluent copper concentrations may allow the recovery of the native mussel fauna in the river reach downstream of the power plant.

Point source industrial discharges can introduce significant amounts of toxic chemicals into streams in a very localized fashion. Two major industrial discharges occur in the Clinch River basin of Virginia (EPA, 1996). The AEP Clinch River Steam Plant was responsible for two catastrophic toxic events in the river, in 1967 and 1970, which caused total mussel kills in the river up to 18 km downstream (Crossman et al., 1973). The Cypress Foote Mineral Company has impaired the benthic fauna in Stock Creek, a tributary to the Clinch River at Speers Ferry, and may be negatively affecting the mussel fauna in the Clinch River (EPA, 1996). Other small industrial discharges also may have local impacts, but are not considered likely to influence the biota in large reaches of stream or river (EPA, 1996). Traffic accidents can also be devastating to mussels. In August of 1998, a tank truck overturned and spilled some of its load of foam rubber manufacturing agent into the Clinch River immediately upstream of the town of Cedar

Bluff, Virginia. The resulting chemical spill killed mussels and fish up to 3.5 km downstream, including over 230 specimens of federally endangered mussels (L. Koch, personal comm.). This event occurred at one of the sites used in my study, albeit well after the experiments were completed. Point-source contaminants, especially accidental spills, have had major negative impacts on the Clinch River.

Land-use practices also degrade mussel habitat in the Clinch River basin.

Logging activities on steep slopes, such as the ridges in the Clinch River watershed, lead to sedimentation of streams, altered hydrologic characteristics, and sometimes channel alteration and destabilization (Chamberlain et al., 1991). Almost 60% of the hydrologic units in the Clinch and Powell River watersheds were ranked as having a “high” potential for non-point source pollution impacts by the Virginia Department of Forestry and the Soil and Water Conservation Districts, mostly due to sedimentation and erosion (EPA, 1996). Excess sediment loads are detrimental to unionid populations through disturbance of recruitment, altered siphoning behavior, physical habitat degradation, and loss of host fish habitat (Ellis, 1936; Mohan, 1992). Sedimentation and pesticide run-off from the 28% of the watershed used to grow crops, most of which lie in floodplains, are believed to negatively influence the benthic biota of the Clinch River (EPA, 1996). In addition, cattle pasture is responsible for nutrient enrichment, bank destabilization, and siltation of the stream corridors (EPA, 1996). Cattle also affect mussel habitat by compacting stream beds and physically damaging mussels through crushing. Such non-point source impacts were identified as the most significant water pollutants in the Clinch River watershed by the Bi-State Task Force Report to the Governors of Virginia and Tennessee (EPA, 1996).

Sewage effluents have also had a major effect in the Clinch River. There are 119 municipal discharges in the Clinch/Powell watershed, including municipal sewage treatment plants, community effluents, and mining facilities (EPA, 1996). Since chlorine and ammonia are common components of sewage treatment plant effluents (White, 1986; Belanger, 1991), these facilities can be detrimental to mussel populations (Horne and McIntosh, 1979). Goudreau et al. (1993) reported depauperate or absent mussel assemblages below the sewage treatment outfalls at Tazewell and Richlands in the Clinch River. Asian clams are also negatively influenced by exposure to sewage treatment effluents, exhibiting reduced respiration rates, slower growth, increased mortality, and lower population densities (Belanger, 1991).

Assessment of Impacts with Biomarkers

Biomarkers, defined as physiological, biochemical, and genetic parameters affected by exposure to contaminants or other xenobiotic stressors, have been sought by researchers and managers to help effectively protect natural environments. There is a need to develop reliable methods to assess the effects of anthropogenic stresses in aquatic systems through the use of biomarkers (Fox, 1993). Effective biomarkers can allow managers to determine contaminant exposures, assess the integrated effects of stressors, detect when contaminants reach levels that cause harm to individuals or populations, and set priorities for remediation or clean-up activities (Fox, 1993). Physiological and biochemical parameters are usually most effective when used as a group, or suite, of biomarkers that support and complement each other (Melancon, 1995). Measuring the response of a range of factors allows the investigator to assess the integrated impact of a

broad range of stressors (Melancon, 1995). Ideally, linkages to higher level effects, such as growth, reproduction, survival, population declines, community, or ecosystem effects, can be made (Huggett et al., 1992). For these reasons, tests of potential biomarkers need to be conducted in the field at sites believed to be influenced by anthropogenic stressors.

Biomarkers in individuals can be measured at a range of organizational levels: molecular, enzymatic pathway, organelle, cell, tissue, organ, and individual (Stegeman et al., 1992). One approach to assessing biomarkers at the organismal level of organization is by measuring biochemical parameters that affect the whole body, such as glycogen content or RNA/DNA ratio (Venu Gopal et al., 1990; Peakall, 1992; Patterson et al., 1997). These types of assessments integrate the effects over the entire body but usually do not immediately respond to stressors (Mayer et al., 1992). Another approach to assessing health of mussels is by measuring the status of the energy metabolism. This type of response occurs more quickly than whole body responses following the onset of stressful conditions. The most rapidly responding and potentially most sensitive bioindicators would be enzyme activity levels (Stegeman et al., 1992). Certain enzyme systems exhibit significant changes quickly upon exposure to stressors (Stegeman et al., 1992). By developing these bioindicators, a process for determining suitable habitat locations for preservation or restoration of mussel populations would be available for management agencies.

Mussels and Clams as Test Organisms

The problem with using organisms that are in decline for biomarker measurement is that most of the methods require the sacrifice or mutilation of animals. For mussels

with populations that are threatened or endangered, this is a very real impediment to their use for environmental monitoring. One way around this problem is to identify suitable surrogate test organisms. The Asian clam (*Corbicula fluminea*) is a logical option for investigation as a potential substitute (Farris et al., 1989). These clams are frequently abundant, easily caught, and belong to the same taxonomic class as freshwater mussels. Therefore, Asian clams may serve as suitable test organisms to assess the health conditions likely to be experienced by resident mussels.

Freshwater bivalves are well suited for use as a biomarker of contaminants, as they meet the criteria of sedentary, relatively long-lived, available, and amply large organisms (Moore, 1966). Because mussels are relatively immobile, physiological and biochemical responses are the result of the local environmental conditions and not some distant exposure. Mussels, as benthic filter feeders, are exposed to contaminant through particulates, sediment pore water, and the overlying water column (Naimo, 1995). Many of the commonly found aquatic pollutants preferentially partition to the sediment fraction of the aquatic environment. Compounds such as polycyclic aromatic hydrocarbons (PAH's) and polychlorinated biphenyls (PCB's) are hydrophobic and quickly adsorb to particulate matter that settles to the bottom (Connell and Miller, 1984). Metals, as well, tend to accumulate in bottom sediments achieving concentrations greater than that of the overlying water (Connell and Miller, 1984, Naimo, 1995). A large fraction of the pesticides in use today are also highly lipophilic and preferentially partition into the organic matter that settles to the bottom of waterways (Connell and Miller, 1984). Concentrations of these types of pollutants in the interstitial water are often greater than

that in the water column (Chapman, 1989). Since mussels live in this part of the aquatic environment, they are subjected to relatively high levels of pollutants.

General Biomarkers

Glycogen stores serve mussels as the ready reservoir of convertible energy. Whenever excess food is obtained and absorbed, the portion that is not immediately required for respiration, growth, or reproduction is converted to glycogen for storage until future need (de Zwaan, 1979; Zandee et al., 1980; Bayne and Newell, 1983). Mussels store glycogen in many tissues, including foot and mantle (Gabbott and Whittle, 1986). By using foot or mantle tissue, samples may potentially be taken from individuals without permanently harming them. This is obviously a desirable feature of any biomarker test. Glycogen levels in bivalves have been shown to decrease in response to a variety of environmental stresses including anoxia, reduced food availability, ammonia exposure, organic contaminants, sewage effluents, and quarantine conditions (Cantelmo-Cristini et al., 1985; Gaffney and Diehl, 1986; Bidwell et al., 1995; Chetty and Indira, 1995; Patterson et al., 1997). Therefore, glycogen content has the potential to be a good biomarker for chronic anthropogenic stress.

Glycogen reserves fluctuate throughout the year in bivalve mollusks in temperate climates, which interferes with its use as a biomarker of anthropogenic stress. Glycogen has been shown to change in correlation with reproductive cycles in both freshwater (McMahon, 1991) and marine bivalves (Gabbott, 1983). Unionids, sphaeriids, and corbiculids all exhibit changes in the glycogen content with reproductive cycles (McMahon, 1991). Glycogen content generally decreases with gametogenesis and

embryonic development, and increases in nonreproductive intervals. Zebra mussels also exhibit strong seasonal variations in carbohydrate content among seasons (Sprung, 1995). Temperature also affects the metabolic rate of mussels which can cause changes in the glycolytic and gluconeogenic rates (Zandee et al., 1980; McMahon, 1991). Therefore, interpretation of changes in glycogen content must be made with respect to seasonal cycles.

Related to the total glycogen reserves, the amount of assimilated energy that can be converted to energy stores versus the amount used for present metabolism is an indication of overall well being. The flow of carbohydrates through an organism is dependent on the balance of the nutrient uptake and the metabolic costs of maintenance, reproduction, and growth (Ibarguren et al., 1990; Ibarguren and Ramos-Martinez, 1991). Excess energy is stored in mussel tissues as glycogen through the processes of gluconeogenesis and glycogenesis (Barcia et al., 1991). Energy deficits lead to the breakdown of glycogen through the process of glycolysis (de Zwaan, 1979). Since these processes are multi-step physiological reactions, a large number of enzymes and intermediate compounds are involved (de Zwaan and Wijsman, 1976). The assessment of these chemicals has the potential to provide information about the direction of carbohydrate metabolism in mussels. Fructose-1,6-bisphosphatase (FBPase) and phosphofructokinase (PFK) are competing enzymes in the breakdown and synthesis of fructose-6-phosphate (F6P) and fructose-1,6-bisphosphate (F16BP) (Bohinski, 1987). The reaction has been determined to be a controlling point for the gluconeogenic and glycolytic pathways in mammals and mollusks (Bohinski, 1987; Aoyama et al., 1992;

Barcia et al., 1993).

Evidence suggests that the activity of PFK may be a predictor of the glycolytic rate in other vertebrates as well (Ferguson and Storey, 1992; Churchill and Storey, 1994; Kiessling et al., 1995). This enzyme is allosterically inhibited by the presence of ATP, NADH, and citrate, which are high when the animal has adequate mobilized energy (Bohinski, 1987). Conversely, as these compounds are depleted in the cells, the inhibition of PFK is released, and the glycolytic reaction is favored (Michaelidis et al., 1993). In fact, there is some evidence to suggest that PFK activity increases with the concentration of 5'-AMP and F26BP in the marine bivalve *Mytilus edulis* and the gastropod *Helix pomatia* (Biethinger et al., 1991). These controls have the effect of increasing the activity of PFK during times of energetic stress when the levels of ATP decline and the levels of AMP rise. Increases in the activity of PFK have been reported in a variety of aquatic invertebrates as a response to hypoxia or anoxia (Ramaiah, 1974; Brooks et al., 1991; Hardewig et al., 1991). However, some studies on marine intertidal bivalves have found decreases in the activity of PFK in response to anoxia (Storey, 1985; Meinardus-Hager and Gade, 1992). Michaelidis and Athanasiadou (1994) reported that the PFK activity levels in the freshwater mussel *Anodonta cygnea* decreased in response to hypoxia. Either way, there is evidence to suggest that the activity levels of PFK are likely to change in response to environmental stresses.

The complementary enzyme for PFK in the glycolysis/gluconeogenesis process is fructose-1,6-bisphosphatase. Fructose-1,6-bisphosphatase catalyzes the reverse reaction of F16BP to F6P, which is one component in the gluconeogenic sequence (Tejwani,

1983). As stated before, this pair of reactions is believed to be one of the primary control points of glycolysis and gluconeogenesis (Aoyama et al., 1992; Barcia et al., 1993). This reaction system can set up a futile cycle in which substrates are alternated back and forth between the two reactions (Bohinski, 1987). However, the activity level of PFK is usually much higher than that of F16BPase (Barcia and Ramos-Martinez, 1992; Ferguson and Storey, 1992; Soengas et al., 1993). Because of the higher activity level of PFK, it is commonly believed to be the controlling enzyme in this pair of reactions. Cameselle et al. (1980) found that the PFK and FBPase reaction system had a major regulatory role of carbohydrate metabolism in the marine mussel *Mytilus edulis*.

The use of cellulolytic activity, the collective activity of enzymes responsible for breaking down cellulose in the digestive system of animals, has been shown to be a useful indicator of pollutant stress in bivalves under controlled or severe exposures (Farris et al., 1988). The assay was sensitive enough to detect differences in the responses of *C. fluminea* that were exposed to metals in the laboratory and field. Since this assay measures the response of digestive enzymes, it serves also as a useful indicator of alterations in feeding characteristics (Farris et al., 1988). Cellulolytic enzyme activity was also depressed upon exposure to acid mine effluent and zebra mussel infestation (Haag et al., 1993; Milam and Farris, 1998). Therefore, cellulase activity appears to have potential use as a general indicator of stress. However, validation of the assumption that seasonal influences on the activity levels do not alter the results is needed. Field trials are also needed to determine if this assay effectively detects the more subtle, sublethal impacts that are commonly experienced in rivers.

The use of the ratio of RNA to DNA also has been investigated as a general biomarker (Peakall, 1992). Various studies with fish have shown positive relationships between RNA:DNA and growth (Haines, 1973; Buckley, 1979; Wilder and Stanley, 1983). The ratio has also been shown to respond to environmentally relevant contaminants (Mohapatra and Noble, 1992; Roesijadi et al., 1995). However, reports are conflicting on the response of RNA:DNA to food availability and chemical stresses, suggesting that corroborating assays should be used in support of this assay (Peakall, 1992).

Biomarkers of Specific Contaminants

A more specific biomarker, the measurement of acetylcholinesterase activity, has been employed to determine exposures to organophosphate and carbamate pesticides (Mineau, 1991). Acetylcholinesterase activity has been used recently to detect low level exposures of fish to pesticides in a lake (Gruber and Munn, 1998). Mixtures of pesticides caused cumulative, synergistic effects in fish, prawns, and oysters (Bocquene et al., 1995). Additional metal contaminants did not reduce acetylcholinesterase significantly (Bocquene et al., 1995). Other studies, however, have reported decreases in acetylcholinesterase activity due to exposure to metals (Suresh et al., 1992; Reddy and Venugopal, 1993). Further complicating the issue, Day and Scott (1990) reported that acetylcholinesterase activity levels in aquatic invertebrates were not decreased after exposure to realistic environmental concentrations of organophosphate pesticides, and only became inhibited when pesticide concentrations approached lethal levels. Recently, a freshwater mussel die-off was attributed to pesticide exposure after acetylcholinesterase

analyses revealed that the dead mussels had acetylcholinesterase activities reduced by 63-75% (Fleming et al., 1995). The specificity of acetylcholinesterase activity responses to a single class of contaminants may be debatable, but the apparent usefulness of this assay in field exposures shows promise.

Objectives of the Study

The objectives of my study were to test the suitability of several biomarkers as indicators of stress in mussels and as a means to evaluate the suitability of the chosen habitats for freshwater mussels. The bulk of the physiological parameters chosen for my study were general indicators of stress, and as such were not intended to identify sources of contaminants or the specific pollutants that might degrade a habitat. Determining whether a subset of the measured parameters would serve as an effective suite of biomarkers, correlated with the status of mussel populations at those sites, was the first objective. Secondly, natural variation in the biomarkers over the course of the year was investigated to develop the background data for future use of these biomarkers in field situations. The final objective was to evaluate the appropriateness of using Asian clams as surrogates for unionids, because of their abundance and sympatric distribution with unionids in nearly all river systems.

MATERIALS AND METHODS

Field Exposure of Mussels and Clams

Two species of freshwater bivalves, rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*), were deployed in cages along several sites within the Clinch River basin. Deployment sites were chosen to represent stream reaches upstream and downstream of suspected adverse impacts or to investigate sites in which unionid populations have recently declined or have been extirpated. The deployment was conducted three times to investigate seasonal patterns in the responses of the bivalves at each site. Experiments were conducted in fall (September-October, 1995), spring (April-May, 1996), and summer (July-August, 1996). Winter was omitted due to near-constant high water conditions.

Site Selection and Descriptions

Eight sites were used for deployment of mussels during the course of the experiments. They were as follows: Clinch River immediately upstream of the confluence with Middle Creek, Clinch River immediately upstream of the Richlands-Raven sewage treatment plant, Clinch River approximately 1.6 km downstream of the Richlands-Raven STP, Clinch River immediately upstream of the Clinch River Steam Plant (CRSP) at Carbo, VA, Clinch River approximately 500 m downstream of CRSP fly ash dump, Copper Creek approximately 500 m upstream from the confluence with the Clinch River, Stock Creek approximately 2 km upstream of the confluence with the Clinch River, and Little River approximately 0.7 km upstream of the confluence with the Clinch River. Study sites are identified on the map of the Clinch River basin (Figure 8).

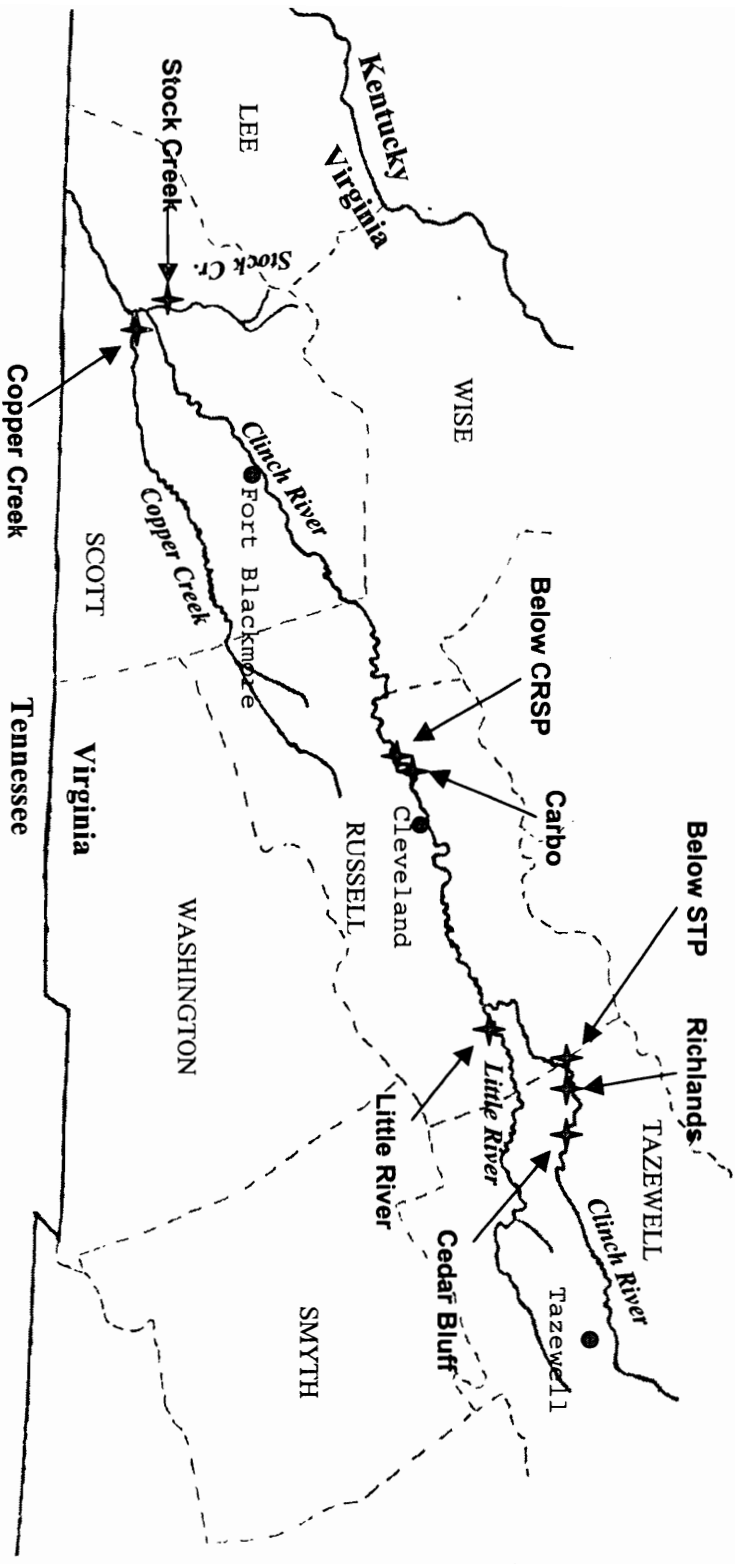


Figure 8. Location of field exposure sites for mussels and clams in the Clinch River watershed. Cage sites are identified by stars. County names are in capital letters and counties are marked with dashed lines. Towns are marked with circles.

The most upstream site in the Clinch River (referred to as Cedar Bluff) was immediately upstream of the confluence with Middle Creek, lying between the towns of Cedar Bluff and Richlands. This site was added as a 'reference' site during the final experiment, because early analyses indicated that the site upstream of the Richlands-Raven sewage treatment plant (STP) may have been suffering from the effects of urbanization as the Clinch River flowed through the town of Richlands. The site was located 15 m upstream of a low water bridge over the Clinch River, approximately 100 m upstream of the U.S. Route 460 Bypass bridge, adjacent to U.S. Route 460 Business. The site had substantial numbers of mussels. At this location, the Clinch River was approximately 13 m wide. The dominant substratum was gravel with some sand in the interstitial spaces. There was no cobble in the immediate area. A thin layer of silt was present on the substratum, and flow was slow to moderate. The cage was placed in water 0.5 m deep under low flow conditions. Native rainbow mussels occurred at the site where the cage was placed.

The next site downstream in the Clinch River (referred to as Richlands) was 0.4 km upstream of the Richlands-Raven STP, which is downstream of the town of Richlands, immediately downstream of a medical clinic complex. The Clinch River was approximately 18 m wide at the site. The substratum was a mixture of cobble and gravel, with little silt present. Flow was moderate throughout the entire shoal where the cage was placed. Water depth was only 0.1 m under summer low flow conditions. The river had a tendency to become turbid at this site after even relatively low levels of rainfall. This site was selected to separate any adverse effects caused by the town of Richlands

from effects of the STP. Since the Richlands site was downstream and the Cedar Bluff site was upstream of the town of Richlands, comparing the responses of mussels and clams at the two sites would allow the effects of the town on mussel physiology to be determined. Similarly, comparisons with the next site downstream would allow the determination of the effects of the STP on the mussels and clams. Only a few native rainbow mussels were found at this site, but Asian clams were common.

The deployment site below the Richlands-Raven STP in the Clinch River (referred to as Below STP) was 2.0 km downstream of the plant effluent. The site was adjacent to Route 67, at a pullover 0.5 km from the intersection with U.S. 460. The river was approximately 15 m wide, with a substratum of gravel and sand. Very little silt was present, and the physical habitat was similar to that at the Richlands site. Depth during the lowest flow conditions was 0.1 m, and averaged 0.3 m during the experiments. Flow velocity was moderate. Turbidity also caused a sampling problem at this site, as small rainfall events resulted in significant increases in turbidity, water velocity, and water depth. During sampling prior to the experiment, no mussels were found but Asian clams were abundant.

Two sites were chosen bracketing the American Electric Power Clinch River Steam Plant (CRSP) at Carbo, VA, a facility that caused devastating mussel die-offs in 1967 and 1970. This facility was still introducing potentially harmful levels of copper and zinc into the Clinch River in the late 1980's (Cherry et al., 1991).

The site upstream of the CRSP (referred to as site Carbo) was chosen to serve as a reference for comparison with the site downstream of the plant. The site was

immediately adjacent to Route 664 and the CRSP, 80 m upstream of the first effluent pipe. The river at this site was approximately 23 m wide under low flow conditions. The maximum depth was 1.0 m, and the mean depth was approximately 0.6 m. The substratum was a mixture of cobble, gravel, and sand in mid-channel, with sand and silt forming the substratum along the left ascending bank. There was also exposed bedrock in the stream channel. The substratum provided a diversity of microhabitats in the reach, including protected areas under large rocks, gravel shoals, and interstitial sand. Water velocity was moderate. Twelve species of unionids occurred at this site, including numerous rainbow mussels. Asian clams also were present at the site. The density of mussels was moderate when compared to other sites I sampled in the Clinch River.

The site downstream of the CRSP and its fly-ash landfill (referred to as Below CRSP) was 3.2 km downstream of the Carbo site and 0.6 km downstream of the landfill, adjacent to Route 665. The river is split into two channels in this reach, and the site chosen was in the left ascending channel, on the landfill side. The substratum at this site was a mixture of cobble, gravel, and sand, with almost no silt present in the channel. However, the apparent complexity of the habitat was not as great as that at Carbo. The left channel was approximately 12 m wide, and had a mean depth of 0.5 m. During preliminary surveys, only 1 live mussel was found within 25 m of the site, a wavy-rayed lampmussel (*Lampsilis fasciola*). Relic mussel shells were abundant, but no fresh dead mussel shells were found. Asian clams were present at the site. The physical habitat appeared to be suitable for unionids.

The Copper Creek site was chosen as one of the potentially impacted sites. The

selected site was 0.3 km upstream of the confluence of Copper Creek and the Clinch River (CRM 211 (CRK 339)), adjacent to Route 627. In 1980, the lower portion of Copper Creek was reported to be relatively pristine and had suitable substrata for unionids (Ahlstedt, 1986). However, by 1994, the mussel fauna in the stream had declined drastically and no specific cause was determined (Ahlstedt, pers. comm.). During surveys performed in 1980, a site 0.32 km upstream of the confluence with the Clinch River had three species of mussels present, and a total of 19 species occurred in the lower 3.4 km of this stream. During preliminary sampling in 1995, no mussels were found at a site 0.3 km upstream of the confluence with the Clinch River. Additionally, other benthic fauna, such as crayfish, insects, and snails, also were uncommon. At this site, Copper Creek was 11 m wide and had a gravel and sand substratum. The maximum depth of the channel under low flow conditions was 0.8 m, and the mean depth was approximately 0.5 m. Flow was moderate to fast. The banks were stable, there was very little silt in the stream, and the riparian zone was regenerating woodland. A gravel bar had formed along the left ascending side of the channel. The physical habitat appeared to be suitable for mussels.

Stock Creek joins the Clinch River from the northwest at CRM 212 (CRK 341), 2.6 km upstream of the confluence of the Clinch River and Copper Creek. Stock Creek was selected as a cage site based on conversations with fisheries biologist John Jesse, Virginia Department of Game and Inland Fisheries. A battery manufacturing facility (Foote Mineral Co.) was adjacent to Stock Creek, and it was believed to have caused fish kills in the stream (Jesse, pers. comm.; EPA, 1996). During preliminary surveys, no live

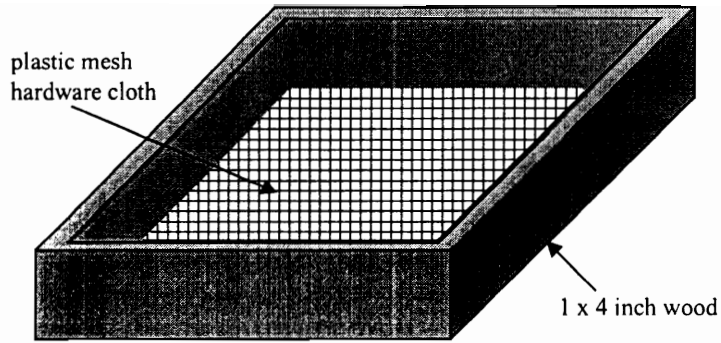
mussels were found in Stock Creek, and only one relic shell was found. The site chosen was 2 km upstream of the confluence with the Clinch River. The stream was 0.5 m wide at the site and up to 9 m wide in the immediate vicinity. The substratum was predominantly gravel with some sand, and was very similar to that of the Copper Creek site. The maximum depth at the site was 1.0 m, and the mean depth was approximately 0.3 m. Flow velocities were moderate, and very little silt occurred in the stream. The banks were covered by grass and weeds and appeared to be subject to erosion during high water events. The water temperature in Stock Creek tended to be cooler than at any of the other sites.

The final site was the reference site and the source population of mussels for the study. Little River is a tributary of the Clinch River at CRM 298 (CRK 480), in the upper portion of the drainage. The river was 18 m wide at the site, with a maximum depth of 0.6 m and a mean depth of 0.4 m under low flow conditions. Little River has a mixed substratum at this site, comprised of cobble, gravel, and sand. The substratum ranged from large, flat cobbles with sandy substrata underneath to patches of sand and gravel. This diversity of microhabitats provides suitable habitat for mussels, as evidenced by the large numbers of rainbow mussels present in the reach. Asian clams also were abundant at this site. Flow velocities were moderate, and very little silt was present in the river, with the exception of one large backwater area upstream of the ford. The riparian area was mostly pasture along the left ascending bank and road, with a tree line along the right ascending side. Land use in the watershed is mostly agricultural, with a large tract through which the river passes owned by the Stuart Land and Cattle Company.

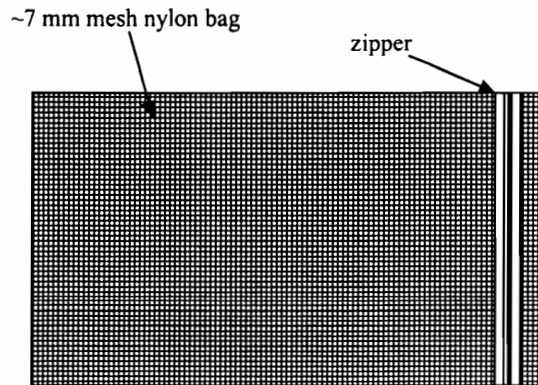
Field Exposure of Mussels and Clams

All mussels and clams used for the deployments were collected from the same site in the Little River. The collection site was approximately 0.7 km upstream of the confluence with the Clinch River, at a gravel ford along Route 640 in Russell County, VA. Mussels and clams were collected by snorkeling and maintained in aerated coolers filled with river water for transport. Animals were transported to Blacksburg, VA and maintained in Living Streams (Frigid Systems, Inc., Toledo, OH) until they could be deployed in the cages, which was within 10 days. The bivalves were then randomly mixed and assigned to a given site.

The animals were deployed in cages anchored to rebar stakes at the eight designated sites. Ten *V. iris* and 30 *C. fluminea* were deployed at each location. The cages used for each species were different, due to differences in mobility and ecology of the animals (Figure 9). Rainbow mussels were held in 0.5 m square wood frame cages with mesh bottoms and open tops. The cages were 9 cm deep. Each cage was set into the substratum so that the top of the cage was flush with the surrounding substratum. The excavated material was then placed in the cage to provide suitable substrate for the mussels to burrow and stabilize. The cage substratum was deep enough so that animals were able to burrow completely below the surface. Cages were set in place at least one week prior to deployment of the animals and were filled with instream substrata so that any associated biota would become established prior to the introduction of the mussels. Asian clams were held in nylon mesh bags (approximately 0.5 cm mesh size) which could be sealed with a zipper. These bags were filled one-half to two-thirds full with



Cage used for holding rainbow mussels (*Villosa iris*) at exposure sites



Bag (cage) used for holding Asian clams (*Corbicula fluminea*) at exposure sites.

Figure 9. Diagram of the two types of cages used for field exposure of rainbow mussels and Asian clams.

natural substrata from the site. This fill substratum was sorted to remove any Asian clams so that only test clams would be used for analysis. The thirty clams were then introduced into the bag and the bags were buried to the surface of the surrounding substratum and anchored to a rebar stake. The clam bags were placed immediately adjacent to the mussel cages. The animals were held in these cages for 30-40 days. In addition, 8 rainbow mussels and 30 Asian clams were sacrificed and dissected as described below within 3 days of the start of the deployment to provide reference point samples of physiological parameters. The beginning (T_0) samples were not taken during the fall exposure based on the assumption that the LR samples would serve as the reference.

At the end of the exposure periods, the animals were collected by snorkeling, and cages were excavated if necessary. The entire area within approximately 0.5 m upstream, 1.0 m beside, and 2.0 m downstream of the cages was searched so that as many of the mussels as possible were found, since some individuals could have escaped or been removed by predators. The *C. fluminea* bags were removed from the stream, emptied, and the clams present in the bag were collected. The animals from each site were placed in 3.8 L jars filled with river water from the collection site. These jars were then placed in a cooler with ice water to minimize metabolism. The mussels and clams were then transported back to the laboratory where they were processed.

Sampling of Tissues

Processing of the animals included measurement, sacrifice, and dissection. Each mussel and clam was measured for length (maximum distance parallel to hinge), depth

(maximum distance from dorsum to ventrum), and width (maximum distance from right valve to left valve). After taking these measurements, they were sacrificed and dissected. Rainbow mussels were dissected into nine tissue fractions: 3 pieces of foot tissue, 2 pieces of digestive gland, mantle, gut/stomach/gonad, adductor muscles, and remaining tissues (including gills, kidneys, and heart). Asian clams were dissected into three tissue fractions as follows: foot, adductor muscles, and body (including all other soft tissues). Tissue samples were placed in freezer-safe plastic 1.5 ml centrifuge tubes and were sealed. As each animal was dissected, the tissue samples were put on ice for no more than 20 min and were then placed in a -60°C freezer for storage.

Biochemical Assays

Six biochemical assays were performed on the field-deployed animals, and each assay is described in the following sections.

Glycogen Content

Glycogen content was performed by the enzymatic digestion method of Roehrig and Allred (1974). Tissue samples were homogenized at a 1:10 or 1:20 weight/volume ratio in 0.05M sodium phosphate buffer at pH 4.8 using a Biospec Tissue Tearor® hand-held homogenizer at setting 2-3 for 15-30 sec. This resulted in a sample that was well homogenized. Samples were then centrifuged at 1200 x g in a refrigerated floor centrifuge for 45 min. The supernatants were poured off for use to determine glycogen content, while the pellet was saved for dry weight determination. The pellets were dried in a 105°C drying oven for 48 hr and were weighed to determine the dry weight of the sample. Duplicate 100 µl aliquots of the supernatant were placed in tubes with 3.5 Units

of Amyloglucosidase (Sigma Chemical Co.) and vortexed for 3 sec. Another 100 ml aliquot of the supernatant was placed in a tube with the appropriate volume of pH 4.8 0.05M PO₄ buffer and vortexed. These mixtures were incubated in a 50°C water bath for 2 hr. After removal from the water bath, 4 ml of a mixture of peroxidase, glucose oxidase, and O-dianosidine was added to each of the incubated samples. The mixture was vortexed for 3 sec and then incubated in a 50°C water bath for 30 min. The samples were then removed from the water bath, allowed to cool, and absorbance was measured on a spectrophotometer (Milton Roy Model 520) at a wavelength of 450 nm.

The first incubation step allows amyloglucosidase to convert all of the glycogen in the sample to glucose while the sample with only buffer serves as a control. The final incubation of this procedure allows the O-dianosidine to bind with free glucose and form an absorbant complex in proportion to the amount of glucose present. Since the samples without amyloglucosidase only absorb proportional to the initial amount of glucose, and the samples with amyloglucosidase absorb proportional to the initial amount of glucose and the glycogen which has been broken down, the difference between the two values reflects the amount of glycogen in the samples. The actual amounts and concentrations of glycogen and glucose were quantified by the generation of a standard curve using *Mytilus edulis* glycogen (Sigma Chemical Co.), which were processed through both incubation steps. Concentrations were calculated as µg of glucose or glycogen per mg of tissue dry weight in the sample. The tissues used for this assay were mantle tissue for *V. iris* and foot tissue for *C. fluminea*. Mantle tissue is a major glycogen storage organ (Gabbott and Whittle, 1986). Asian clam foot tissue was used because the amount of

mantle tissue in each was inadequate for analysis.

RNA:DNA Ratio

The ratio of RNA to DNA was measured by a modification of the method of Heath et al. (1996). Foot tissue samples were used for both species in performing this assay. Approximately 50 mg samples of tissue were minced with a razor blade and then placed in a dounce homogenizer, where it was gently homogenized. Then 50 μ l of 1 M NaCl was added to the tissue, and the mixture was gently homogenized again. Another 50 μ l volume of 1 M NaCl was added, and the process was repeated. The sample was transferred to a 1.5 ml centrifuge tube and the homogenizing tube was rinsed with 1 ml of Tris/MgCl₂/CaCl₂. An aliquot of 10 μ l of proteinase K (Sigma Chemical Co.) solution was added to the sample, and it was gently mixed by inversion three times. The sample was incubated with the proteinase K for 1 hr at 37°C. The sample then was centrifuged at 16,000 x g for 15 min to precipitate large proteins and particulates, and the supernatants were decanted and stored in a -60°C freezer until analysis.

The amounts of RNA and DNA were determined using an ethidium bromide cocktail and fluorescence measurement. An aliquot of 200 μ l of each sample was mixed with 2 ml of cocktail (0.050 M Tris, 3.2 mM CaCl₂, 4 mM MgCl₂, 8 mg proteinase K per 100 ml, and 0.2 mg of ethidium bromide per 100 ml) and incubated for 1 hr at 25°C. The fluorescence of the sample at 328 nm excitation and 592 nm emission was recorded as the total nucleoside and nucleotide quantity in the sample. An aliquot of 5 μ l RNAase was added to each sample and incubated for 30 min at 25°C. Then, another fluorescence reading was taken to determine the amount of initial fluorescence due to RNA. This step

was followed by the addition of 10 μ l DNAase to the sample, followed by another fluorescence reading to determine the quantity of DNA in the sample. Amounts of RNA and DNA were quantified with changes in fluorescence through the regression of standard curves for both RNA and DNA.

Cellulolytic Enzyme Activity

Cellulolytic enzyme activity was determined using a modification of the method of Farris et al. (1998). This assay incorporates two separate determinations of the activity of this group of enzymes. One of the procedures measures the change in the viscosity of a solution of a suitable substrate for the enzymes. The other procedure measures the amount of substrate that is digested by the enzyme mixture.

Homogenization of tissues was carried out once for both procedures. Tissue samples were homogenized in 0.05 N sodium acetate buffer at pH 6.0, at a weight to volume ratio of 1:5. Whole visceral mass (except for foot) and mantle was used for *C. fluminea* samples, while samples of digestive gland tissue were used for *V. iris*. Tissues were homogenized using a Tissue Tearor at a setting of 2-3 for 15-30 sec. This effectively homogenized the samples. The samples were transferred from homogenizing tubes to pre-weighed 1.5 ml centrifuge tubes and were centrifuged at 11,000 x g for 20 min. The supernatants were decanted and stored in 1.5 ml tubes in a -60 °C freezer until analyzed. The pellets and tubes were placed in a drying oven at 105 °C for 48 hr to dry completely. These pellets then were removed and weighed to determine dry weights of the samples for use in calculations of enzyme activity normalized by weight.

The viscosity change procedure followed a modification of the method of Sinsabaugh et al (1980). The substrate solution consisted of 2.5% carboxymethylcellulose (CMC) (Sigma Chemical Co., low molecular wt.) in 0.05 N sodium acetate buffer at pH 6.0. A 200 μ l aliquot of thawed sample was mixed with 2 ml of substrate solution or buffer by vortexing for 3 sec. The sample/substrate and sample/buffer mixtures were incubated at 25 °C for 30 min. Viscosity measurements then were made on each sample. The measurement of viscosity consisted of determining the amount of time necessary for an aliquot of the mixture to drain from one designated mark to another in a standardized 1 ml graduated glass pipet. The “effluent time”, the time required for the fluid to drain from one mark to another in the pipet, for the unreacted substrate was determined by measuring the time immediately after a sample was added. After 30 min (for *C. fluminea*) or 60 min (for *V. iris*), effluent times and total elapsed times from the beginning of incubation were measured. Since the change in effluent time was determined to be an exponential decay with elapsed time, a regression based on the natural log of the elapsed time was calculated so that all viscosity measurements could be normalized to a standard incubation time. Different incubation times were used for the two species because the Asian clam samples decreased the viscosity of the solution more quickly than those of rainbow mussel samples.

The other procedure for this assay measured the amount of sugar released by the enzyme when it was incubated with CMC. This determination followed the method of Somogyi (1945). The above mixture of enzyme and CMC was allowed to incubate for 15 min (for *C. fluminea*) or 120 min (for *V. iris*). Incubation times for the two species were

different because the reaction rates differed. A 100 μ l aliquot of the mixture was added to 900 μ l of 0.10 M sodium phosphate buffer at pH 6.0. This was mixed with 1 ml of copper reagent (4 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 12 g sodium potassium tartrate, and 24 g of sodium carbonate dissolved in 40 ml of H_2O). This mixture was vortexed and incubated for 10 min at 100 °C. An aliquot of 500 μ l of color reagent (25 g $\text{NH}_4\text{MoO}_3 \cdot 4\text{H}_2\text{O}$ in 450 ml deionized water, 21 ml concentrated H_2SO_4 , and 3 g $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in water) was added to each sample, followed by vortexing. The absorbance of the samples was then read on a spectrophotometer at 520 nm. Calculation of sugar released was based on the generation of a standard curve using known concentrations of glucose. Each sample was measured in pairs, one with substrate and one without substrate, to account for any free glucose present in the sample that was not due to the action of the enzymes. This assay allowed the measurement of glucose quantities as low as 1 μ g per sample.

Phosphofructokinase Activity and Fructose-1,6-bisphosphatase Activity

The measurements of the activities of phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (F-1,6-bPase) were carried out by modifications of the methods of Brooks et al. (1991) and Storey (1982), respectively. Samples of foot tissue were used for these assays with both species. Samples of foot tissue from approximately 30 to 100 mg wet weight were homogenized in an imidazole buffer (1.702 g imidazole, 930 mg EDTA, 952 mg EGTA, 90 mg dithiothreitol, and 2.100 g sodium fluoride in 500 ml of distilled deionized water) at a ratio of 1:20-40 weight to volume, with a Tissue Tearor hand-held homogenizer at setting 2-3 for 15 sec. The homogenized mixture was centrifuged at 15,000 x g for 15 min and the supernatant was used for both assays.

Phosphofructokinase

The PFK assay was conducted by monitoring the decrease in absorbance of the sample over time. In a 1 ml cuvette (polystyrene with a 1 cm light path), 20-60 μl of sample, 20 μl of ATG, 10 μl of ATP, 15 μl of NADH, and enough reaction buffer (imidazole, magnesium chloride, and potassium chloride) to make 990 μl were gently mixed with a pasteur pipet. The absorbance at 340 nm was recorded every 30 sec for 3 min to determine a background reaction rate of the sample without substrate for the enzyme. Then, 10 μl of fructose-6-phosphate, the substrate for PFK, was added to the cuvette, and the mixture was again gently mixed with a pipet. The absorbance was recorded every 30 sec for 3 min to determine the reaction rate due to PFK. These rates were standardized by regression with known concentrations of PFK.

Fructose-1,6-bisphosphatase

F-1,6-bPase activity was determined by measuring the amount of inorganic phosphorus released by the enzyme during a fixed incubation time. For this assay, 100 μl of sample was placed in incubation buffer (50 mg MnCl_2 , 18.5 mg EDTA, 617 mg MgSO_4 , 3.305 g Tris-HCl, 485 mg Tris-base, dissolved in 500 ml H_2O , add 170 mg F-1,6-BP to half samples). Each sample was run in pairs, one aliquot in buffer without substrate and one in buffer with substrate to separate phosphorus released due to F-1,6-bPase activity from background phosphorus liberation. After incubating at 37 °C for 16-24 hr, a 250 μl aliquot from each sample was mixed with 1.0 ml of 5% TCA to stop the reaction. A 330 μl aliquot of this sample/TCA mixture was added to 1 ml of copper reagent (39 ml of acetic acid, 261 ml of distilled deionized water, 750 mg of

CuSO₄·5H₂O, and 13.8 g of sodium acetate) and 167 µl of 5 % ammonium molybdate, and was vortexed. Then, 167 µl of Enol solution (50 ml H₂O, 2.5 g Na₂SO₃, and 1.0 g of methylaminophenol) was added; the sample was vortexed again, and the absorbance of the mixture was measured at 870 nm on a spectrophotometer. A standard curve of phosphorus was generated using known concentrations of inorganic phosphorus in incubation buffer.

Acetylcholinesterase Activity

The activity of acetylcholinesterase was measured by the method of Hill and Fleming (1982). Adductor muscles of both species were used for this assay. The small size of the adductors in Asian clams required the use of tissues from two individuals. Adductor muscles were homogenized in 0.05 M Tris buffer (pH 8.0) at a weight to volume ratio of 1:10 using a Tissue Tearor at setting of 2-3 for 15 sec. The samples then were put in 1.5 ml tubes and centrifuged at 4,000 x g for 30 min. In a polystyrene disposable cuvette, 3.0 ml of DTNB solution (0.05 M Tris at pH 7.4, 99 mg/liter DTNB), 100 µl of acetylthiocholine iodide (45 mg/ml), and 20 µl of sample were mixed by inversion three times. The absorbance at 405 nm was recorded every 30 sec for 3 min. The slope of the increase in absorbance versus time was calculated, and the activity of acetylcholinesterase was determined by use of a standard curve generated by using known concentrations of acetylcholinesterase. Data were converted to mUnits (1 unit is the enzyme required to convert 1 µmole of acetylthiocholine per min) of activity per 1 mg of wet weight or per 1 mg protein.

Protein Content

Protein assays were performed using the Lowry method (Schleif and Wensink, 1981). The incubation buffer was mixed as follows: 0.10M sodium hydroxide with 3% sodium carbonate with 2 ml of 2% copper sulfate and 2 ml of 4% potassium tartrate added to make a final volume of 100 ml. A 2 ml aliquot of this solution was mixed with an appropriate amount of sample to produce an absorbance in the linear range (up to 100 µg of protein) and allowed to stand for 10 min. After this incubation, 100 µl of Folin:Ciocalto's reagent (diluted 1:1 with ddi water) was added to the samples and mixed. This mixture was incubated for 30 min at room temperature to allow color development. The absorbance of the samples was then measured at a wavelength of 650 nm. Calculation of protein concentration was based on a standard curve using bovine serum albumin solutions at known concentrations.

Statistical analyses

Statistical analyses were performed on the physiological parameters using ANOVA procedures. Parametric tests were used because normality tests of the data indicated that the sample distributions approximated normality. Comparisons were made among sites within a single experiment, among experiments, and between species. Additionally, interaction terms were used to determine whether the variation among sites was consistent among experiments (seasons). Best subsets regression analyses were conducted to choose parameters which would serve as suitable biomarkers. These analyses were performed on the data from each species for each trial. A compilation of the inclusion of each parameter in the "best regression" model and the best two parameter

model was made to determine which variables were most consistently useful as potential biomarkers. All statistical analyses were performed in Minitab version 10.5 (Minitab, Inc., State College, PA).

RESULTS

Six separate biochemical assays were performed on the tissues of both rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) which were held in cages at designated locations within the Clinch River and its tributaries. The variations in these parameters were evaluated for differences among sites and among seasons of the year.

During the fall (September-October) trial, mussels and clams were placed in cages at four Clinch River sites (hereafter referred to as Richlands, Below STP, Carbo, and Below CRSP, respectively, from upstream to downstream), Copper Creek, Stock Creek, and Little River. Mussels were recovered from Richlands, Below STP, Carbo, Below CRSP, Copper Creek, and Little River, while clams were recovered from Richlands, Below Raven/Doran STP, Carbo, Below CRSP, and Copper Creek. All the mussels in the cage at Stock Creek died within 30 days, and the clam cages were removed from the stream and emptied by vandals at both Stock Creek and Little River. The death of the mussels at Stock Creek was confirmed by the recovery of nine pairs of valves out of ten mussels placed in the cage. Only two mussels held at Little River were recovered, as the cage appeared to have been scoured by high flows; the substratum was partially washed out, and the rest of the mussels were lost. Mussels ranged in length from 34.1 to 61.1 mm with a mean of 43.3 mm. There was no difference in length among sites ($p=0.46$).

Lengths of Asian clams ranged from 10.6 to 26.0 mm, and clams Below CRSP were smaller than at other sites ($p=0.004$).

Mussels and clams were placed at Carbo, Below CRSP, Copper Creek, Stock Creek, and Little River during the spring (May-June) trial. No animals were deployed at Richlands or Below Raven/Doran STP due to high flows and very turbid water conditions for the entire time of the trial. Both mussels and clams were recovered from each of the deployment sites, except Copper Creek, where the mussel cage was scoured and the mussels were lost. Only two mussels were recovered from Stock Creek for the 30 day sampling time. Mussel lengths ranged from 29.7 to 55.3 mm with a mean of 40.0 mm. There was no statistical difference in lengths among sites ($p=0.184$). Asian clams ranged in length from 14.3 to 24.1 mm with a mean length of 18.4 mm. Clams at Carbo were statistically smaller than those at Stock Creek and Little River ($p=0.007$).

The summer (July-August) trial was initiated by placing animals in cages at Cedar Bluff, Richlands, Below Raven/Doran STP, Carbo, Below CRSP, Copper Creek, Stock Creek, and Little River. Recovery of experimental animals was accomplished at all sites except Stock Creek. The cages at Stock Creek were washed away during a high flow event which was strong enough to substantially shift the substratum in the vicinity of the cages. The range of lengths for mussels was 30.6-57.7 mm, with a mean of 41.8. Lengths were not different among sites ($p=0.492$). The range of lengths for clams was 14.1-23.8 mm, with a mean of 19.4 mm. Clams at Cedar Bluff were smaller than those at Richlands and Carbo. Measurements of clams Below CRSP, Copper Creek, and Little River were erroneous and not used.

All significance levels are $p < 0.05$ unless otherwise stated. Water quality parameters are given in table 4.

Glycogen Assay

Several parameters were measured in the course of determining the total amount of glycogen present in tissues. The amount of free glucose present in the tissue, the dry weight of the tissue, and the ratio of free glucose to glycogen were determined for each sample processed. Different tissues were used for mussels and clams due to size constraints. Analyses were performed on the entire mantle tissue of mussels, while whole foot tissues of clams were used for this assay. Data were not corrected for size or sex because regression and ANOVA analyses indicated that these factors did not significantly influence glycogen or glucose values ($p = 0.12 - 0.91$). The glucose content during the fall experiment was the exception, with the regression coefficient of length being statistically different from zero ($p = 0.021$), but the low R^2 value (0.172) indicated that the relationship was weak. In addition, when glucose content was compared to tissue dry weight, there was no relationship ($p = 0.498$). Direct comparisons of the actual carbohydrate contents of clams and mussels were not performed because the assays used different tissues for the two species as explained in methods, and glycogen content is generally lower in foot tissue than mantle (Zandee et al., 1980; Naimo and Monroe, unpublished data).

Glycogen Content

Glycogen levels (mg of glycogen/g dry tissue weight) during the fall trial were not significantly different among sites for either mussels or clams (Table 5). The mean values for mussels ranged from 15.2 mg/g at Little River to 168.9 mg/g at Below

Table 4. Water quality parameters at cage sites. Parameters are listed as: value at start of experiment, value at end. Asterisks indicate data that are unavailable. Chlorine was also measured; free chlorine was always below detection, and total chlorine never exceeded 17 µg/L.

Site	Fall			Spring		Summer	
	Temp. (C)	pH	DO	Temp. (C)	DO	Temp. (C)	pH
Cedar Bluff						24.0, 27.0	8.3, *
Richlands	*, 15.0	*, 7.9	*, 9.6			24.0, 22.0	8.0, *
Below STP	*, 15.0	*, 7.7	*, 9.3			*, 21.0	7.9, *
Carbo	*, 14.0	*, 8.0	*, 10.0	13.5, *	10.0, *	26.0, *	8.1, *
Below CRSP	*, 19.0	*, *	*, 8.1	13.5, *	6.2, *	26.0, 23.0	8.2, 8.4
Copper Creek	*, 17.0	*, 8.2	*, 9.7	15.0, *	11.4, *	*, 22.5	*, 8.2
Stock Creek	*, 15.5	*, 8.4	*, 10.3	13.5, *	9.6, *	21.0, *	6.7, *
Little River	*, 15.0	*, 7.4	*, 9.9	15.0, *	9.8, *	25.0, 22.0	8.2, 8.5

Table 5. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the fall experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	Glucose (mg/g wet wt)	Glycogen (mg/g wet wt)	Glucose:Glycogen	RNA:DNA
Richlands				
<i>Villosa iris</i>	45.7±8.2 (5)	102.5±55.3 (5)	1.364±0.739 (5)	0.63 (1)
<i>Corbicula fluminea</i>	7.8±3.8 (6)	121.5±14.0 (6)	0.058±0.027 (6)	^{ab,c} 0.72±0.04 (6)
Below STP				
<i>Villosa iris</i>	58.9±13.8 (5)	168.9±45.4 (5)	0.841±0.506 (5)	---
<i>Corbicula fluminea</i>	9.4±3.3 (6)	72.4±19.6 (6)	0.168±0.069 (6)	^{b,c} 0.78±0.07 (6)
Carbo				
<i>Villosa iris</i>	49.1±8.9 (6)	129.2±41.0 (6)	0.711±0.287 (6)	1.21 (1)
<i>Corbicula fluminea</i>	10.56±3.38 (6)	126.5±14.4 (6)	0.111±0.058 (6)	^c 0.89±0.05 (6)
Below CRSP				
<i>Villosa iris</i>	56.7±9.8 (6)	136.4±43.0 (6)	0.582±0.165 (6)	1.85±0.49 (5)
<i>Corbicula fluminea</i>	25.1±8.3 (6)	173.0±60.9 (6)	0.206±0.048 (6)	^a 0.60±0.08 (6)
Copper Creek				
<i>Villosa iris</i>	38.6±5.4 (5)	68.4±23.2 (5)	1.251±0.560 (5)	3.28±2.50 (5)
<i>Corbicula fluminea</i>	13.8±3.6 (6)	70.2±13.9 (6)	0.216±0.046 (6)	^{ab} 0.66±0.04 (6)
Little River				
<i>Villosa iris</i>	22.6±10.2 (2)	15.2±5.5 (2)	1.427±0.158 (2)	---
<i>Corbicula fluminea</i>	---	---	---	---

Table 5 continued. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the fall experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha<0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	PFK activity (mU/mg wet wt)	FBPase activity (mU/mg wet wt)	ACHase activity (mU/mg wet wt)	Water content of digestive gland or visceral mass (%)
Richland:				
<i>Villosa iris</i>	4.02±0.54 (5)	173±9 (5)	10.9±3.8 (5)	92.7±0.4 (5)
<i>Corbicula fluminea</i> Below STP	^{ab} 2.43±0.46 (6)	* ^{ab} 2830±83 (6)	19.2±2.1 (6)	*89.4±0.6 (6)
<i>Villosa iris</i>	5.33±0.54 (6)	160±27 (5)	5.6±2.2 (5)	93.5±0.8 (5)
<i>Corbicula fluminea</i> Carbo	* ^a 2.76±0.46 (6)	* ^b 2960±98 (6)	*20.1±1.0 (6)	*89.3±0.6 (6)
<i>Villosa iris</i>	6.50±1.23 (7)	145±14 (6)	8.8±2.7 (6)	93.2±0.2 (7)
<i>Corbicula fluminea</i> Below CRSP	* ^{bc} 1.38±0.64 (6)	* ^{ac} 2500±170 (6)	16.9±2.9 (6)	*89.1±1.0 (6)
<i>Villosa iris</i>	6.90±1.89 (7)	145±18 (7)	9.0±3.0 (7)	92.3±0.5 (7)
<i>Corbicula fluminea</i> Copper Creek	* ^{abc} 1.78±0.60 (6)	* ^c 3300±157 (6)	17.5±2.4 (6)	*89.3±0.5 (6)
<i>Villosa iris</i>	5.73±0.45 (5)	154±25 (5)	12.1±2.7 (4)	91.2±0.6 (5)
<i>Corbicula fluminea</i> Little River	* ^c 0.85±0.11 (6)	* ^{ab} 2670±103 (6)	20.5±2.3 (6)	89.9±0.7 (6)
<i>Villosa iris</i>	3.61±0.50 (2)	125±24 (2)	13.4±4.8 (2)	91.6±0.4 (2)
<i>Corbicula fluminea</i>	---	---	---	---

Table 5 continued. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the fall experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). An asterisk (*) subscript marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	Change in effluent time of CMC sol'n (s)	Sugar released from CMC sol'n (mg/g dry wt/hr)	Endocellulase x exocellulase activity ($\times 10^5$)	Endocellulase/ exocellulase activity ($\times 10^4$)
Richlands				
<i>Villosa iris</i>	^{a,b} 18.3±1.7 (5)	7.80±1.41 (5)	8.90±1.98 (5)	77.86±9.30 (5)
<i>Corbicula fluminea</i>	*6.65±0.65 (6)	7.73±1.24 (6)	11.96±3.32 (6)	65.97±2.41 (6)
Below STP				
<i>Villosa iris</i>	^a 21.3±2.3 (5)	8.03±1.01 (5)	9.58±1.21 (5)	76.05±10.72 (5)
<i>Corbicula fluminea</i>	*7.17±0.34 (6)	7.56±1.69 (6)	12.02±2.88 (6)	58.65±12.31 (6)
Carbo				
<i>Villosa iris</i>	^a 21.3±0.7 (7)	9.19±1.13 (7)	11.50±1.67 (7)	81.29±8.62 (7)
<i>Corbicula fluminea</i>	*9.44±1.69 (6)	11.3±2.25 (6)	24.32±8.36 (6)	71.90±3.25 (6)
Below CRSP				
<i>Villosa iris</i>	^b 15.1±2.1 (7)	6.26±0.94 (7)	6.70±1.45 (7)	69.61±5.51 (7)
<i>Corbicula fluminea</i>	*7.92±1.16 (6)	8.00±1.70 (6)	13.66±4.80 (6)	64.10±3.75 (6)
Copper Creek				
<i>Villosa iris</i>	^b 15.4±1.4 (5)	6.44±0.88 (5)	6.56±1.19 (5)	71.57±6.08 (5)
<i>Corbicula fluminea</i>	*6.70±0.66 (6)	7.15±1.19 (6)	10.67±2.91 (6)	62.78±3.27 (6)
Little River				
<i>Villosa iris</i>	^b 14.2±1.8 (2)	5.09±2.07 (2)	4.78±2.57 (2)	61.13±15.63 (2)
<i>Corbicula fluminea</i>	---	---	---	---

Raven/Doran STP. For clams, the values ranged from 70.2 mg/g to 173.0 mg/g, with Copper Creek and Below Raven/Doran STP having the lowest values and Below CRSP having the highest value. However, the glycogen level differences among the sites were not statistically significant, and the two-way ANOVA results do not indicate a significant interaction between species and sites. The results do not provide strong evidence of a response difference between the two species.

ANOVA analysis revealed that there were differences in glycogen levels ($p < 0.05$) among sites during the spring trial for mussels (Table 6). However, no differences were detected for clams (Table 6). At sites Little River and Carbo, mussels had higher levels of glycogen in mantle tissue than mussels at sites Below CRSP and Stock Creek, as well as those at the beginning of the trial based on a multiple comparisons test using Fisher's protected LSD method. The mussels deployed at the source population site (Little River) and Carbo were able to store more energy than those at the sites downstream of the CRSP (Below CRSP) and in Stock Creek, indicating that the nutrient supply was significantly better or environmental stress levels were significantly lower for mussels at Carbo and Little River. Coincidentally, Carbo and Little River were also the sites with the highest numbers of native mussels during preliminary surveys. The lower glycogen levels in Stock Creek may have been due to the lower water temperatures present in this stream. The interaction term between species and sites in the two-way ANOVA analysis indicated that the two species had different glycogen levels content among sites. Asian clams exhibited much less variation in the levels of glycogen among sites than did rainbow mussels. The mean glycogen levels in mussels held at site Carbo and Little River were approximately double those at the other sites. In contrast, the differences in

Table 6. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the spring experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	Glucose (mg/g wet wt)	Glycogen (mg/g wet wt)	Glucose:Glycogen	RNA:DNA
Beginning of experiment				
<i>Villosa iris</i>	^b 25.1±5.6 (8)	^b 110.5±39.9 (8)	0.349±0.100 (8)	4.84±1.47 (8)
<i>Corbicula fluminea</i>	---	---	---	*1.12±0.07 (6)
Carbo				
<i>Villosa iris</i>	^a 46.8±5.0 (9)	^a 245.4±44.1 (9)	0.259±0.055 (9)	1.08±0.36 (9)
<i>Corbicula fluminea</i>	^a 7.77±0.81 (6)	116.8±7.9 (6)	0.068±0.008 (6)	0.80±0.04 (6)
Below CRSP				
<i>Villosa iris</i>	^b 30.8±3.9 (8)	^b 110.2±17.2 (8)	0.315±0.049 (8)	1.17±0.49 (8)
<i>Corbicula fluminea</i>	^{ab} 12.5±1.7 (6)	107.3±5.4 (6)	0.118±0.018 (6)	0.90±0.06 (6)
Copper Creek				
<i>Villosa iris</i>	---	---	---	---
<i>Corbicula fluminea</i>	^a 25.1±8.3 (6)	111.8±7.2 (6)	0.094±0.007 (6)	1.00±0.08 (6)
Stock Creek				
<i>Villosa iris</i>	^b 14.0±5.0 (7)	^{ab} 104.4±29.4 (7)	0.240±0.107 (7)	2.21±0.69 (7)
<i>Corbicula fluminea</i>	^a 8.0±1.1 (6)	98.4±15.4 (6)	0.090±0.013 (6)	*0.88±0.08 (5)
Little River				
<i>Villosa iris</i>	^a 49.9±4.3 (7)	^a 236.6±22.8 (7)	0.216±0.016 (7)	2.24±0.54 (7)
<i>Corbicula fluminea</i>	^b 19.5±5.8 (6)	125.7±15.6 (6)	0.156±0.046 (6)	*0.79±0.10 (6)

Table 6 continued. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the spring experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	PFK activity (mU/mg wet wt)	FBPase activity (mU/mg wet wt)	AChEase activity (mU/mg wet wt)	Water content of digestive gland or visceral mass (%)
Beginning of experiment				
<i>Villosa iris</i>	27.13±3.55 (8)	8.3±1.2 (8)	0.401±0.092 (8)	89.6±0.5 (8)
<i>Corbicula fluminea</i>	*0.047±0.047 (6)	*2593±602 (6)	* _a 23.02±0.94 (6)	*92.2±0.2 (6)
Carbo				
<i>Villosa iris</i>	29.23±0.79 (9)	9.3±0.6 (9)	0.503±0.073 (9)	89.6±1.2 (9)
<i>Corbicula fluminea</i>	* _{ab} 2.098±1.894 (6)	*2200±134 (6)	* _a 20.7±0.8 (6)	90.4±0.8 (6)
Below CRSP				
<i>Villosa iris</i>	28.12±1.79 (8)	9.6±0.6 (8)	0.347±0.114 (8)	90.8±0.4 (8)
<i>Corbicula fluminea</i>	* _{ab} 2.098±1.894 (6)	*2441±506 (6)	* _a 17.7±0.3 (6)	*92.4±0.3 (6)
Copper Creek				
<i>Villosa iris</i>	---	---	---	---
<i>Corbicula fluminea</i>	_b 5.030±1.809 (6)	2050±171 (6)	_b 51.7±8.0 (6)	92.2±0.2 (6)
Stock Creek				
<i>Villosa iris</i>	25.22±3.61 (7)	8.3±1.4 (7)	1.117±0.788 (7)	89.3±1.1 (7)
<i>Corbicula fluminea</i>	* _{ab} 0.373±0.285 (6)	*2434±513 (6)	* _a 21.4±1.4 (6)	89.8±0.8 (6)
Little River				
<i>Villosa iris</i>	27.01±2.03 (7)	8.4±0.5	0.474±0.106 (7)	90.8±0.4 (7)
<i>Corbicula fluminea</i>	* _{ab} 0.352±0.253 (6)	*2677±679 (6)	* _a 23.0±0.9 (6)	92.0±0.6 (6)

Table 6 continued. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the spring experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	Change in effluent time of CMC sol'n (s)	Sugar released from CMC sol'n (mg/g dry wt/hr)	Endocellulase x exocellulase activity ($\times 10^5$)	Endocellulase/ exocellulase activity ($\times 10^{-4}$)
Beginning of experiment				
<i>Villosa iris</i>	^{ab} 6.4±0.8 (8)	^b 4.11±0.77 (8)	^a 6.14±1.99 (8)	^b 137.0±10.6 (8)
<i>Corbicula fluminea</i>	^a 7.9±1.3 (6)	* ^a 9.43±1.42 (6)	^a 9.83±3.44 (6)	130.3±12.5 (6)
Carbo				
<i>Villosa iris</i>	^{ab} 7.1±0.6 (9)	^a 7.15±0.85 (9)	^{ab} 12.73±2.33 (9)	^a 188.5±6.4 (9)
<i>Corbicula fluminea</i>	^{ab} 8.60±1.11 (6)	^a 8.91±1.09 (6)	^a 9.74±2.51 (6)	* ^a 108.9±4.3 (6)
Below CRSP				
<i>Villosa iris</i>	* ^{bc} 7.5±0.8 (8)	* ^a 7.46±0.90 (8)	^{ab} 13.53±2.84 (8)	^a 203.0±10.9 (8)
<i>Corbicula fluminea</i>	^{bcd} 11.91±0.71 (6)	^{abc} 11.91±0.71 (6)	^{ab} 18.87±2.27 (6)	* ^a 106.3±2.4 (6)
Copper Creek				
<i>Villosa iris</i>	---	---	---	---
<i>Corbicula fluminea</i>	^{cd} 12.5±0.5 (6)	^{bc} 13.61±1.01 (6)	^{ab} 21.85±2.78 (6)	104.1±3.0 (6)
Stock Creek				
<i>Villosa iris</i>	^a 4.8±0.8 (7)	^b 3.19±0.61 (7)	^a 3.7±1.22 (7)	^{abc} 142.9±8.9 (7)
<i>Corbicula fluminea</i>	^{abc} 8.3±2.2 (6)	* ^{ab} 9.63±2.16 (6)	^a 14.06±5.22 (6)	107.7±8.7 (6)
Little River				
<i>Villosa iris</i>	^c 9.5±1.0 (7)	* ^a 9.07±0.88 (7)	^b 21.88±4.27 (7)	^{ac} 175.1±4.4 (7)
<i>Corbicula fluminea</i>	^d 14.7±1.3 (6)	^c 16.04±1.55 (6)	^b 33.95±6.13 (6)	* ^a 96.6±2.5 (6)

mean glycogen content among sites for clams varied by only 27% from the highest to lowest mean value. Therefore, Asian clams appeared to be capable of obtaining enough nutrients at all sites to store similar amounts of energy, while mussels were able to store more energy at two sites than at the others. The glycogen content in mussels from Below CRSP, Copper Creek, and the beginning of the experiment were within 15% of the overall mean glycogen content measured during the fall trial. The glycogen contents in Asian clams during this trial also were approximately equal to those measured in the fall. The fact that Asian clams were capable of maintaining energy reserves at all sites provided further evidence that their energy acquisition was not limited by the conditions at the sites chosen.

The summer trial showed one marginal difference among treatments for mussels (Table 7). The animals analyzed at the start of the experiment had higher glycogen levels than all of those at the end of the experiment ($P < 0.10$). The mean glycogen content for animals at the beginning of the experiment was 355.6 mg/g compared to 139.8-271.3 mg/g for samples from the field sites. The clams exhibited differences ($p < 0.05$) among treatments for this trial (Table 7). Clams deployed at Copper Creek had lower glycogen levels (117.0 mg/g) than those at Little River, Carbo, and those sacrificed at the beginning of the experiment (151.7, 163.4, and 168.8 mg/g respectively). The mean glycogen content of the clams at the beginning of the trial was significantly higher than those of clams held in cages at Copper Creek, Richlands, and Below CRSP. The remaining sites were not significantly different. There was no difference in the way glycogen levels of the two species varied among sites. Both species tended to have a higher mean glycogen content during this trial than in the spring or fall trials. However,

Table 7. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the summer experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	Glucose (mg/g wet wt)	Glycogen (mg/g wet wt)	Glucose:Glycogen	RNA:DNA
Beginning of experiment				
<i>Villosa iris</i>	^c 71.9±5.9 (8)	^b 355.6±32.5 (8)	0.214±0.026 (8)	0.92±0.31 (8)
<i>Corbicula fluminea</i>	^a 8.95±1.35 (6)	^c 168.8±17.4 (6)	^a 0.052±0.004 (6)	0.49±0.04 (6)
Cedar Bluff				
<i>Villosa iris</i>	^{ab} 53.5±4.8 (8)	^a 195.4±46.4 (8)	0.428±0.107 (8)	1.36±0.39 (8)
<i>Corbicula fluminea</i>	^{abc} 12.87±1.16 (6)	^{abc} 144.0±11.2 (6)	^{bc} 0.091±0.008 (6)	0.545±0.052 (6)
Richlands				
<i>Villosa iris</i>	^{abc} 57.6±18.1 (3)	^a 180.9±63.9 (3)	0.326±0.011 (3)	0.63±0.20 (3)
<i>Corbicula fluminea</i>	^{bc} 14.51±1.60 (6)	^{ab} 126.0±4.3 (6)	^{cd} 0.114±0.010 (6)	0.76±0.10 (6)
Below STP				
<i>Villosa iris</i>	^{bc} 58.5±6.7 (7)	^{ab} 270.3±32.4 (7)	0.231±0.031 (7)	1.74±0.67 (7)
<i>Corbicula fluminea</i>	^{abc} 12.8±2.0 (6)	^{abc} 139.7±9.2 (6)	^{bc} 0.090±0.009 (6)	0.59±0.12 (6)
Carbo				
<i>Villosa iris</i>	^a 35.8±1.1 (5)	^a 190.0±47.5 (5)	0.255±0.071 (5)	0.48±0.05 (5)
<i>Corbicula fluminea</i>	^{ac} 11.5±2.4 (6)	^{bc} 163.4±9.8 (6)	^{ab} 0.069±0.010 (6)	0.48±0.06 (6)
Below CRSP				
<i>Villosa iris</i>	^a 39.65±4.90 (8)	^a 190.5±53.4 (8)	0.465±0.173 (8)	0.78±0.15 (8)
<i>Corbicula fluminea</i>	^{abc} 13.63±2.20 (6)	^{ab} 131.7±3.6 (6)	^{bcd} 0.103±0.016 (6)	0.62±0.11 (6)
Copper Creek				
<i>Villosa iris</i>	^{ab} 44.4±9.4 (4)	^a 139.8±64.2 (4)	0.722±0.301 (4)	0.30±0.05 (4)
<i>Corbicula fluminea</i>	^a 8.75±0.99 (6)	^a 117.0±8.5 (6)	^{ab} 0.075±0.007 (6)	0.59±0.11 (6)
Little River				
<i>Villosa iris</i>	^{ab} 51.1±8.5 (7)	^{ab} 271.3±65.7 (7)	0.228±0.037 (7)	2.58±0.62 (7)
<i>Corbicula fluminea</i>	^h 17.9±2.5 (6)	^{bc} 151.7±19.9 (6)	^g 0.128±0.026 (6)	*0.61±0.08 (6)

Table 7 continued. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the spring experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha < 0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	PFK activity (mU/mg wet wt)	FBPase activity (mU/mg wet wt)	AChEase activity (mU/mg wet wt)	Water content of digestive gland or visceral mass (%)
Beginning of experiment	---			
<i>Villosa iris</i>		129±17 (8)	11.9±2.5 (8)	89.5±0.3 (8)
<i>Corbicula fluminea</i>	^a 0.33±0.06 (6)	* _a 1261±122 (6)	14.1±1.2 (6)	---
Cedar Bluff				
<i>Villosa iris</i>	^b 115.62±6.64 (8)	117±7 (8)	13.9±2.1 (8)	90.8±0.3 (8)
<i>Corbicula fluminea</i>	* _a 0.59±0.11 (6)	* _{ab} 1446±40 (6)	20.6±0.4 (6)	89.9±0.6 (6)
Richlands				
<i>Villosa iris</i>	^{ab} 95.76±0.16 (3)	127±11 (3)	12.2±3.5 (3)	91.5±0.7 (3)
<i>Corbicula fluminea</i>	* _{ab} 1.34±0.33 (6)	* _c 1957±87 (6)	19.2±0.7 (6)	88.8±0.5 (6)
Below STP				
<i>Villosa iris</i>	^a 74.05±9.16 (7)	119±13 (7)	11.9±1.4 (7)	91.3±0.4 (7)
<i>Corbicula fluminea</i>	* _b 1.96±0.44 (6)	* _c 1987±129 (6)	21.8±1.4 (6)	90.4±0.4 (6)
Carbo				
<i>Villosa iris</i>	^b 117.31±11.70 (5)	114±19 (5)	14.3±3.4 (5)	90.5±0.2 (5)
<i>Corbicula fluminea</i>	* _b 2.26±0.65 (6)	* _c 1989±74 (6)	17.62±0.94 (6)	89.8±0.5 (6)
Below CRSP				
<i>Villosa iris</i>	^a 86.77±10.60 (8)	99±9 (8)	10.5±1.9 (8)	90.8±0.3 (8)
<i>Corbicula fluminea</i>	* _{ab} 1.40±0.42 (6)	* _{bc} 1723±99 (6)	15.3±0.9 (6)	91.8±0.3 (6)
Copper Creek				
<i>Villosa iris</i>	^{ab} 100.14±11.36 (4)	102±5 (4)	8.9±2.4 (4)	91.2±0.8 (4)
<i>Corbicula fluminea</i>	* _a 0.79±0.32 (6)	* _{ab} 1529±127 (6)	13.7±1.4 (6)	92.2±0.2 (6)
Little River				
<i>Villosa iris</i>	^{ab} 91.52±11.62 (7)	109±6 (7)	10.6±1.5 (7)	90.8±0.4 (7)
<i>Corbicula fluminea</i>	* _{ab} 1.17±0.39 (6)	* _{ab} 1509±144 (6)	20.5±2.3 (6)	91.7±0.4 (6)

Table 7 continued. Biochemical parameter values for rainbow mussels (*Villosa iris*) and Asian clams (*Corbicula fluminea*) during the spring experiment. Values are given as mean±SE (n). Means within a column that share the same letter superscript are not statistically different ($\alpha<0.05$). An asterisk (*) marks differences between species. Columns with no letter superscripts did not exhibit statistical differences among sites.

Site	Change in effluent time of CMC sol'n (s)	Sugar released from CMC sol'n (mg/g dry w/hr)	Endocellulase x exocellulase activity ($\times 10^5$)	Endocellulase/ exocellulase activity ($\times 10^{-4}$)
Beginning of experiment				
<i>Villosa iris</i>	^a 5.8±0.6 (8)	^a 4.04±0.53 (8)	^a 5.48±1.17 (8)	^a 136.7±7.8 (8)
<i>Corbicula fluminea</i>	---	---	---	---
Cedar Bluff				
<i>Villosa iris</i>	^{cd} 8.4±0.4 (8)	^c 9.14±0.84 (8)	^b 19.20±2.64 (8)	^c 196.6±10.1 (8)
<i>Corbicula fluminea</i>	^a 17.5±1.0 (6)	7.25±0.80 (6)	14.73±2.51 (6)	^b 44.2±2.8 (6)
Richlands				
<i>Villosa iris</i>	^d 10.3±1.2 (3)	^c 11.13±0.72 (3)	^b 26.54±3.78 (3)	^c 209.0±8.7 (3)
<i>Corbicula fluminea</i>	^a 15.8±0.8 (6)	[*] 6.24±0.73 (6)	[*] 11.12±1.80 (6)	^b 43.1±3.4 (6)
Below STP				
<i>Villosa iris</i>	^{bc} 7.4±0.5 (7)	^b 5.96±0.55 (7)	^a 10.32±1.61 (7)	^{ab} 156.4±7.8 (7)
<i>Corbicula fluminea</i>	[*] 18.5±0.9 (6)	7.71±0.67 (6)	16.46±2.17 (6)	^b 44.4±2.8 (6)
Carbo				
<i>Villosa iris</i>	^{abc} 7.1±0.2 (5)	^b 6.50±0.61 (5)	^a 10.76±1.49 (5)	^{abc} 174.4±8.9 (5)
<i>Corbicula fluminea</i>	^{ab} 18.4±2.1 (6)	4.82±0.82 (6)	9.20±2.28 (6)	^a 31.6±4.0 (6)
Below CRSP				
<i>Villosa iris</i>	^{ab} 6.2±0.2 (8)	^{ab} 5.40±0.40 (8)	^a 7.53±0.88 (8)	^{bc} 173.7±5.5 (8)
<i>Corbicula fluminea</i>	[*] 25.7±4.3 (6)	10.48±2.34 (6)	31.18±13.77 (6)	^b 49.5±3.2 (6)
Copper Creek				
<i>Villosa iris</i>	^{cd} 8.7±1.7 (4)	^b 6.62±0.65 (4)	^{ab} 13.18±3.36 (4)	^{abc} 163.4±21.7 (4)
<i>Corbicula fluminea</i>	[*] 35.3±4.8 (6)	8.41±1.84 (6)	34.52±10.89 (6)	^a 27.1±2.6 (6)
Little River				
<i>Villosa iris</i>	^{abc} 7.2±0.4 (7)	^b 5.91±0.43 (7)	^a 9.95±1.32 (7)	^{ab} 157.8±5.6 (7)
<i>Corbicula fluminea</i>	^{ab} 20.5±1.5 (6)	[*] 9.50±1.16 (6)	21.89±5.40 (6)	^b 52.5±1.2 (6)

the samples sacrificed at the beginning of the experiment had the highest mean glycogen content of all treatments, including those animals held in cages at the source site (Little River), indicating that the energy reserves of bivalves may have been declining during this trial in response to seasonal cycles.

The overall pattern of variation in glycogen content indicated that glycogen changes in a predictable fashion among seasons in mussels. Mussels exhibited the highest glycogen levels during the summer trial and increasing levels during the spring trial (Figure 12). Asian clams were much more consistent in glycogen levels among sites and seasons than mussels were (Figure 13), indicating that the clams are more readily adapted to extracting nutrients from a wide range of environments or have lower metabolic energy requirements. The variability among sites exhibited by mussels suggested that habitat characteristics influenced the energy reserves that mussels could maintain or build. From this standpoint, Asian clams appear to have a physiological advantage over rainbow mussels.

Glucose Content

No difference in free glucose content was determined among sites in either mussel mantle tissue or clam foot tissue during the fall trial (Table 5). However, the levels of free glucose were different for the two species. The mussels exhibited higher mean values for glucose content than did Asian clams. Mussels had from 2-7 times more free glucose in mantle tissue than clams had in foot tissue. Glucose is used for the immediate energy needs of the organism in mollusks (de Zwaan, 1979). Therefore, the lower free glucose content in clams indicated that clams have lower metabolic demands than

mussels. The lack of differences among sites for both mussels and clams indicated that the energetic burdens on these animals were similar among sites during this season.

Both mussels and clams exhibited significantly different ($p < 0.05$) levels of free glucose among sites during the spring trial (Table 6). Mussels deployed at Carbo (46.8 mg/g) and Little River (49.9 mg/g) had higher glucose levels than those Below CRSP (30.8 mg/g), Stock Creek, and at the beginning of the experiment (25.1 mg/g). The mussels held at Stock Creek had the lowest glucose levels at both 8 and 30 days (14.0 and 9.8 mg/g, respectively). Clams at Little River had higher levels of glucose (19.5 mg/g) than those at Carbo (7.8 mg/g), Stock Creek (8.0 mg/g), and Copper Creek (10.5 mg/g). Clams held at Carbo and Stock Creek had the lowest glucose levels during this trial. The mean levels of glucose were lower in clams than in mussels again during the spring sampling. Two-way ANOVA analysis revealed that the patterns of variation among sites were different for the two species. The highest glucose levels for mussels occurred at Carbo, the site upstream of the CRSP, and Little River, the source population site. These sites are also the two sites with the best populations of native mussels, including specimens of all age groups. Free glucose has been reported to fluctuate in the haemolymph of marine mussels, being highest during the summer and fall when food resources are greatest (Livingstone and Clarke, 1983). The higher levels of glucose in the animals at Carbo and Little River could be a response to better nutritional conditions earlier in the spring. However, the glucose levels in rainbow mussels during the fall were around 40 mg/g, which was comparable to the higher levels during the spring trial, and over twice the levels of the Stock Creek site during the spring. The lower free glucose

levels during the spring may signal a reduction in metabolic activity or a shift to dependence on glycogen as the major source of glucose (Livingstone and Clarke, 1983). Asian clams exhibited the highest levels of free glucose at Little River, but the levels at Carbo were lower than those Below CRSP, although this difference was not significant. Contrary to the response of the mussels, Carbo had the lowest glucose levels of any site for the clams. The higher glucose levels at Little River indicated that the clams were either able to obtain better nutrition there than at the other locations, or they were more active metabolically.

During the summer trial, differences ($p < 0.05$) in free glucose among sites were detected for both mussels and clams (Table 7). Mussels sacrificed at the beginning of the trial had higher levels of free glucose (71.9 mg/g dry wt.) than mussels held in cages at all sites except Richlands (57.6 mg/g dry wt.) and Below Raven/Doran STP (58.5 mg/g dry wt.). The levels for mussels held at Carbo (35.4 mg/g dry wt.) and Below CRSP (39.7 mg/g dry wt.) were significantly lower than those held Below Raven/Doran STP. Clams held at Little River (17.9 mg/g dry wt.) and Richlands (14.5 mg/g dry wt.) had significantly higher levels of glucose than those held at Copper Creek (8.8 mg/g dry wt.) and those sacrificed at the initiation of the trial (9.0 mg/g dry wt.). The mean value for clams held at Little River was also significantly higher than that for clams held at Carbo. The pattern of fluctuation exhibited by the two species was quite different. Mussels showed an increasing glucose content in the order Carbo, Below CRSP, Copper Creek, Little River, and the beginning samples. Glucose levels of the clams, on the other hand, were lowest at Copper Creek and the beginning samples increasing from Carbo to Below

CRSP to Little River. The overall trend of lower glucose levels in mussels held in the cages compared to those at the beginning of the trial suggested that there was a seasonal shift in the nutritional quality or quantity of the available food items, which was less pronounced in the more headwater reaches of the basin (Clinch River sites around Richlands (Cedar Bluff, Richlands, and Below Raven/Doran STP) and Little River). The clams seemed to exhibit the opposite response, obtaining better nutrition, and higher glucose levels, in the caged animals than at the beginning of the trial.

Glucose:Glycogen Ratio

The ratio of glucose to glycogen was calculated to estimate the amount of available carbohydrate energy in a form ready for immediate use. The only significant differences detected in this ratio were for clams in the summer trial (Tables 5-7). During the summer trial, clams deployed at Richlands (0.114) and Little River (0.128) had significantly higher values for this ratio than clams held at Carbo (0.069), Copper Creek (0.075), and the clams sacrificed at the beginning of the trial (0.052). The ratio for the Little River samples also was significantly higher than that of animals held at Cedar Bluff (0.091) and Below Raven/Doran STP (0.090). Clams held at Little River had both high glucose and high glycogen levels, indicating that the site provided these animals with adequate nutrition, but elevated metabolic activity or stress. Clams from the Richlands site had high glucose levels and low glycogen levels. Such a response might be explained by a metabolically taxing habitat with plentiful food supplies. Such a situation would allow the clams to assimilate adequate nutrients, and hence glucose, but not allow the animal to store much of the energy. Rainbow mussels consistently had higher ratios

of glucose to glycogen than did Asian clams during all seasons, indicating that mussels have higher Krebs's cycle metabolic demands (de Zwaan, 1979).

Seasonal Comparisons

Two-way ANOVA analyses revealed that season influenced all of the above parameters. Glucose content was significantly different among seasons for *V. iris* but not for *C. fluminea* (Figures 10 & 11). Glycogen content was also significantly different among seasons for *V. iris* and for *C. fluminea* (Figures 12 & 13). Glycogen content was higher during the summer than in the fall for *V. iris*, while the spring values were generally intermediate. A similar pattern also was exhibited by *C. fluminea*, except that the values for the fall experiment were more highly variable among sites. Differences in available food resources among the seasons are a possible cause for these fluctuations. The ratio of glucose to glycogen also was different among seasons for both species, with the values from the fall experiment being higher than those during the other seasons (Figures 14 & 15). The higher ratios during fall reflect the increased utilization of glycogen energy reserves to provide glucose for metabolic activity. The glucose levels for rainbow mussels among the seasons did not form a predictable seasonal pattern, and there was no difference among seasons exhibited by the clams. Therefore, the data indicate that the levels of free glucose are maintained more consistently throughout the year than glycogen content.

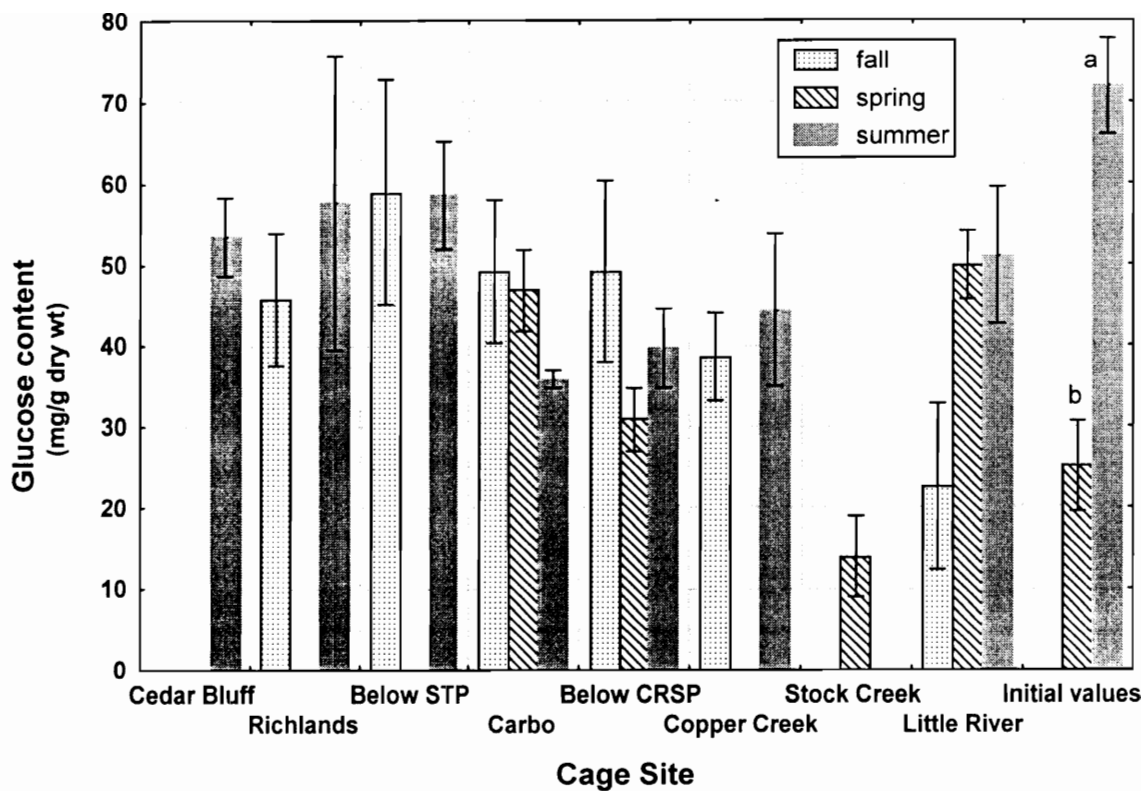


Figure 10. Comparison of glucose content of *V. iris* mantle tissue among seasons.

Columns are labeled with letters to designate statistical differences within a site.

Columns which share a common letter are not statistically different ($\alpha = 0.05$).

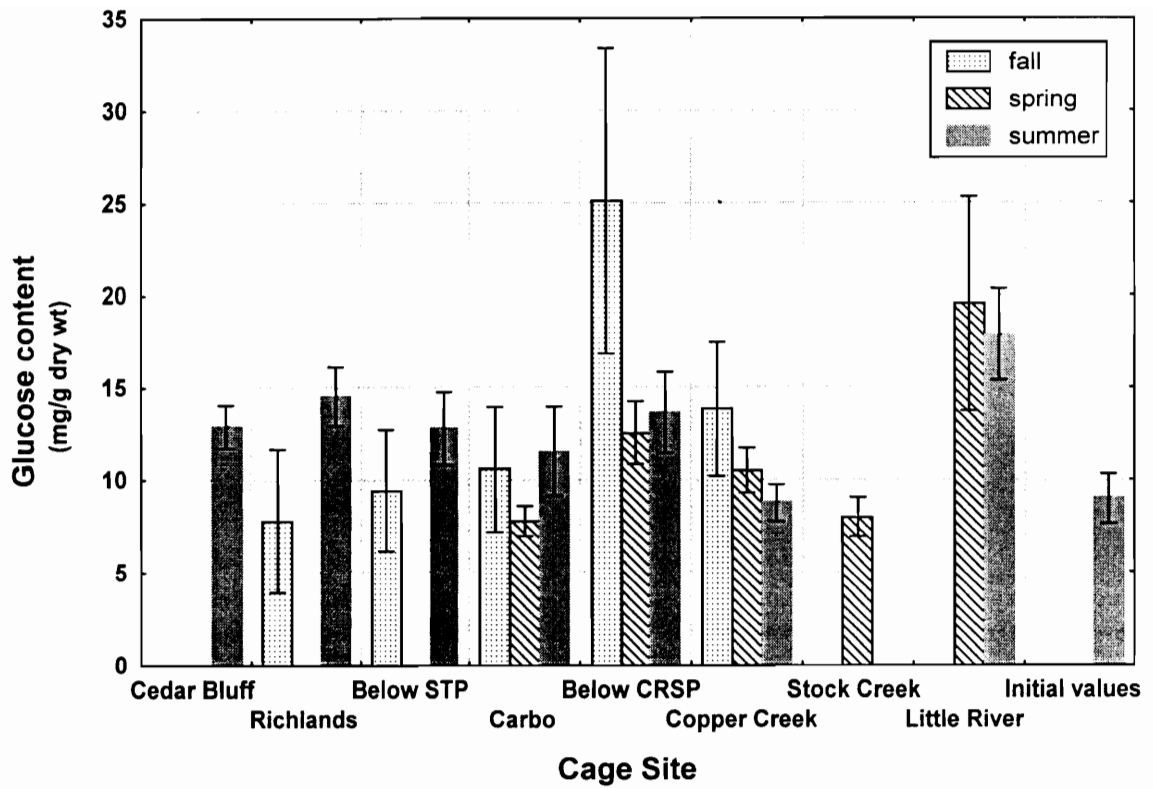


Figure 11. Comparison of glucose content of *C. fluminea* foot tissue among seasons. No statistical differences were detected within a site ($\alpha = 0.05$).

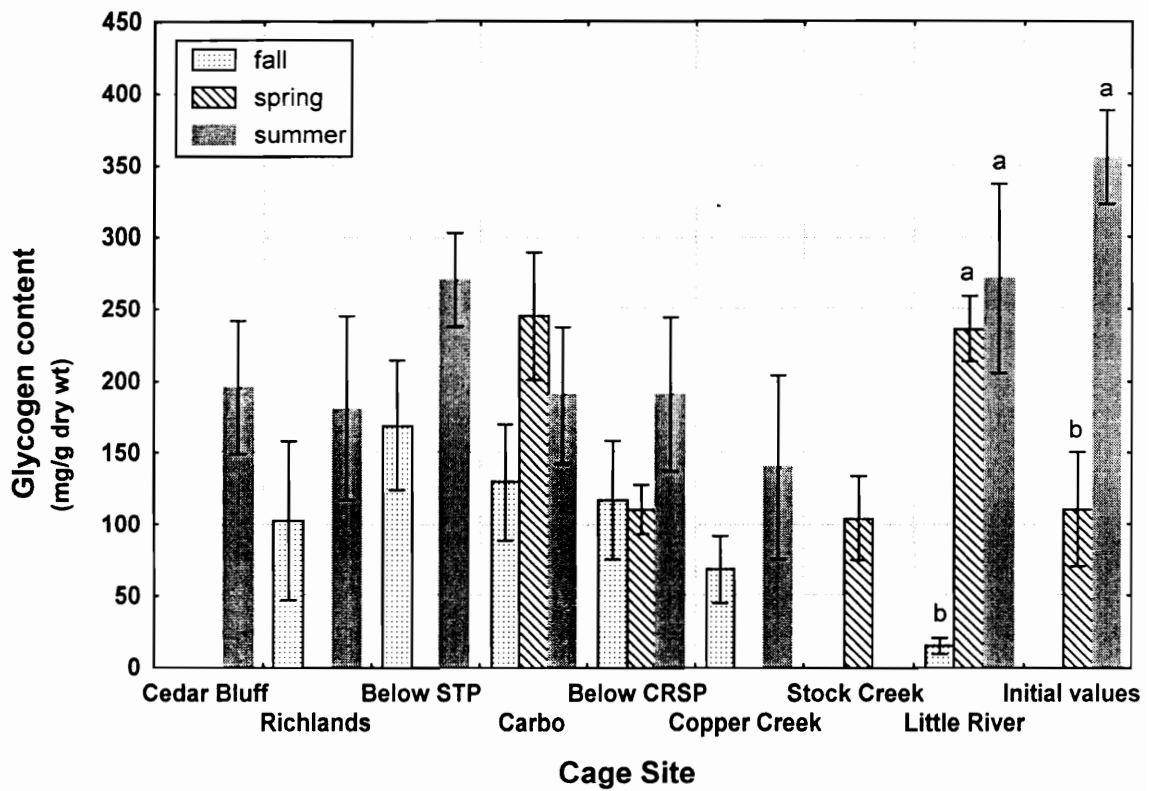


Figure 12. Comparison of glycogen content of *V. iris* mantle tissue among seasons.

Columns are labeled with letters to designate statistical differences within a site.

Columns which share a common letter are not statistically different ($\alpha = 0.05$).

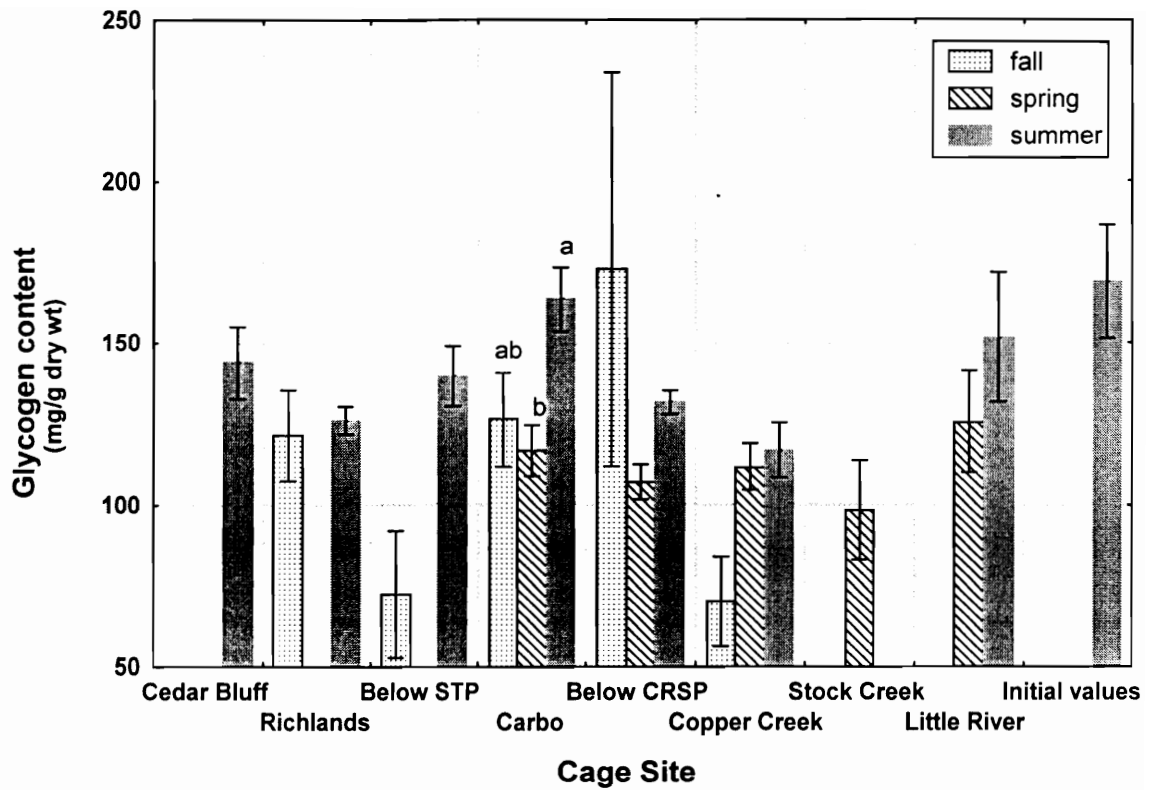


Figure 13. Comparison of glycogen content of *C. fluminea* foot tissue among seasons.

Columns are labeled with letters to designate statistical differences within a site.

Columns which share a common letter are not statistically different ($\alpha = 0.05$).

RNA:DNA Ratio

The ratio of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA) was measured in foot tissue for both mussels and clams. Data were not obtained for mussels at two sites during the fall trial because of technical problems and a lack of adequate tissue samples.

During the fall trial, the clams exhibited differences in RNA:DNA among the sites at which they were deployed (Table 5). Clams caged at Carbo (0.89) had a significantly higher RNA:DNA ratio than those held Below CRSP (0.60) and at Copper Creek (0.66). Clams held Below Raven/Doran STP (0.78) had a significantly higher RNA:DNA ratio than those Below CRSP. The ratio values ranged from 0.60 for clams held Below CRSP to 0.89 at Carbo. There are two reasons to expect the ratio of RNA to DNA to increase, a response to increased growth rates or to increased cell repair and protein synthesis. The above data suggest that the clams Below Raven/Doran STP and at Carbo are growing faster than at the other sites or that these sites were more stressful to the clams. When compared to the glucose and glycogen analyses, the latter explanation seems more likely. Clams held at both sites, Below Raven/Doran STP and Carbo, had relatively low levels of glycogen and glucose, indicating that nutrient acquisition and storage was limited at these two sites. Without the ability to obtain enough food to build glycogen reserves, clams would be unable to put much energy into growth.

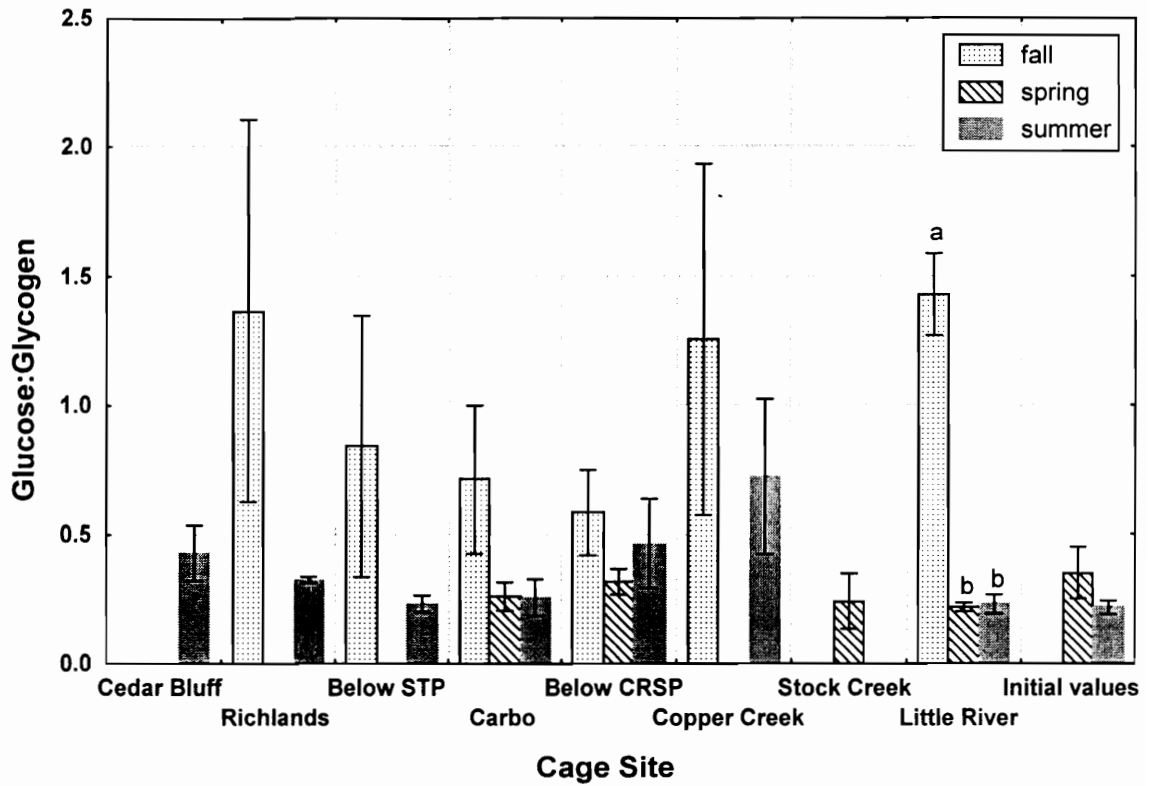


Figure 14. Comparison of the ratio of glucose to glycogen for *V. iris* mantle tissue among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns within a site which share a common letter are not statistically different ($\alpha = 0.05$).

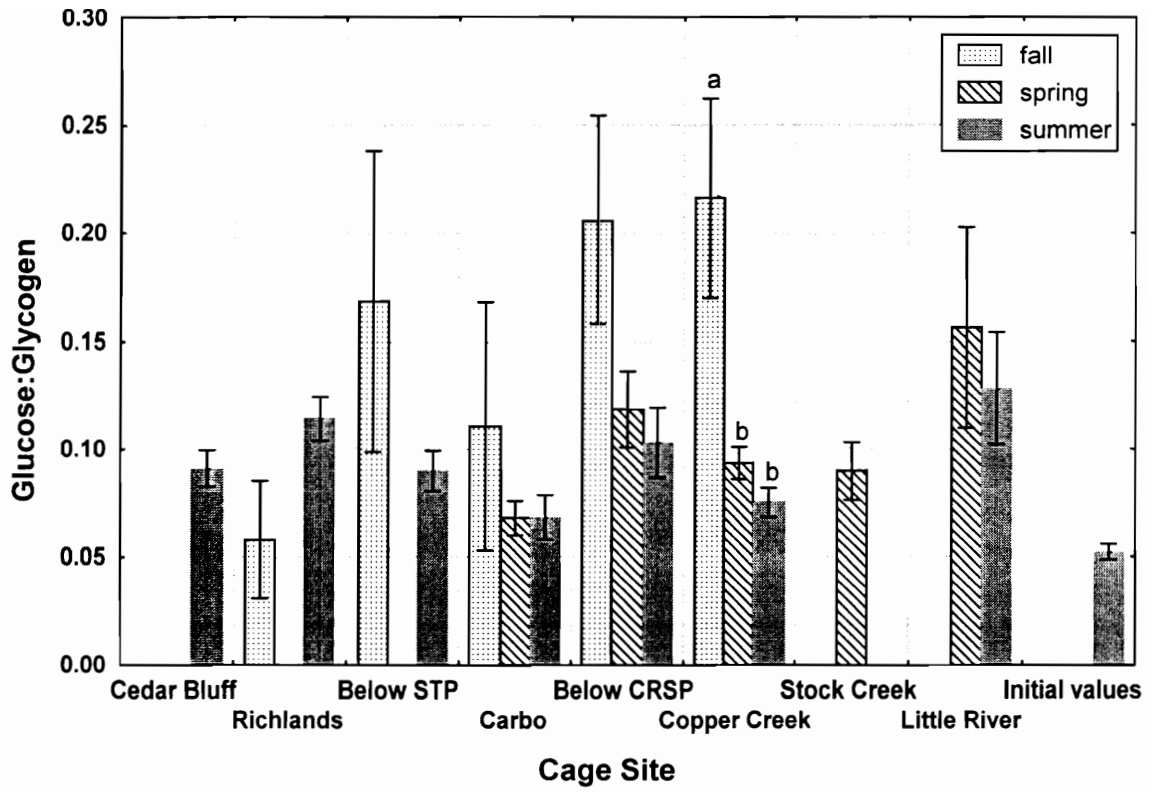


Figure 15. Comparison of the ratio of glucose to glycogen in *C. fluminea* foot tissue among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$).

The spring trial revealed no difference among sites for either mussels or clams (Table 6). However, the means of each site of the field-deployed mussels were lower than the mean of the mussels sampled at the beginning of the exposure. The mean ratio value for the animals sacrificed at the beginning of the experiment was 4.84, as compared to 1.08-2.24 for the field deployed samples. The clams exhibited similar results. The ratio of the beginning samples was 1.12 as compared to 0.79-1.00 for the field-deployed samples. The RNA:DNA ratio for the beginning clam samples was statistically different from all of the field-deployed ratios except for Copper Creek (1.00). The decrease in all of the caged animals implied that placing the mussels and clams in cages decreased growth rates during the spring of the year.

The summer trial revealed differences among sites for the mussels, but not for the clams (Table 7). The mean RNA:DNA ratio value for mussels held at the Little River site (2.58) was higher than all others except those held Below Raven/Doran STP (1.73), while the ratio of the Copper Creek animals was the lowest (0.30), significantly lower than the Below Raven/Doran STP and Little River samples. The fluctuations in RNA:DNA among sites followed a similar pattern to that of glycogen content. Therefore, the variation exhibited was likely due to differences in growth rates among sites. The one exception to the pattern match was that the beginning samples had a moderate RNA:DNA ratio, but high levels of glycogen, which could have been residual stores. The clams had lower RNA:DNA ratios, ranging from 0.48 for Carbo to 0.76 for Richlands, but were not significantly different among sites. The pattern of variation was different for the two species. The greatest differences were exhibited at Cedar Bluff, Richlands, and Below

Raven/Doran STP. At these sites, the clams had lower values at Cedar Bluff and Below Raven/Doran STP than at Richlands, while the mussels had higher values at Cedar Bluff and Below Raven/Doran STP than at Richlands. The other major difference was that the mussels had a high average RNA:DNA ratio at Little River, and the clams had a ratio similar to that at other sites. Interestingly, the pattern of variation in the RNA:DNA ratio among sites for clams was almost the mirror image of the pattern of variation in glycogen content, but the same as the glucose pattern. The combination of these results provided evidence that the changes in RNA:DNA ratio in clams were due to the effects of environmental stresses.

Mussels generally had higher RNA:DNA ratios than clams during all seasons. Since the ratio increases with any increased synthesis of protein, the mussels either have higher growth rates or greater levels of protein synthesis in support of physiological processes and tissue repair. Since the rainbow mussels achieve a larger size, and the length of the experiments was not adequate to assess growth, there is no way to discern between the two possibilities in this case. All mussels and clams used were of adult size, so rapid juvenile growth should not have been a factor in the comparisons.

The season also influenced the RNA:DNA ratio for both species. The species exhibited a pattern of higher ratios during the spring than during the summer (Figures 16 & 17). The pattern does not match that of glycogen or glucose content, but suggests that both species experienced more growth or stress during the spring than summer. For *C. fluminea*, this response was predominantly due to changes in the amount of DNA in the samples among seasons.

Cellulolytic Enzymes Activity

Cellulolytic enzymes activity was measured using the digestive gland tissue of mussels and the body tissue of clams. Two different measurements were made on each sample to assess the activity of the cellulase enzymes. One method measured the change in the viscosity of a solution containing substrate for the enzyme. The other method measured the amount of sugar released by the activity of the enzyme in a solution of substrate. A third method, which measured the amount of cellulase activity on suspended cellulose in the sample, was attempted. However, the activity levels in the samples of both species were so low in the final assay that the reactions could not be accurately measured.

The viscometric assay was corrected for procedural differences between samples from the two species. Note that the incubation time of the enzyme homogenate with the substrate was twice as long for the mussels as for the clams. Shorter incubation times were used for Asian clam samples because the solution viscosity changed too much to accurately measure when incubation times equaled those used for rainbow mussels. Additionally, the clam homogenates used entire visceral masses, while the mussel homogenates used only digestive gland tissues. To correct for potential differences in water content and incubation time, comparisons were made on the change in effluent times, a linear function of change in viscosity, caused by the homogenate. The data were normalized using the dry weight of the particulate material collected after centrifugation of the homogenate and effluent times corrected for the differences in sample incubation,

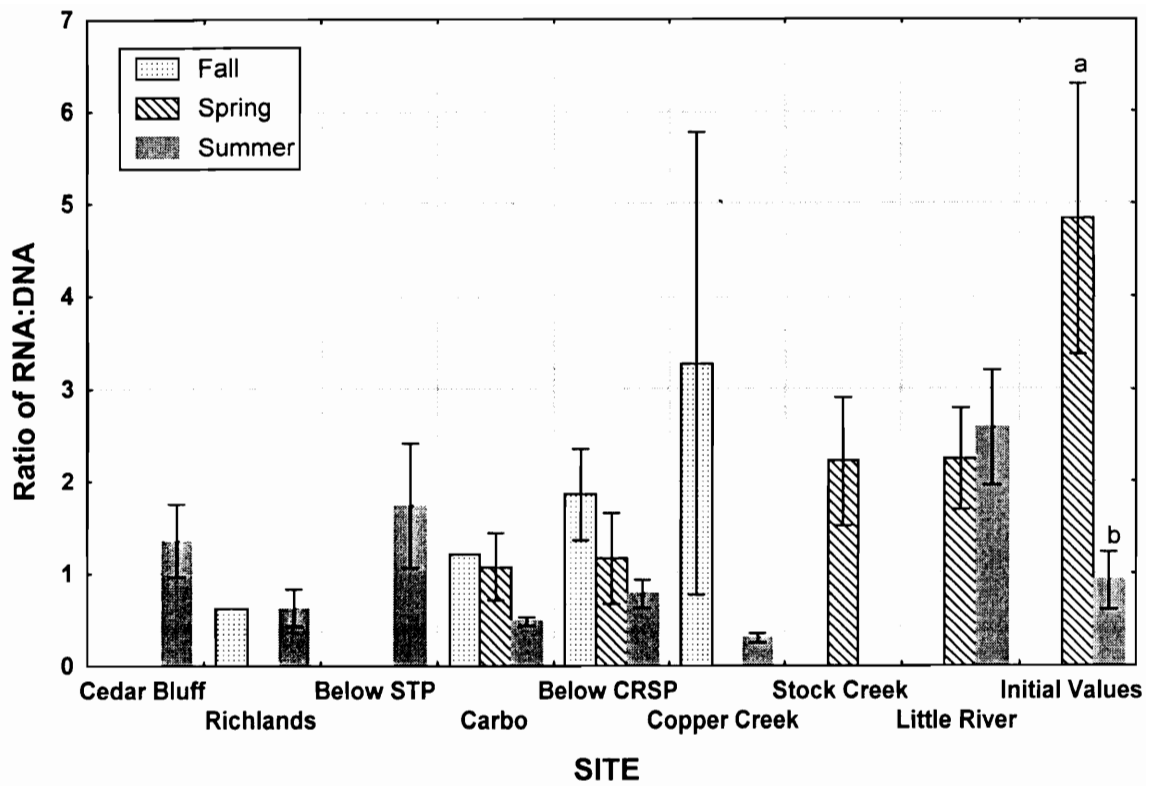


Figure 16. Comparison of the ratio of RNA to DNA in *V. iris* foot tissue among seasons.

Columns are labelled with letters to designate statistical differences within a site.

Columns which share a common letter are not statistically different ($\alpha = 0.05$).

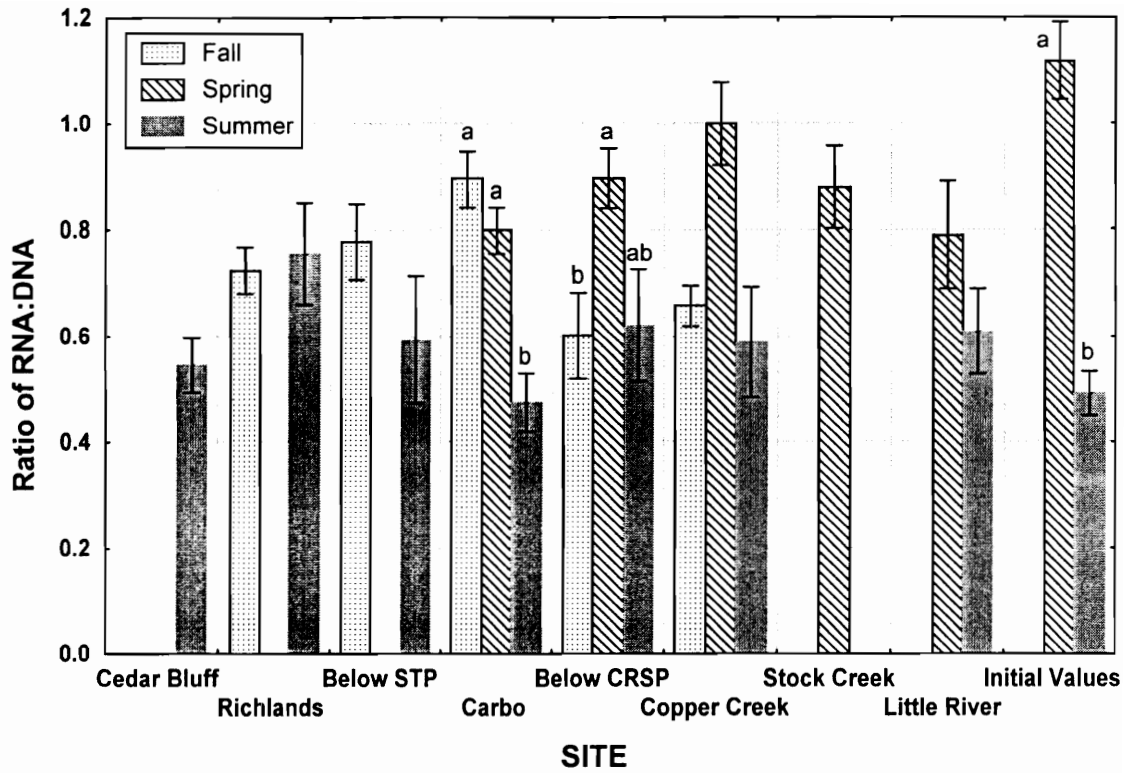


Figure 17. Comparison of the ratio of RNA to DNA in *C. fluminea* foot tissue among seasons. Columns are labelled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$).

unavoidable due to the handling time for each sample. No differences were detected in water content. Higher values indicated greater activity of the enzyme on the substrate.

Comparisons for the fall experiment showed a difference among sites for mussels, but not for clams (Table 5). Mussels held Below Raven/Doran STP and at Carbo yielded significantly higher values (21.3 and 21.3 sec/mg dry wt./hr, respectively) than the remaining sites except Richlands (18.3 sec/mg dry wt./hr). The values ranged from 14.2 sec/mg dry wt/hr at Little River to 21.3 sec/mg dry wt./hr Below Raven/Doran STP for mussels, and from 6.6 sec/mg dry wt./hr at Copper Creek to 9.4 sec/mg dry wt./hr at Carbo for clams. The apparent switch in the relative activity of cellulolytic enzymes between clams and mussels was due to the higher dry weights of the clam tissues. The sugar-releasing assay did not show significant differences for *V. iris* or *C. fluminea* (Table 5). The product and ratio of the two assays also were not different among sites (Table 5), indicating that there were no significant impairments of cellulolytic enzyme activity.

The spring experiment yielded differences among sites for both assays and species. For *V. iris*, the change in effluent time was greater at Little River than at all other sites except Below CRSP, while the mussels from Stock Creek exhibited lower enzyme activity than Below CRSP (Table 6). Asian clams also exhibited the highest values at Little River, which were higher than those at the beginning of the experiment, Carbo, and Stock Creek. The change in effluent time at Carbo was also lower than at Copper Creek. The sugar-releasing assay produced almost the same results as the viscometric assay, with Little River having the highest enzyme activity levels (Table 6).

Both of the combined measures yielded differences among sites for *V. iris* (Table 6), while only the product of the two assays yielded differences among sites for *C. fluminea* (Table 6).

Likewise, the summer experiment yielded differences among sites for both assays. *V. iris* from Richlands had the highest enzyme activity, which was higher than all sites except Cedar Bluff and Copper Creek (Table 7). Cedar Bluff and Copper Creek also produced higher values than Below CRSP and the beginning samples. The sugar-releasing assay produced a similar pattern of results, with the highest values for Richlands and Cedar Bluff and the lowest for the beginning samples (Table 7). Asian clams exhibited the highest activity based on the viscometric assay at Copper Creek, which was higher than at all other sites (Table 7). Cedar Bluff and Richlands had lower values than Below CRSP as well. The sugar-releasing assay produced differences only at the $p < 0.10$ level in Asian clams. At this level, Carbo and Richlands were lower than Below CRSP, Little River, and Copper Creek. Both the product and ratio (Table 7) of the two assays yielded differences among sites for mussels while only the ratio revealed differences among sites for Asian clams.

There was a clear seasonal influence on the activity of this enzyme system for both species. This influence was most pronounced in the endocellulase assay based on viscometry. The cellulolytic enzymes of mussels exhibited a much greater level of activity in the fall than during the spring and summer, which were comparable (Figure 18). Asian clams had the highest endocellulase activity during the summer (Figure 19). The sugar-releasing enzyme activity did not produce a distinct seasonal response, but

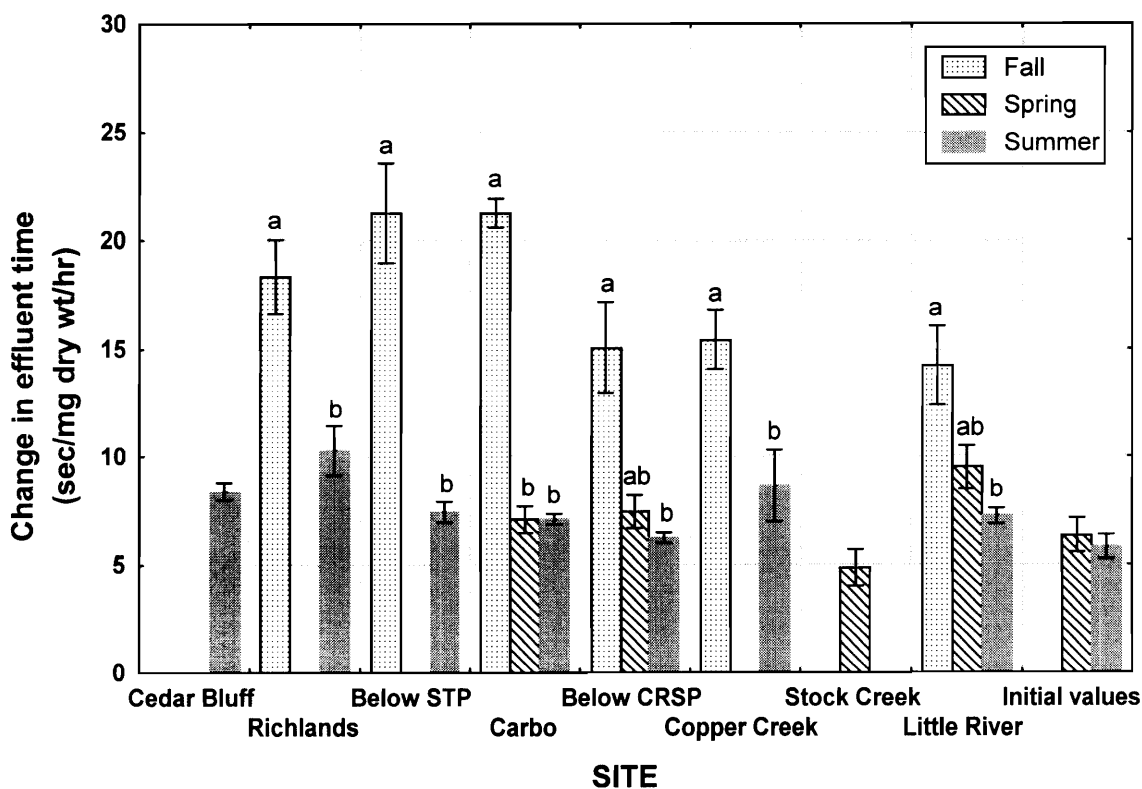


Figure 18. Comparison of the change in effluent time (viscosity) of carboxymethyl cellulose solution after incubation with *V. iris* digestive gland tissue homogenate among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$).

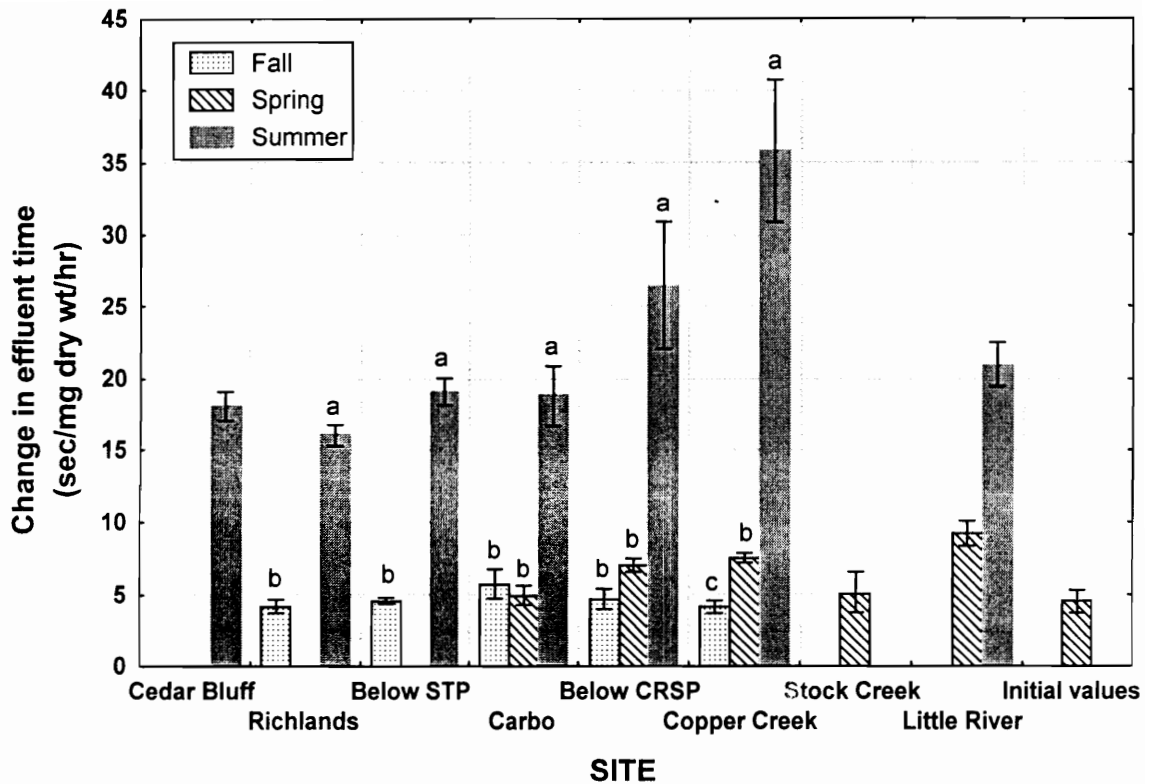


Figure 19. Comparison of the change in effluent time (viscosity) of carboxymethyl cellulose solution after incubation with *C. fluminea* visceral mass tissue homogenate among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$).

there was a difference among seasons for both species (mussels at $p < 0.10$).

Fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase (FBP) is one of the enzymes responsible for the synthesis of glucose. The activity of this enzyme was assayed as a potential control point of the metabolism of carbohydrates. Differences among sites were noted only for clams. However, there were differences among seasons for both mussels and clams, with the fall samples having the highest activities. There were also very large differences in the activity levels exhibited by the two species. The clams had enzyme activity levels more than 10 times greater than mussels. One unit of activity is defined as the release of one μ mole of inorganic phosphorous per mg of tissue per hour.

During the fall experiment, site differences were detected for the clams (Table 5). Clams held Below CRSP had higher levels of FBP activity (3302 mUnits) than those held at Carbo, Richlands, and Copper Creek (2503, 2833, and 2671 mU, respectively). The FBP activities of clams held at Carbo were also lower than Below Raven/Doran STP (2961 mU). The higher FBP activity in clams Below CRSP and Below Raven/Doran STP suggest that these specimens were in the process of synthesizing more glucose, and hence storing more glycogen, than clams at the other sites. The mussels did not show differences among sites (Table 5). The pattern of variation of FBP was different between the two species. Whereas the mussels had gradually decreasing activity among sites in the order of Richlands (173 mU), Below Raven/Doran STP (160 mU), Copper Creek (154 mU), Carbo (146 mU), and Below CRSP (145 mU), the clams exhibited an up and

down pattern. Activity levels were high Below Raven/Doran STP and Below CRSP, low at Carbo and Copper Creek, and intermediate at Richlands. This difference in patterns of variation suggests alternative responses to environmental conditions. It appeared that the FBP activity was varying with differences in environmental conditions among sites for the clams. Since the activity did not exhibit any steady trend within the four Clinch River sites, there is not a trend to increase or decrease FBP activity with river size or order. However, the fluctuations could have been a response to temperature, as the two sites with the highest activity levels were downstream of a sewage treatment plant and a power plant. The activity level of FBP in clams was 16-22 times higher than that of mussels. Such a difference in FBP activity levels suggests that, compared to mussels, clams are capable of using more assimilated nutrition for building glycogen reserves.

During the spring experiment, differences among sites were not measured for either species (Table 6), but the widely disparate activity levels between species was even more pronounced. The clams had FBP activity levels which were more than 200 times greater than those of mussels. Clams had activities of 2050 to 2677 mUnits, while mussels had activity levels of 8.3 to 10.6 mUnits. Interestingly, the activity levels for this enzyme were lower during the spring than during the fall, implying that the balance between nutrient acquisition and demand left less energy to be stored. The reproductive status of the mussels could have required most of the nutrient energy assimilated, since the late spring is the time of glochidial release for rainbow mussels (Zale and Neves, 1982). The gender of the mussel and gravidity were not correlated with FBP activity.

The summer experiment showed differences among sites for the clams, but not for

mussels (Table 7), again indicating that clams use this enzyme as a control point in carbohydrate metabolism. Clams held at Richlands, Below Raven/Doran STP, and Carbo had higher activity levels (1957, 1987, and 1989 mUnits, respectively) than those at all other sites except Below CRSP (1723 mU). The FBP activity of the animals Below CRSP was higher than that of animals sacrificed at the beginning of the experiment (1261 mU). The clams continued to have much higher FBP activity than the mussels. The pattern of variation was also different between the two species (two-way ANOVA interaction $p < 0.001$), providing further evidence that these two species respond to the environmental conditions differently. The activity levels of the mussels were intermediate at Cedar Bluff (117 mU), increased at Richlands (127 mU), then decreased from Richlands through Below Raven/Doran STP (119 mU), Carbo (114.2 mU), and Below CRSP (99 mU). FBP activity was low at Copper Creek (102 mU) and Little River (109 mU), but was high at the beginning of the experiment (129 mU). The clams had activity levels which increased sharply from Cedar Bluff to Richlands, remained high Below Raven/Doran STP and Carbo, then decreased Below CRSP, were low at Copper Creek and Little River, and were lowest at the beginning of the experiment. The low values at the beginning of the experiment for clams might reflect the use of energy for reproductive efforts as they reproduce during the summer (McMahon, 1991). Asian clams generally reproduce twice annually, once in the early summer and again in the late summer. Asian clams in the New River in southwest Virginia release juveniles from June to September, Possibly affecting nutrient allocation (Doherty et al., 1987). Species differences in the response to sites indicate that clams had an energetic advantage at the

Clinch River sites and rainbow mussels were energetically favored at the Little River site.

The seasonal differences in the activity of FBP were obvious for both species. The mussels exhibited the highest levels of FBP activity during the fall and the lowest levels, approximately 1/8 of the fall levels, during the spring (Figure 20). Since spring is the natural time of glochidial release for this species, the reduction in FBP activity during the spring was likely due to reproductive energy demands. However, no difference in FBP activity was determined between sexes. Asian clams also had the highest activity levels of FBP during the fall, but spring values were intermediate, and the low activity levels during the summer were only half the fall values (Figure 21). The low summer values were most likely a response to higher metabolic costs due to increased temperatures or reproductive activity.

Phosphofructokinase Activity

Phosphofructokinase (PFK) is the competing enzyme for FBP in the cycling of fructose-1,6-bisphosphate and fructose-6-phosphate in the pathway of glucose metabolism (de Zwaan, 1979; Bohinski, 1987). PFK activities are higher during times of increased glycolysis (Storey and Storey, 1990). This enzyme was also assayed as a potential control point of the synthesis/breakdown of glycogen (Biethinger et al., 1991). Differences among sites were measured during all seasons for the clams and during the summer experiment for the mussels. The mussels also exhibited differences in activity

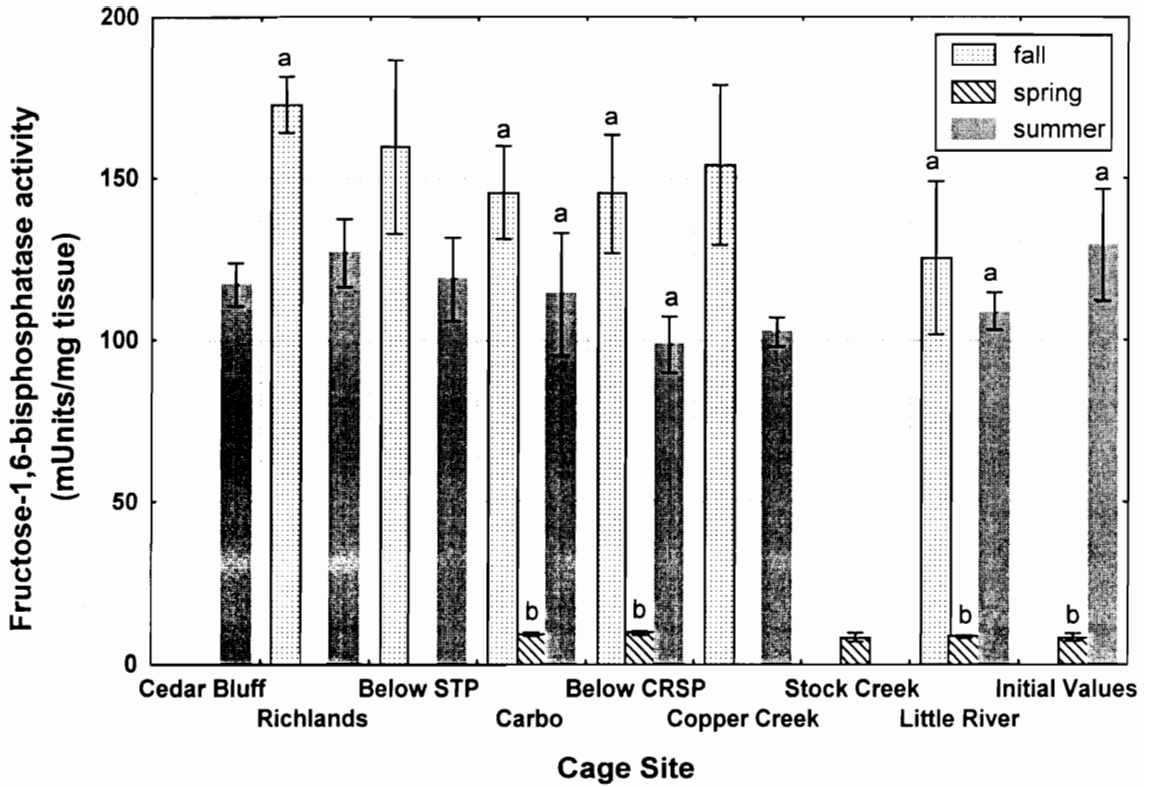


Figure 20. Comparison of fructose-1,6-bisphosphatase activity of *V. iris* foot tissue among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$). One unit of activity is the enzyme required to release 1 μ mole of phosphorous per minute.

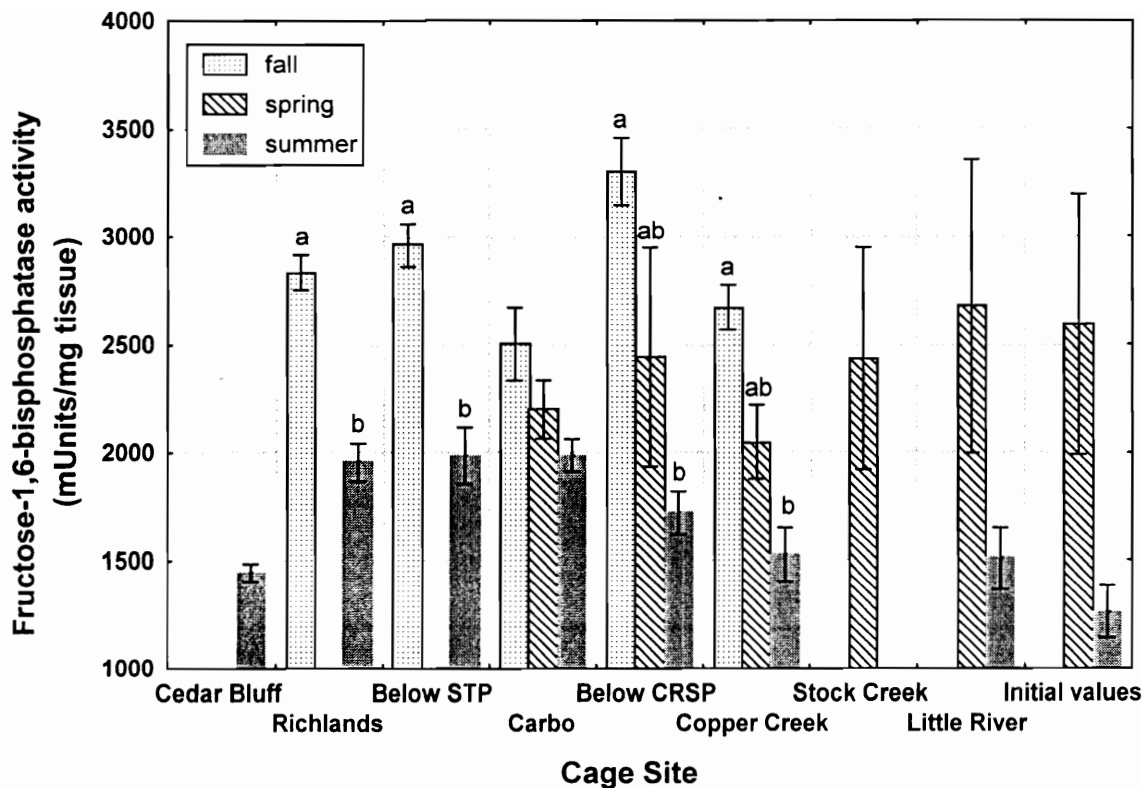


Figure 21. Comparison of fructose-1,6-bisphosphatase activity in *C. fluminea* foot tissue among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$). One unit of activity is the enzyme required to release 1 μ mole of phosphorous per minute.

levels of PFK among seasons. In addition, with the exception of the fall experiment, the PFK activity in mussels was more than an order of magnitude greater than that of the clams.

During the fall experiment, only clams exhibited differences among sites (Table 5). Clams held at Richlands and Below Raven/Doran STP had higher PFK activity levels (2.43 and 2.76 mU/mg wet wt, respectively) than those held at Copper Creek (0.83 mU/mg wet wt). The enzyme activity for animals Below Raven/Doran STP was also higher than the activity for animals held at Carbo (1.38 mU/mg wet wt). The mean values for PFK activity in mussels were 1.5 to 6.5 times greater than the mean values of clams at the same sites. This difference was in the opposite direction of the FBP activities, which were higher in clams than in mussels. The pattern of variation in PFK activity among sites was inversely related to that of FBP for mussels, while these activity levels varied similarly for clams. The inverse relationship between FBP and PFK suggested that these two enzymes work in a competing fashion to regulate the glycolytic flow in rainbow mussels. The similarity in the pattern of variation of these two enzymes in clams indicated that the control point for this species is probably at some other metabolic step.

The spring experiment also yielded site differences in PFK activity only for clams (Table 6). Clams held at Copper Creek had higher PFK activity levels (5.03 mU/mg wet wt) than those at all other sites except Below CRSP (2.10 mU/mg wet wt). No differences were determined among the other sites. The pronounced difference in activity levels between mussels and clams was exhibited again with the ratio of mussel to clam

activity ranging from 13.5 to 577. In general, the difference in PFK activity between the species was greater during this experiment than the fall trial. Each species had similar variation patterns for PFK and FBP. However, comparing the patterns between species revealed differences. The mussels had high levels of activity at Carbo (29.23 mU/mg wet wt), moderate levels Below CRSP (28.12 mU/mg wet wt) and Little River (27.01 mU/mg wet wt), and low levels at Stock Creek (25.22 mU/mg wet wt). In contrast, clams exhibited low activity levels at Carbo (0.42 mU/mg wet wt) and Little River (0.35 mU/mg wet wt). Clams and mussels exhibited different patterns of carbohydrate metabolism, increasing or decreasing glycolytic rates at contrasting sites. Continued separation of the PFK activity levels between the species supported the hypothesis that the glycolytic control points and demands for these two species are different.

The summer trial revealed differences among sites for both clams and mussels (Table 7). Clams from Below Raven/Doran STP and Carbo had higher PFK activities than those from Cedar Bluff and Copper Creek, as well as the beginning samples. Mussels held at Cedar Bluff and Carbo had higher PFK activity levels than ones held Below Raven/Doran STP and Below CRSP. The large difference between activity levels of the two species was also exhibited during this trial. Mussels at the various sites had PFK activity levels that were 52-196 times greater than that of the clams. The pattern of variation among sites followed by the two species also was different. The mussels had high activity levels at Cedar Bluff, Carbo, and Copper Creek, while the activity levels at Richlands, Below Raven/Doran STP, and Little River were lower. The clams had low PFK activity levels for Cedar Bluff, Copper Creek, and the beginning samples, moderate

activities for Richlands, Below CRSP, and Little River, and high values for Below Raven/Doran STP and Carbo.

Season influenced the activity levels of PFK in mussels but not in clams (Figures 22 & 23). The greatest PFK activity levels in mussels were during the summer. Spring activity levels were approximately one third of the summer levels, and fall levels were only one tenth of the summer levels. This is a very large seasonal variation and, coupled with the large seasonal fluctuation in FBP activity, indicates that the PFK/FBP enzyme coupling is a control point of glucose metabolism. However, Asian clams appear to have a different mechanism for seasonally controlling glycolytic and gluconeogenic rate, as the PFK activity does not vary among seasons.

Acetylcholinesterase Activity

Acetylcholinesterase activity was measured in both species as a potential indicator of exposure to organophosphate or carbamate pesticides. These chemicals suppress the activity of acetylcholinesterase, causing malfunctions in the nervous system of the exposed animal. Differences among sites were exhibited by clams during all three experiments. There were no differences among sites for mussels. Clams generally had higher levels of acetylcholinesterase activity than mussels during all three experiments.

During the fall experiment, clams exhibited a difference among sites for acetylcholinesterase activity (Table 5). Clams held at Copper Creek had higher total AChE activity levels (20.5 mU/mg wet wt) than those held at all other sites except Below Raven/Doran STP (20.1 mU/mg wet wt). However, when this was normalized on the basis of protein content, this difference disappeared. Mussels showed no difference

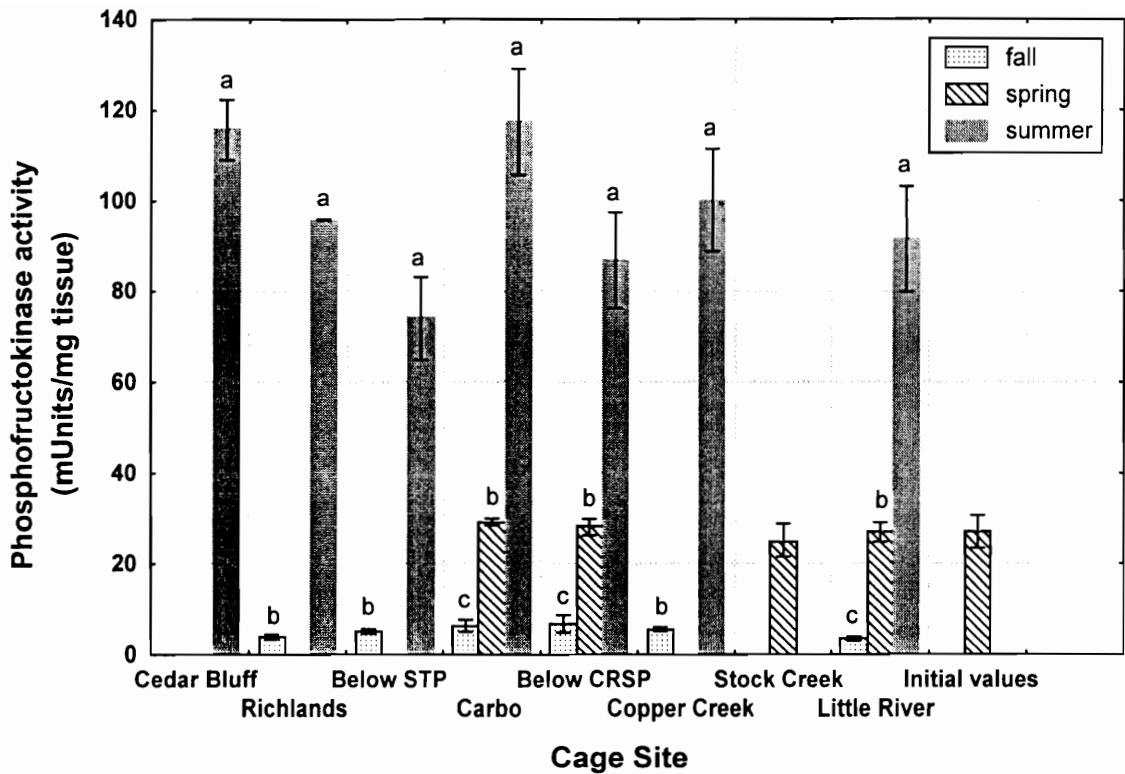


Figure 22. Comparison of phosphofructokinase activity in *V. iris* foot tissue among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$). One unit of activity is the enzyme required to reduce 1 μ mole of NADH per minute.

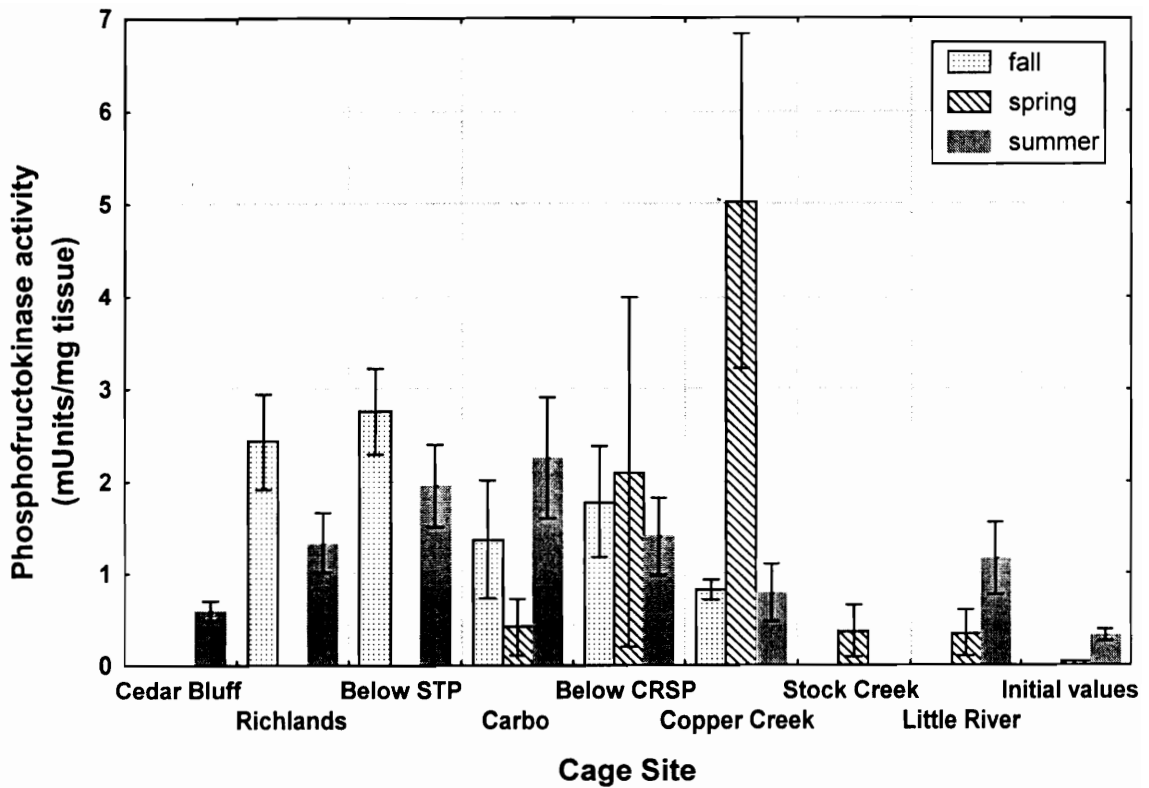


Figure 23. Comparison of phosphofructokinase activity in *C. fluminea* foot tissue among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$). One unit of activity is the enzyme required to reduce 1 μ mole of NADH per minute.

among sites for acetylcholinesterase activity. Clams did have significantly higher activity levels than mussels. The difference ranged from 1.7-3.7 times higher activity in the clams when normalized to protein content. The higher activity levels present in clams indicated that Asian clams were more physically active than rainbow mussels during the fall. This could have been due to a general tendency of clams to be more active or to an adaptation which allows clams to retain activity levels as temperatures decrease.

During the spring experiment, differences among sites were again evident for clams but not mussels. Site differences were determined for AChE activity whether based on mU/mg wet weight or mU/mg protein (Table 6). When normalized to the protein content, clams held at Copper Creek had higher AChE activity levels (636 mU/mg prot) than those from any other sites. Clams from Carbo (434 mU/mg prot) and the beginning samples (453 mU/mg prot) had higher activity levels than those from Below CRSP (358 mU/mg prot). The order of activity levels from lowest to highest was Below CRSP, Stock Creek (391 mU/mg prot), Little River (411 mU/mg prot), Carbo, beginning samples, and Copper Creek. If the activity levels were normalized on the basis of mg of wet weight, the only site difference measured was that clams from Copper Creek had higher AChE activity levels (51.7 mU/mg wet wt) than those from all other sites. Clams did not have higher activity levels than mussels during this experiment on the basis of activity per mg protein. Therefore, it could be inferred that mussels and clams likely have similar activity levels during this season. The higher AChE activity levels for clams held at Copper Creek indicated that Copper Creek did not suffer from pesticide runoff during the exposure period. The low activity levels at Stock Creek and Below

CRSP suggested that these sites caused a general stress on the animals or were subject to pesticide runoff. Since these sites were not adjacent to agricultural fields, the potential for pesticide impacts was low.

The summer experiment also revealed differences among sites for clams but not mussels (Table 7). AChEase activity levels normalized to mg of protein showed that clams held at Cedar Bluff, Richlands, and Below Raven/Doran STP had higher activity levels (849, 729, and 795 mU/mg prot, respectively) than Copper Creek (406 mU/mg prot), Below CRSP (452 mU/mg prot), and beginning samples (538 mU/mg prot). Clams from Copper Creek and Below CRSP had lower activity levels than all other samples except those sacrificed at the beginning of the experiment. Clams held at Cedar Bluff had higher activity levels than those from all other sites except Richlands and Below Raven/Doran STP. When normalized on the basis of wet weight, clams from Cedar Bluff, Richlands, Below Raven/Doran STP, and Little River had higher activity levels (20.6, 19.2, 21.8, and 20.5 mU/mg wet wt, respectively) than clams from Below CRSP (15.3 mU/mg wet wt), Copper Creek (13.6 mU/mg wet wt), and the beginning of the experiment (14.1 mU/mg wet wt). Clams held at Copper Creek had lower activity levels than those from all other sites except Below CRSP and the beginning samples. In addition, clams held Below Raven/Doran STP had higher levels of AChEase activity than Carbo (17.6 mU/mg wet wt). Unlike the fall and spring experiments, clams deployed at Copper Creek had the lowest AChEase activity levels during the summer. The decrease of 40-50% from the highest activity levels might have signaled an exposure to agricultural pesticide runoff. During this trial, clams caged at two of the sites believed to

be the most impacted, Copper Creek and Below CRSP, exhibited the lowest AChEase activity levels. Clams had approximately twice the level of AChEase activity as mussels during this experiment. Clams had higher AChEase activity levels than mussels during the two trials at the temperature extremes. Such a response suggests that clams have greater temperature adaptability for maintaining physical activity capabilities.

Seasonal differences were detected in AChEase activity for both species. When normalized on the basis of mg of protein, Asian clams had generally higher activity levels during the summer than during the spring, but this was not consistent among all sites. The exception to this was at Copper Creek, which had a higher level of activity during the spring experiment than the summer experiment. The seasonal influence in mussels is exhibited when activity is normalized on the basis of wet weight (Figure 24). The striking result of this comparison is the very low AChEase activity levels during the spring experiment, less than one eighth of the activity levels during the other seasons.

Identification of Impacted Sites with the Biomarkers

One of the objectives of this project was to investigate biomarkers which could detect environmental problems at sites for mussels. None of the individual parameters measured consistently indicated that particular sites were favorable or poor among the seasons tested. Therefore, an analysis was performed using all of the measured parameters as a single composite biomarker. By combining the biomarkers, differences among sites which were not statistically different when evaluated with a single parameter, might be discernable. Within each species and season, the individual results for each biomarker were ranked. These ranks were summed for each individual mussel and for the

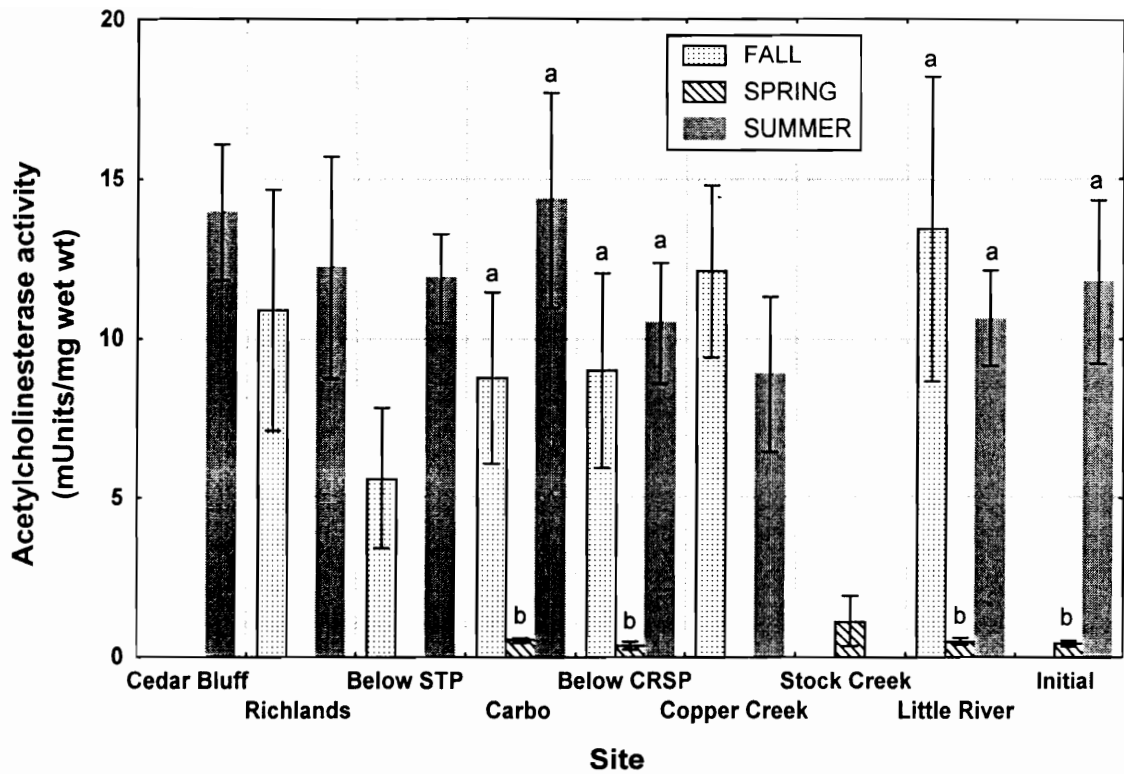


Figure 24. Comparison of acetylcholinesterase activity of *V. iris* adductor muscle among seasons. Columns are labeled with letters to designate statistical differences within a site. Columns which share a common letter are not statistically different ($\alpha = 0.05$). One unit of activity is the enzyme required to reduce 1 μ mole of acetylthiocholine per minute.

individual clam samples. Kruskal-Wallis analysis was employed to compare the ranks among sites, identifying whether any sites were statistically different from each other. If the Kruskal-Wallis test proved significant, a Moods median test with pairwise comparisons was performed to identify which sites were different.

The results of these analyses provided some evidence that the composite of biomarkers identified poor sites. Using mussel results, Below CRSP (the site downstream of the CRSP) and Copper Creek were chosen among the poorest sites in all seasons (Tables 8-10). While statistical differences among sites were only obtained for the spring experiment, the order of rank medians always placed Below CRSP in the poorest two sites and Copper Creek among the poorest 3 sites. Additionally, Carbo, the Clinch River site upstream of the the CRSP, was always selected as the best site based on biomarker results. The analytical results with Asian clams were not quite as clear, but Carbo was generally selected among the best sites and Below CRSP among the worst. There were no significantly different results among sites for clams. Tables of these analyses are included in Appendix 1.

Given the potential for identification of habitat quality using a compilation of all potential biomarkers, best subset regression analyses were performed in an attempt to identify which parameters were most useful in predicting habitat quality. The sites were ranked for habitat suitability according to preliminary survey data and literature reports (Ahlstedt, 1986; Farris et al., 1988; Goudreau, 1988; Yeager, 1994; EPA, 1996). The order selected based on the available information was, from worst to best, Stock Creek, Copper Creek, Below CRSP, Below Raven/Doran STP, Richlands, Cedar Bluff, Little

River, and Carbo. Best subsets regressions were executed with the site rank order as the independent variable and the physiological parameters as the dependent variables. The subroutine selects the best subsets of parameters based on adjusted-R² values and the number of parameters in the model. The analyses for mussels revealed four parameters that were included in the best models during more than one season (Appendix 2). These were endocellulase activity, exocellulase activity, FBP activity, and water content of digestive gland. Based on the results from my study, these would be the physiological parameters of most interest for further investigation as biomarkers. The analyses for clams produced four parameters that were included in the best models for all three seasons; water content of visceral mass, FBP activity, PFK activity, and AChE activity (Appendix 2). These physiological parameters warrant further investigation for their usefulness as biomarkers of environmental stress in Asian clams.

DISCUSSION

There was little consistency between the physiological responses of Asian clams and rainbow mussels to the same biomarker. The use of Asian clams as surrogates for native mussels has been proposed by Farris et al. (1994). However, my study provides evidence that Asian clams are unsuitable as surrogates for rainbow mussels without prior validation of response similarity. The biochemical and physiological parameters measured did not respond similarly in both species.

No single physiological measurements in my study clearly indicated that either clams or mussels were stressed at any site during all periods tested. However, the absence of such consistent negative physiological responses does not necessarily mean that all sites are equally suitable habitats for mussels and clams. Habitats that are unsuitable during part of the year may not be capable of supporting mussel populations. Any adverse impacts could be severe enough to negatively effect juvenile mussel survival or growth but not cause a clear response in adults. Juvenile mussels are seemingly more sensitive to certain toxicants than adults (Jacobsen et al., 1993). Alternatively, intermittent habitat or water quality degradations can be missed by discrete time studies, even though these episodic events may negatively impact populations (Yeager, 1994).

While none of the physiological measurements, individually, identified particular sites as consistently good or bad, they collectively provided evidence of intermittent stresses on mussels and clams at certain sites. The use of groups, or suites, of measurements has been recommended in the assessment of contaminant impacts,

especially when the identity of the critical stressor(s) is unknown (Melancon, 1995). Fox (1993) stresses the need for a chosen suite of biochemical parameters to encompass as many of the major physiological functions of an organism as possible. Such an approach provides an assessment of a broad range of potential pollutants, since the specificity of one assay does not entirely determine the assessment. A 'composite index' was devised in which each of the sites was ranked from worst to best for the biomarkers measured, and the sums of these ranks for each site were compared. Even though composite indices of the biomarkers did not indicate that any site was inhospitable at all times, they did indicate that each of the sites without substantial mussel populations, with the exception of below the Raven/Doran STP, was inferior compared to other sites during at least one season. Thus, it appears that the suite of biomarkers was more effective at identifying poor sites than any of the individual measures. The attempts to identify the most promising biomarkers for determining habitat suitability through best subsets regressions produced marginal results. However, these analyses did suggest parameters that may be worth pursuing in future research.

Comparative Response of the Species: Do Asian Clams Make Good Surrogates for Mussels?

The use of Asian clams as surrogates for determining environmental impacts on native freshwater mussels has been proposed (Farris et al., 1994; Milam and Farris, 1998). However, there are few data to support or refute this recommendation. I am unaware of any study that has assessed the field responses of a native mussel species and Asian clams under similar conditions and times. While using the ubiquitous Asian clam

for biomonitoring in place of representatives of declining mussel faunas is appealing, without this link, inferences are scientifically tenuous. Data from my study address this question, as well as identifying physiological differences between rainbow mussels and Asian clams.

The ratio of exocellulase to endocellulase activity provides some indication of the available nutrition for bivalves. Since the two enzyme groups accomplish different portions of digestion in the guts of invertebrates, the ratio indicates which fractions of ingested food can be effectively digested and assimilated (Schulz et al., 1986; Hogan et al., 1988). The exocellulase primarily hydrolyzes cellobiose chains to glucose, while endocellulases break the β -1,4-glycosidic linkages, forming smaller polysaccharide and disaccharide chains (Schulz et al., 1986; Farris et al., 1989). Therefore, higher ratios of exocellulase to endocellulase would suggest that the animals are relying more heavily on dissolved organic material or ruptured cells as food. Rainbow mussels exhibited significantly higher ratios of exocellulase to endocellulase in all seasons, indicating a reliance on different nutritional sources. In fact, the mussels had up to 4 times higher ratios during the summer than did Asian clams. The ratios measured for Asian clams in my study were similar to those reported by Farris et al. (1989). The comparative difference in the ratio of exocellulase to endocellulase suggests that, while some overlap in food resources is inevitable, resource partitioning may occur.

The relative response of the two species can change with season, fluctuating similarly during certain times of year, while differing during others. This was the case for rainbow mussels and Asian clams with my assays. Comparing results among studies

of freshwater bivalves reveals differences in activity levels and biochemical parameters. Reports of cellulolytic activity in freshwater bivalves range from 0.058 to 41.9 mU/ μ g dry wt (Farris et al., 1988; Farris et al., 1989; Haag et al., 1993; Berg, 1994; Farris et al., 1994). The cellulase activities for Asian clams in my study were lower than those reported by Farris et al. (1988, 1989, 1994) by a factor of 10-100. However, the treatments, times of year, and sites were different. The cellulase activity levels reported for *Quadrula quadrula* were approximately 10 times lower than the activities for *V. iris* in my study (Berg, 1994). These results underscore the wide range of possible activity levels for this assay both within and among species of freshwater bivalves. The differences in absolute cellulolytic activity levels of the two species does not prevent the use of the Asian clam as a surrogate, if the patterns of variation among sites and seasons are similar for both species. This was not the case for Asian clams and rainbow mussels. During both the spring and summer sample times, two-way ANOVA showed that the patterns of variation among sites were not the same for the two species. Therefore, Asian clams could not reliably be used as surrogates for rainbow mussels to predict how cellulolytic enzyme activity would respond to environmental conditions.

Similar results were found for other assays during the spring and summer experiments. Glucose content, RNA:DNA ratio, F16BPase activity, PFK activity, AChE activity, and water content all showed differences between the two species in the responses to sites during the summer. The spring experiment resulted in differences between species for RNA:DNA ratio, glucose content, and glycogen content. The fall experiment produced differences between the species only for F16BPase activity. The

seasonal variation in the behavior of many of the measured parameters was also inconsistent between the clams and mussels. Such differences suggest that these two organisms interact very differently with their environments. Therefore, they are not likely to be equally tolerant of, or susceptible to, contaminant insults or habitat alterations. The Asian clam is a relatively short-lived animal that is adapted for rapid colonization of new habitats and frequent reproduction (McMahon, 1991). In contrast, mussels are generally long-lived animals, with annual reproductive efforts, and slow colonization and recovery rates (McMahon, 1991). Since the two species have such different life histories, it is not surprising that the measured physiological and biochemical parameters would behave differently in these taxa.

The seasonal patterns of PFK and FBPase activity were different in Asian clams and rainbow mussels. The clams exhibited a clear seasonal variation in the activity level of FBPase, but not in PFK activity. Gluconeogenic activity may be regulated at this locus, but glycolysis is probably regulated at some other point in the reaction sequence. An alternative possibility is that the glycolytic rate is relatively constant throughout the year. Given the evidence that Asian clams are relatively 'energy efficient', this species may be able to maintain a homeostatic metabolic rate (McMahon, 1991). The similarity of soft tissue mass to shell size in Asian clams (Doherty et al., 1990) supports such an hypothesis. Such a lack of variation in PFK activity differs from the rainbow mussels and the marine mussel *Mytilus galloprovincialis* (Ibarguren et al., 1990). The rainbow mussel exhibited approximately 5 and 20 times greater PFK activity during the summer than the spring and fall, respectively. However, the range of species investigated to this

point is small, and generalizations about taxonomic groups cannot be made.

The differences in the absolute magnitudes of PFK and FBPase activities in Asian clams and rainbow mussels were pronounced. The PFK activity was approximately 10-fold higher in mussels than in clams, indicating a much greater level of glycolytic metabolism. The Asian clam activity levels from 2-5 mU/mg tissue are close to the values reported for mantle tissue of the sea mussel *Mytilus galloprovincialis* (7.7 mU/mg tissue), cockle (*Cardium turberculatum*) foot muscle (6.1 mU/mg tissue), *Patella caerulea* foot muscle (9.6 mU/mg tissue), and freshwater mussel (*Anodonta cygnea*) ventricles (2.9 mU/mg tissue) (Villamarin, 1990, Lazou, 1991; Meinardus-Hagar and Gade, 1992; Michaelidis et al., 1993). The higher PFK activity levels measured in rainbow mussels also have precedence in the literature. PFK activity in foot tissue from the blue mussel *Mytilus edulis* and the snail *Helix pomatia* have been reported as 39 and 120 mU/mg tissue, respectively (Biethinger et al., 1991). These activity levels are similar to the values of 25-120 mU/mg tissue in rainbow mussels. Rainbow mussels, blue mussels, and *H. pomatia* appear to be more dependent on glycolytic energy to meet metabolic demands than some other animals, including molluscs. The FBPase activities measured in my study were higher than others reported in the literature. The fall and summer values for the mussels in this study are 2-10-fold higher than that reported for *M. galloprovincialis*, 17.6-85 mU/mg tissue (Barcia et al., 1992; Barcia and Ramos-Martinez, 1992). Most other reported values are significantly lower than the above activity levels (Barcia et al., 1991; Soengas et al., 1992; Soengas et al., 1993; Garcia-Rejon et al., 1997). While the FBPase activity levels in rainbow mussels from this study

were on the high side of those previously reported for mollusks, those of Asian clams were more than 10-fold greater. Such a high activity level for FBPase suggests that a much greater amount of assimilated energy is shunted toward glycogen synthesis in clams than in mussels (de Zwaan, 1979). An increased efficiency in energy utilization or food assimilation may provide Asian clams the competitive advantage needed to invade areas inhabited by unionids and to occupy habitats that are marginal for unionids.

In order to fully understand the implications of the PFK and FBPase activity results, further research is needed on the metabolic pathways of these two species. Since there are other possible control points for glycolysis/gluconeogenesis (Bohinski, 1987), the two species may simply have different mechanisms for regulating carbohydrate metabolism. Also enlightening would be the determination of the total glycolytic and non-glycolytic energy consumption of the two species. It is possible that unionids depend more heavily on glycolytic pathways to address metabolic needs than Asian clams. This could also have energetic ramifications because aerobic metabolism produces a much higher yield of ATP per molecule of carbohydrate than anaerobic (glycolytic) metabolism (Bohinski, 1987).

Future studies which focus on the differences in physiological responses of unionids and exotic bivalves would provide critical information needed to understand the dynamics of interactions between these possible competitors. Ideally, such studies would include laboratory components with controlled exposures to known stressors or conditions, and field studies to validate the results. The results of these studies would help managers predict where future invasions of exotics will likely occur and how severe

the problems will be. Additionally, this information could lead to the discovery of control strategies for exotics, such as Asian clams and zebra mussels, that do not adversely effect native unionids and sphearids.

Developing an understanding of the differences in the physiological responses of Asian clams and native unionids is valuable from an evolutionary viewpoint, as well as a management/conservation viewpoint. Current theory is that unionids evolved the complex life-history strategy, requiring a parasitic phase on fish, under the environmental conditions of a relatively stable habitat, while Asian clams evolved in much more variable habitats with strategies beneficial for invading new habitats (McMahon, 1991). The parasitic glochidial stage in unionids si believed to be an adaptation for increasing dispersal rates ((McMahon, 1991). However, Asian clams appear to have much greater dispersal potential in habitats where both occur. Asian clams may also have advantages in food assimilation, energy utilization, and habitat tolerance over unionids. Answers to these questions could help address how life-history and physiological adaptations are influenced by environmental conditions.

Seasonal Patterns in the Measured Biochemical Parameters

Closer examination of the individual biochemical parameters among sites and through time is useful for understanding the environmental physiology of freshwater bivalves. Very little information is available in the literature addressing the seasonal fluctuations in physiological or biochemical parameters of freshwater bivalves. My study addressed parameters that reflected the integrated energy status, glycolytic/gluconeogenic direction, protein synthesis, nutrient assimilation activity, and nervous system capacity.

When viewed collectively, these parameters begin to outline some of the environmental physiology of unionids and clams. Seasonal patterns were determined for some of these parameters. Understanding how natural environmental changes influence the physiology and biochemistry of freshwater bivalves will help in understanding how these animals will respond to anthropogenic stressors.

Glycogen and Glucose Content

Glycogen and glucose analyses provide information on the long-term energy status of the animals. Since mollusks depend on glycogen as the primary energy storage mechanism (de Zwann and Zandee, 1972; Pandian, 1975; Bayne and Newell, 1983, Patterson et al., 1997), the amount of glycogen stores present can strongly influence the fitness of the mussel or clam. No trend in glycogen content, free glucose content, or glucose:glycogen ratio was present that corresponded with stream size or river mile. Since my study used mussels deployed at five sites in the Clinch River and three sites in tributaries of the Clinch River, any such trends which existed should have been apparent.

The seasonal differences in the glycogen content of mussels provided evidence that they were capable of assimilating and storing more energy during the summer than during the fall. The summer build-up of glycogen coincides with the period of gametogenesis for *V. iris*, a bradyctictic species. Several other studies have reported such increases in glycogen content during non-reproductive and early gametogenic periods and declines toward the time gametes are released, implying the dependence on glycogen-derived energy for reproductive output (Hickman and Illingworth, 1980; Cantelmo-Cristini et al., 1985; Hummel et al., 1988; Sarkis, 1993). Asian clams did not exhibit a

strong seasonal variation in glycogen at any site. Reports that *C. fluminea* are capable of spawning throughout the year are supported by the lack of a definite tendency of glycogen to correlate with seasons (Cantelmo-Cristini et al., 1985). The variability among sites was greater in *C. fluminea* during the fall, suggesting that demands on the clams' energy reserves, due to limited food resources, reproductive activity, or environmental degradation, were accentuated during the fall. Additional factors can also influence the levels of glycogen in bivalves. Available food supplies affect glycogen stores in bivalves through the availability of excess or inadequate nutritional energy (Gabbott, 1983; Hummel et al., 1988). In many systems, chlorophyll a concentrations and phytoplankton densities increase in the spring and continue at relative high levels through summer, coinciding with the glycogen levels in marine mussels and clams (Zandee et al., 1980; Hummel et al., 1988). Therefore, separating the effects of gamete production, egg maturation, and food supplies is difficult. A bradyctictic mussel, such as *V. iris*, would be expected to exhibit low levels of glycogen during the spring, which is the time immediately before glochidial release (Cantelmo-Cristini et al., 1985; Hummel et al., 1988). However, increases in the quantity or quality of food resources could have offset the declines in glycogen stores.

Glucose content reflects the amount of carbohydrate energy available for immediate metabolic action and has been suggested as a sensitive marker for the flow of energy through the glycolytic pathway in marine bivalves (Ibarguren and Ramos-Martinez, 1991). Season did not affect the free glucose content of either mussels or clams consistently among sites. The fact that glucose did not vary consistently among

seasons indicated that the two test species did not alter the free glucose concentrations in response to coarse level seasonal influences, such as temperature, light cycle, or food availability.

The ratio of glucose to glycogen was clearly different among seasons for both species. The higher ratios during the fall, intermediate ratios in the spring, and low ratios in the summer are indications that the energetic demands, relative to nutritional resources, for freshwater bivalves are greatest during the fall of the year. Since I have found no other studies to evaluate the ratio of glucose to glycogen, there are no other data with which to compare my results. At first glance, it would appear that the ratio would be a mirror image of the glycogen content, due to the fact that glucose did not significantly vary among seasons. However, analytical results were more complicated. The variation present among individuals at a given site was much greater during the fall than during the summer. Since hyperglycemia has been shown to occur in some animals during environmental stress (Joosse and Geraerts, 1983; Fletcher, 1984), the extreme variability in these ratios provides evidence that the level of stress varies among individuals. The high level of variability was present in both mussels and clams. Interestingly, the data from the spring experiment exhibited levels of variability between that of fall and summer. The variability may reflect adaptation to different optimal temperature regimes (Bayne and Newell, 1983) or differences in feeding rates. The lack of an influence of gravidity on glycogen, glucose, or glucose:glycogen indicates that glochidial brooding does not significantly interfere with feeding or tax energy reserves.

Phosphofructokinase/Fructose-1,6-bisphosphatase Activity

The activities of the competing enzymes PFK and FBPase have been shown to be important regulators of the glycolytic and gluconeogenic rate in poikilotherms (Horecker et al., 1975; Tejwani, 1983; Biethinger et al., 1991; Ferguson and Storey, 1992).

Therefore, the activities of these two enzymes have the potential to indicate the glycolytic/gluconeogenic responses of freshwater bivalves to environmental changes. My results support the hypothesis that FBPase and PFK are control points in the gluconeogenic and glycolytic pathways of mussels.

Data in previous studies suggest that FBPase concentrations in marine bivalves peak during early spring and late summer/early autumn (Goromosova, 1976; Barcia et al., 1992). The variations in FBPase activity coincided with the fluctuation of protein content, indicating that FBPase responded to the balance between nutrient availability and environmental stress (Ibarguren et al., 1990; Barcia et al., 1992). PFK also has been shown to vary seasonally in bivalves (Ibarguren et al., 1990).

Seasonal variations for both enzymes were evident in my study. Mussels exhibited greater than 10-fold differences in FBPase activity between spring and fall samples. The low activity levels of FBPase in the spring coincide with the timing of glochidial maturation and with the increase of primary production in natural streams. The added metabolic costs of reproduction would prevent mussels from having excess nutrient reserves, and keep FBPase activities low (Goromosova, 1976). The high FBPase activity levels during the fall possibly indicate a preparatory response by mussels to generate energy reserves for overwintering. The pattern of PFK activity changes among

seasons supports this scenario. Unlike the marine mussels, which had a peak in PFK activity in fall and early spring (Ibarguren et al., 1990), the freshwater mussels exhibited a peak in PFK activity during the summer, intermediate activity in the spring, and low activity in the fall. Such a pattern indicated that glycolytic activity was highest in the summer when the temperature was highest. A positive relationship between temperature and PFK activity has been reported for the marine mussel *Mytilus edulis* (Churchill and Livingstone, 1989). Increasing temperatures are also generally associated with increases in metabolic rate in poikilotherms. Increased metabolic rates would tend to release more inorganic phosphorus as ATP is metabolized into ADP and AMP, which activates PFK in molluscs (Biethinger et al., 1991). Therefore, there is not enough information to determine whether the seasonal variations in PFK activity are physiologically controlled through covalent modification, allosteric inhibition, phosphorylation, or cellular particulate binding (Storey and Storey, 1990). Similarly, the cause of the seasonal variation in FBPase is not clear. However, it appears that these enzymes do play a role in determining the balance between glycolytic rates and gluconeogenic rates in the foot tissue of the rainbow mussel.

Cellulolytic Enzymes Activity

Seasonal differences in cellulolytic enzyme activity were apparent in the exocellulolytic activity, but not the endocellulolytic fraction. Mussels had highest exocellulase activities during the fall at all sites, while summer activity levels were generally lowest. In contrast, Asian clams had the highest exocellulase activities during the summer and lowest activities during the fall. The opposing nature of seasonal

exocellulase activity changes supports the hypothesis that rainbow mussels and Asian clams utilize food resources differently. The differences may be adaptively significant, giving one species an advantage over the other at critical times of year. Further studies in which species are supplied with foods that do or do not require exocellulytic activity would be needed to understand the biological significance of the difference.

Acetylcholinesterase Activity

The disparate AChEase activity levels present in rainbow mussels and Asian clams among seasons underlines the necessity for using concurrent controls for any field determinations of AChEase inhibition. Both clams and mussels tended to have greater AChEase activities during the summer than during the other two seasons. Such responses correspond to the positive relationship between temperature and AChEase activity reported in the literature (Edwards and Fisher, 1991). Reproductive status can also influence the activity of AChEase, either raising or lowering the activity (Rattner and Fairbrother, 1991). Therefore, comparing solely among seasons or sites, without the supporting information of adequate controls or normal seasonal trends at other sites, is ill advised.

RNA:DNA Ratio

The ratio of RNA to DNA can be indicative of protein synthesis in response to growth, repair due to stresses, or metabolic induction in response to contaminant exposure. The assay has been reported to be a useful parameter for determining recent nutritional condition and growth (Peakall, 1992). RNA:DNA ratio has been shown to

decrease upon exposure to contaminants in aquatic animals, probably associated with growth suppression (Mohapatra and Noble, 1992; Roesijadi et al., 1995). Since protein synthesis and enzyme induction during exposure to contaminants can cause increases in the RNA:DNA ratio in adult animals, interpretation of this assay is difficult. During my study, only mussels exhibited a difference in RNA:DNA among sites, and these differences were inconsistent. Since the differences detected during the summer were decreases in RNA:DNA, the possibility of higher ratios being caused by protein synthesis in response to induction or repair was dismissed. The lower ratio at Copper Creek during the summer indicated lower growth rates than at other sites, followed closely by Carbo, a site with a healthy mussel assemblage. The higher ratio in mussels caged at Little River in the spring suggests that the nutrient supply improves earlier here than at other sites. Glycogen content and cellulolytic activity were also high, relative to other sites, providing support for the conclusion. Due to the difficulties of interpretation and the variability and inconclusive nature of the results from this study, RNA:DNA ratio is not recommended as a useful biomarker in adult mussels.

Use of the Measured Biochemical Parameters as Biomarkers

The differences in glucose concentration among sites showed that environmental conditions could influence glucose. The direction of change was to decrease the glucose levels at the impacted sites during the spring, the season with the most pronounced differences. The freshwater pulmonate *Lymnaea stagnalis* has been reported to respond to stressors such as anoxia, NaCl, CuSO₄, and anesthetics by increasing hemolymph glucose (Joosse and Geraerts, 1983). Hyperglycemia was a rapid response to the stressor,

reaching high levels within six hours. Hyperglycemia also has been shown to occur in fish (*Limanda limanda*) exposed to air emersion, but these fish exhibited a hypoglycemic rebound (Fletcher, 1984). The decreased glucose levels in mussels held Below CRSP (relative to Carbo) and at Stock Creek may have reflected such a rebound, or may be explained by reductions in overall metabolic activity. However, glucose content is not likely to become a useful biomarker.

Glycogen content could prove to be a useful indicator of overall mussel or clam health with further understanding of the variability exhibited in the field. Glycogen concentration has been shown to decrease in the threeridge (*Amblema plicata*) upon exposure to zebra mussels (Haag et al., 1993). Patterson (1997) also found decreases in glycogen concentration in mussels placed in captive quarantine. However, these conditions were extremely altered from the mussels 'natural' condition. The seasonal and individual variability exhibited by animals sampled from existing wild populations has proven to be substantial, and is often large enough to mask more realistic impacts in rivers and streams (Naimo, pers. comm.). Further understanding of the dynamics of carbohydrate metabolism in freshwater bivalves may allow the detection of more subtle anthropogenic impacts on mussels and clams, but currently only severe impacts are detectable with this assay.

The differences in FBPase and PFK activities among sites during my experiments were inconsistent, indicating that either FBPase and PFK activities are ineffective at determining the energetic stressors occurring at the eight sites investigated or the sites were all suitable, from an energetic standpoint, during the experiments. There are few

studies in the literature which have reported on the responses of PFK and FBPase to contaminant or habitat changes, even though both enzymes have been shown to be control points for the gluconeogenic/glycolytic flux (De Zwaan, 1979; Cameselle et al., 1980; Ibarguren et al., 1990; Churchull and Storey, 1994). However, there are many studies that have investigated the response of these enzymes, especially PFK, to anoxia and exercise and have found marked changes in enzyme activity (Brooks et al, 1991; Lazou, 1991; Whitwam and Storey, 1991; Meinardus-Hagar and Gade, 1992; Michaelidis et al., 1993; Michaelidis and Athanasiadou, 1994). Thus, this pair of enzymes may be a better biomarker for acute impacts or major habitat quality shifts. Seasonal shifts in the enzyme activity must also be taken into account when comparing results.

The use of cellulolytic enzyme activity as a biomarker of environmental stress to freshwater bivalves has been supported by studies on exposure to heavy metals and zebra mussel (*Dreissena polymorpha*) fouling (Farris et al., 1988; Farris et al., 1989; Haag et al., 1993; Farris et al., 1994). Unionids (*Amblema plicata* and *Lampsilis radiata*) encrusted with zebra mussels exhibited decreases in cellulolytic enzyme activity of 44-66% in late summer, with 200± zebra mussels attached (Haag, et al., 1993). Farris (1989) reported decreases in cellulolytic activity of up to 90% upon exposure of Asian clams to zinc at concentrations of 34 µg/l and higher. Studies such as these have shown that cellulolytic activity is depressed under severely stressful conditions. However, in field deployment of Asian clams in the Clinch River, Farris et al. (1994) could find no statistical differences in cellulolytic enzyme activity among sites, even though some sites showed evidence of declining populations of native mussels. My study identified

differences among sites, but these differences were not consistent among seasons. For example, mussels held at Little River had the highest total cellulolytic activity levels during the spring experiment, but the lowest during the fall experiment. Similarly, Asian clams exhibited the highest total cellulolytic activities during summer at Copper Creek, while this site had the lowest levels during fall. Farris et al. (1994) also reported that animals caged in tributaries to the Clinch River tended to have lower levels of cellulolytic enzyme activity than those in the main river. The same trend was not evident in my study and may have been a function of the sampling season during the previous study. The results of this assay in field-deployed mussels and clams did not identify sites with good mussel populations from those with poor mussel populations. While cellulolytic enzyme activity may be effective at identifying contaminant exposure under lab conditions, it has yet to be proven under more variable, subtle field conditions.

The activity of acetylcholinesterase, the enzyme that degrades acetylcholine after neural transmission across a synapse, has been used as a biomarker for exposure to pesticides of the organophosphate and carbamate classes (Connell and Miller, 1984; Peakall, 1992). Reductions in cholinesterase activity have been widely accepted as a useful indicator of exposure to these pesticides post-mortem (Melancon, 1995). Mussels and clams in streams draining watersheds that are farmed are at risk of exposure to such compounds. Differences among sites and seasons were measured in clams and among seasons in mussels during my study. However, the relative site differences were not consistent among seasons. Clams held at Copper Creek had the highest activity levels of AChE during the spring and the second highest activity level during the fall, while the

activity level was the lowest during the summer. The activity level at Copper Creek in the summer was only about 40% of the expected value, based on the response of the clams at other sites. This is consistent with an episodic exposure to a cholinesterase inhibitor such as organophosphate or carbamate pesticides commonly used in agriculture (Edwards and Fisher, 1991).

The clams held Below CRSP, just downstream of the AEP power plant at Carbo, had AChEase activities among the lowest during all seasons, a possible indicator of chronic exposure to metals (Suresh et al., 1992; Reddy and Venugopal, 1993). Chronic exposure to pollutants or stressors can cause suppression of AChEase (Suresh et al., 1992; Reddy and Venugopal, 1993; Gruber and Munn, 1998). Several recent studies have reported suppression of AChEase activity upon exposure to contaminants other than pesticides. Suresh et al. (1992) reported that zinc and mercury concentrations of 6 and 0.1 mg/l, respectively, caused reductions in the AChEase activity of carp after only 1 day, and the effect continued for 30 days. Exposure to urban runoff, pulp and paper mill effluents, and PAH's may also cause AChEase activity levels to decrease in the muscle tissue of fish (Payne et al., 1996). The AEP power plant has a history of releasing zinc and copper in effluent waters, and the current fly ash burial facility is adjacent to the Clinch River, immediately upstream of the Below CRSP site in my study. The suppression of AChEase activity exhibited by clams caged Below CRSP, relative to those at Carbo, indicates that some stressor influenced these clams. Since many stressors can influence the activity of AChEase, the cause of the enzyme activity suppression cannot be identified (Rattner and Fairbrother, 1991). The close proximity of Carbo and Below

CRSP do suggest a stressor originating within this river reach, rather than a more widespread environmental stress. AChEase in marine organisms has been used as a successful biomonitor of pollution gradients (Bocquene et al., 1993; Bocquene et al., 1995). Recently, Fleming et al. (1995) reported evidence that a freshwater mussel die-off was attributable to cholinesterase-inhibiting compounds. However, no chemical evidence was found implying the presence of pesticides or metals, the two classes of pollutants that have been linked to decreases in AChEase activity. The total absence of pesticide residues in sediments and water is possible, even if this was the cause of the die-off, since the persistence of organophosphate and carbamate pesticides is short (Mineau, 1991). It is also possible that the decreased AChEase activities were the result of post-mortem degradation instead of contaminant exposure. This assay has exhibited promise in vertebrates as a biomarker for environmental stresses from pesticide or metals exposure, as well as dietary, seasonal, and reproductive stresses.

Acetylcholinesterase activity in my study showed minimal promise as a biomarker in freshwater bivalves. Other researchers have also found acetylcholinesterase activity to be marginally useful in unionids under field conditions (Augsbarger, pers. comm.). After accounting for the seasonal trends in AChEase activity, the only site that showed a decrease in activity was Copper Creek, a watershed which has a small amount of land tilled for tobacco and corn. The decline of 25-60% from the expected activity, based on the seasonal fluctuations at other sites, may approach the level of biological significance (Edwards and Fisher, 1991). Mussels caged in Little River also exhibited lower AChEase activities during the spring and summer than expected, based on the fall

activity levels and the health of the mussel population. This site also was downstream of a heavily utilized agricultural area, predominantly used as pasture land for cattle. The response of AChEase at these two sites during the seasons of agricultural activity indicated that this assay was useful in detecting some exposure to an environmental stressor, likely from agricultural run-off or activity.

While no single physiological parameter correlated with poor mussel populations among all seasons, the composite of all of them did indicate that sites with depauperate mussel populations were less suitable. Determining which of the parameters should be included in a chosen suite of biomarkers is difficult. The selection of endocellulase activity, exocellulase activity, FBPase activity, and water content of digestive gland as the best biomarkers for mussels by the best subsets regression analysis was tenuous. The four parameters were not included in the best subsets during all three seasons. Perhaps adult rainbow mussels are very tolerant of degraded habitat conditions and do not exhibit large physiological or biochemical changes in response to moderate perturbations. However, best subsets regression analysis for Asian clams identified FBPase and water content as two of the parameters during all three seasons. Therefore, FBPase and water content should be considered in any biomarker development program. After measuring a broad range of potential biomarkers, it is clear that the cost and complexity of developing a reliable suite of biomarkers for adult unionids would be substantial in both money and manpower. The species specific and seasonal variability inherent in many physiological parameters adds to the enormous difficulty of this endeavor. I recommend that future efforts be focussed on developing biomarker protocols for the juvenile life stage.

Juvenile mussels are more sensitive to some contaminants than adults (Jacobson, 1993), and this is likely to hold for many pollutants. One likely candidate biomarker is the measurement of heatshock proteins, which requires very little tissue and responds to many types of stressors (Peakall, 1992).

Summary

The measured physiological parameters vary as a result of environmental differences among both sites and seasons. Seasonal fluctuations were markedly evident in most of the biomarkers tested, rendering them dependent on prior determination of their seasonal dynamics. None of the biomarkers clearly identified all of the impacted sites at all times, but some did show promise such as cellulolytic enzyme activity and fructose-1,6-bisphosphatase activity. The differences in the response of the physiological parameters among sites is likely due to different stressors or conditions at these sites. There is now clear evidence that the rainbow mussel and Asian clam respond very differently to site-specific environmental stresses and to seasonal variation. For this reason, Asian clams cannot serve as surrogate organisms to detect anthropogenic stress on native unionids. In addition, differences in the control points and dynamics of carbohydrate metabolism were detected between these two species. The apparently greater ability of Asian clams to build and maintain energy reserves likely gives them a competitive advantage over native unionids.

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APPENDIX 1

Rank scores of sites for each assay used in the composite biomarker analysis. For each assay, sites were ranked relative to each other and the sum of the ranks was used as the composite score.

FALL													
Site	glucose	glycogen	glu/gly	RNA:DNA	d efflu	rel. sugar	F16bPase	PFK	AChEase	AChEase	AChEase	TOTAL	
					uent				wet wt	prot	prot		
Mussels													
CR1	4	3	5	4	4	4	4	6	5	4	4	3	42
CR2	1	6	3	3	6	5	5	5	4	1	1	1	32
CR3	3	5	2	3	5	6	3	2	2	2	2	4	35
CR4	2	4	1	2	2	2	2	2	1	3	3	2	21
CC	5	2	4	1	3	3	3	4	3	5	5	5	35
LR	6	1	6	6	1	1	1	1	6	6	6	6	34
Clams													
CR1	5	3	5	3	2	3	3	3	2	3	3	3	29
CR2	4	2	3	2	3	3	2	4	1	4	4	4	25
CR3	3	4	4	1	5	5	1	4	4	1	2	1	28
CR4	1	5	2	5	4	4	4	5	3	3	2	2	31
CC	2	1	1	4	1	1	1	2	5	5	5	5	22
SPRING													
Site	glucose	glycogen	glu/gly	RNA:DNA	d effluent	rel. sugar	F16bPase	PFK	AChEase	AChEase	AChEase	TOTAL	
									wet wt	prot	prot		
Mussels													
CR3	4	5	3	5	3	3	4	4	1	4	4	4	36
CR4	3	2	2	4	4	4	4	5	2	1	1	1	28
SC	1	1	4	3	1	1	1	1	5	5	5	5	27
LR	5	4	5	2	5	5	5	3	4	3	3	3	39
START	2	3	1	1	2	2	2	1	3	2	2	2	19
Clams													
CR3	1	4	5	5	2	1	2	2	3	3	3	4	30
CR4	4	2	2	3	4	4	4	4	2	2	1	1	27
CC	3	3	3	2	5	5	5	1	1	6	6	6	35
SC	2	1	4	4	3	3	3	3	4	4	4	2	30
LR	5	5	1	6	6	6	6	6	5	5	2	2	45
START	5	5	1	1	1	2	2	5	6	5	5	5	25

SUMMER													
Site	glucose	glycogen	glu/gly	RNA:DNA	d effluent	rel. sugar	F16bPase	PFK	ACHase wet wt	ACHase prot	TOTAL		
Mussels													
CRO	4	5	3	3	6	7	5	2	7	7	7	49	
CR1	3	2	4	6	8	8	7	4	6	6	3	51	
CR2	2	6	6	2	5	4	6	7	5	5	6	49	
CR3	8	3	5	7	3	5	4	1	8	8	8	52	
CR4	7	4	2	5	2	2	1	6	2	2	4	35	
CC	6	1	1	8	7	6	2	3	1	1	1	36	
LR	5	7	7	1	4	3	2	5	3	3	5	43	
START	1	8	8	4	1	1	8		4	4	2	37	
Clams													
CRO	4	5	4	6	2	3	2	7	7	7	8	48	
CR1	2	2	2	1	1	2	6	4	5	5	6	31	
CR2	5	4	5	4	4	4	7	2	8	8	7	50	
CR3	6	7	7	8	3	1	8	1	4	4	4	49	
CR4	3	3	3	2	6	7	5	3	3	3	2	37	
CC	8	1	6	5	7	5	4	6	1	1	1	44	
LR	1	6	1	3	5	6	3	5	6	6	5	41	
START	7	8	8	7		6	1	8	2	2	3	44	

APPENDIX 2

Minitab output of best subsets regressions for *Villosa iris* during the fall experiment. The X indicates when each parameter was included in the model. The best two models with each number of parameters are presented. Higher adjusted-R² values indicate better models. glu/mg=glucose/mg tissue; gly/mg=glycogen/mg tissue; dry/wet=mantle dry weight/wet weight; glu/gly=(glucose/mg)/(glycogen/mg); F16bP=fructose-1,6-bisphosphatase activity; PFK=phosphofructokinase activity; AChE=acetylcholinesterase activity; F16/PFK=fructose-1,6-bisphosphatase activity / phosphofructokinase activity; F16*PFK= fructose-1,6-bisphosphatase activity X phosphofructokinase activity; exen10⁵=exocellulase activity X endocellulase activity X 10⁵; ex/en-4= exocellulase activity / endocellulase activity X 10⁴; ratio/w=ratio of glucose to glycogen / tissue weight.

Vars	R-sq	Adj. R-sq	C-p	s	F P A e												
					d	g	l	F	C	F	F	x	e	r			
					g	g	r	l	6	K	h	1	1	e	x	a	
					l	l	y	u	b	(E	6	6	n	/	t	
					u	y	/	/	P	m	(/	*	1	e	i	
					/	/	w	g	(U	m	P	P	0	n	o	
					m	m	e	l	U	/	U	F	F	^	-	/	
					g	g	t	y)	m	/	K	K	5	4	w	
1	10.0	6.3	-4.5	1.6203													X
1	3.6	0.0	-3.3	1.6773					X								
2	15.3	8.0	-3.6	1.6058	X												X
2	14.6	7.2	-3.4	1.6125													X
3	19.9	9.0	-2.5	1.5967					X		X						X
3	18.2	7.0	-2.1	1.6140					X								X
4	23.4	8.9	-1.1	1.5979	X				X		X						X
4	23.4	8.8	-1.1	1.5985						X	X	X	X				
5	27.2	9.0	0.1	1.5970	X					X	X	X	X				
5	25.9	7.4	0.4	1.6104	X				X	X	X	X	X				
6	28.8	6.3	1.8	1.6204	X	X				X	X	X	X				
6	28.1	5.4	2.0	1.6282	X					X	X	X	X				X
7	30.4	3.3	3.5	1.6458	X		X	X		X				X	X	X	
7	29.5	2.1	3.7	1.6559	X	X		X	X	X	X	X	X				
8	31.3	0.0	5.3	1.6825	X		X	X	X	X	X	X	X	X	X	X	
8	31.0	0.0	5.4	1.6858	X	X	X	X	X	X	X	X	X	X	X	X	
9	32.1	0.0	7.2	1.7241	X		X	X	X	X	X	X	X	X	X	X	
9	32.0	0.0	7.2	1.7257	X	X	X	X	X	X	X	X	X	X	X	X	
10	32.8	0.0	9.0	1.7715	X	X	X	X	X	X	X	X	X	X	X	X	
10	32.5	0.0	9.1	1.7753	X	X	X	X	X	X	X	X	X	X	X	X	
11	33.0	0.0	11.0	1.8307	X	X	X	X	X	X	X	X	X	X	X	X	
11	32.8	0.0	11.0	1.8336	X	X	X	X	X	X	X	X	X	X	X	X	
12	33.0	0.0	13.0	1.8997	X	X	X	X	X	X	X	X	X	X	X	X	

Minitab output of best subsets regressions for *Villosa iris* during the spring experiment. The X indicates when each parameter was included in the model. The best two models with each number of parameters are presented. Higher adjusted-R² values indicate better models. glu/mg=glucose/mg tissue; gly/mg=glycogen/mg tissue; dry/wet=mantle dry weight/wet weight; glu/gly=(glucose/mg)/(glycogen/mg); F16bP=fructose-1,6-bisphosphatase activity; PFK=phosphofructokinase activity; AChE=acetylcholinesterase activity; F16/PFK=fructose-1,6-bisphosphatase activity / phosphofructokinase activity; F16*PFK= fructose-1,6-bisphosphatase activity X phosphofructokinase activity; exen10⁵=exocellulase activity X endocellulase activity X 10⁵; ex/en-4= exocellulase activity / endocellulase activity X 10⁻⁴; ratio/w=ratio of glucose to glycogen / tissue weight; H2Ocont=percent water content of foot tissue; RNA:DNA=ratio of RNA to DNA.

Vars	R-sq	Adj. R-sq	C-p	s	(d g l F C F F x e a H R g g r l 6 K h 1 1 e x t 2 N l l y u b (E 6 6 n / i O A u y / / P m (/ * 1 e o c : / / w g U m P P 0 n / o D m m t l m) U F F ^ - w n N g g) y U /) K K 5 4 t t A
1	20.9	18.8	-0.6	1.3040	X
1	14.2	11.9	2.4	1.3587	X
2	25.4	21.3	-0.5	1.2839	X X
2	23.2	18.9	0.5	1.3030	X X
3	27.1	20.8	0.8	1.2876	X X X
3	26.4	20.1	1.1	1.2937	X X X
4	30.3	22.1	1.4	1.2773	X X X X
4	29.9	21.7	1.5	1.2810	X X X X
5	40.6	31.6	-1.1	1.1971	X X X X X
5	36.7	27.1	0.6	1.2358	X X X X X
6	41.6	30.7	0.4	1.2050	X X X X X X
6	41.2	30.1	0.6	1.2097	X X X X X X
7	42.8	29.9	1.9	1.2121	X X X X X X
7	42.4	29.3	2.1	1.2164	X X X X X X X
8	43.5	28.4	3.6	1.2245	X X X X X X X
8	43.3	28.2	3.7	1.2262	X X X X X X X
9	44.3	27.0	5.3	1.2369	X X X X X X X
9	44.0	26.6	5.4	1.2400	X X X X X X X
10	44.6	24.8	7.2	1.2553	X X X X X X X
10	44.4	24.5	7.2	1.2571	X X X X X X X
11	44.8	22.2	9.1	1.2762	X X X X X X X
11	44.6	22.1	9.1	1.2774	X X X X X X X
12	44.9	19.4	11.0	1.2991	X X X X X X X
12	44.8	19.3	11.1	1.2999	X X X X X X X
13	44.9	16.2	13.0	1.3245	X X X X X X X
13	44.9	16.2	13.0	1.3247	X X X X X X X
14	44.9	12.8	15.0	1.3516	X X X X X X X

Minitab output of best subsets regressions for *Villosa iris* during the summer experiment. The X indicates when each parameter was included in the model. The best two models with each number of parameters are presented. Higher adjusted-R² values indicate better models. glu/mg=glucose/mg tissue; gly/mg=glycogen/mg tissue; dry/wet=mantle dry weight/wet weight; glu/gly=(glucose/mg)/(glycogen/mg); F16bP=fructose-1,6-bisphosphatase activity; PFK=phosphofructokinase activity; AChE=acetylcholinesterase activity; F16/PFK=fructose-1,6-bisphosphatase activity / phosphofructokinase activity; F16*PFK= fructose-1,6-bisphosphatase activity X phosphofructokinase activity; exen10⁵=exocellulase activity X endocellulase activity X 10⁵; ex/en-4= exocellulase activity / endocellulase activity X 10⁻⁴; ratio/w=ratio of glucose to glycogen / tissue weight; H2Ocont=percent water content of foot tissue; RNA:DNA=ratio of RNA to DNA.

Vars	R-sq	Adj. R-sq	C-p	s	(d g l F C f f x e a H R g g r l 6 K h l l e x t 2 N l l y u b (E 6 6 n / i O A u y / / P m / * 1 e o c : / / w g U (p p 0 n / o D m m t l m) m f f ^ - w n N g g) y U / U k k 5 4 t t A
1	9.8	7.5	-0.1	2.0693	X
1	8.5	6.3	0.4	2.0833	X
2	17.7	13.5	-1.4	2.0010	X X
2	14.9	10.5	-0.2	2.0357	X
3	20.8	14.5	-0.7	1.9892	X X X
3	20.6	14.3	-0.6	1.9915	X X X
4	24.6	16.5	-0.3	1.9668	X X X
4	24.4	16.2	-0.2	1.9698	X X X
5	28.6	18.7	0.0	1.9406	X X X X
5	27.2	17.1	0.6	1.9586	X X X X
6	29.7	17.7	1.5	1.9522	X X X X X
6	29.1	17.0	1.8	1.9609	X X X X X
7	31.1	16.9	3.0	1.9620	X X X X X
7	30.6	16.3	3.2	1.9681	X X X X X
8	32.1	15.6	4.5	1.9764	X X X X X
8	31.9	15.4	4.6	1.9788	X X X X X
9	34.2	15.7	5.7	1.9759	X X X X X
9	33.6	14.9	5.9	1.9849	X X X X X
10	34.8	13.8	7.4	1.9980	X X X X X
10	34.7	13.7	7.4	1.9994	X X X X X
11	35.3	11.6	9.2	2.0227	X X X X X
11	35.2	11.4	9.2	2.0257	X X X X X
12	35.5	8.8	11.1	2.0546	X X X X X
12	35.5	8.8	11.1	2.0552	X X X X X
13	35.7	5.8	13.0	2.0879	X X X X X
13	35.6	5.7	13.1	2.0900	X X X X X
14	35.7	2.4	15.0	2.1255	X X X X X

Minitab output of best subsets regressions for *Corbicula fluminea* during the fall experiment. The X indicates when each parameter was included in the model. The best two models with each number of parameters are presented. Higher adjusted-R² values indicate better models. glu/mg=glucose/mg tissue; gly/mg=glycogen/mg tissue; dry/wet=mantle dry weight/wet weight; glu/gly=(glucose/mg)/(glycogen/mg); eff chng=change in effluent time of CMC solution after incubation with visceral homogenate; rel/wt=sugar released in CMC solution after incubation with visceral homogenate; F16bP=fructose-1,6-bisphosphatase activity; PFK=phosphofructokinase activity; AChE=acetylcholinesterase activity; F16/PFK=fructose-1,6-bisphosphatase activity / phosphofructokinase activity; F16*PFK= fructose-1,6-bisphosphatase activity X phosphofructokinase activity; exen10⁵=exocellulase activity X endocellulase activity X 10⁵; ex/en-4= exocellulase activity / endocellulase activity X 10⁻⁴; ratio/w=ratio of glucose to glycogen / tissue weight; H2Ocont=percent water content of foot tissue; RNA:DNA=ratio of RNA to DNA.

Vars	R-sq	Adj. R-sq	C-p	s	g	g	e	r	(F	P	A	e
1	25.5	22.9	7.5	1.2632									
1	15.2	12.2	12.2	1.3477									
2	35.9	31.2	4.9	1.1932									
2	31.3	26.2	6.9	1.2356	X								
3	39.3	32.3	5.3	1.1832	X								
3	38.5	31.4	5.7	1.1915	X								
4	45.9	37.3	4.4	1.1392	X								
4	45.3	36.6	4.6	1.1457	X								
5	55.2	45.8	2.2	1.0585	X								
5	54.0	44.5	2.7	1.0719	X								
6	59.2	48.6	2.4	1.0313									
6	58.9	48.2	2.5	1.0352									
7	63.5	51.9	2.4	0.99765									
7	63.5	51.8	2.5	0.99840	X								
8	66.9	54.3	2.9	0.97283	X								
8	66.3	53.5	3.2	0.98137	X								
9	67.7	53.1	4.6	0.98499	X								
9	67.3	52.6	4.7	0.99037	X								
10	68.0	51.2	6.4	1.0050	X								
10	68.0	51.2	6.4	1.0050	X								
11	68.8	49.7	8.0	1.0197	X								
11	68.7	49.6	8.1	1.0209	X								
12	68.9	46.9	10.0	1.0485	X								
12	68.8	46.8	10.0	1.0490	X								
13	68.9	43.6	12.0	1.0803	X								
13	68.9	43.6	12.0	1.0806	X								
14	68.9	39.9	14.0	1.1152	X								
14	68.9	39.8	14.0	1.1156	X								
15	68.9	35.6	16.0	1.1543	X								

Minitab output of best subsets regressions for *Corbicula fluminea* during the spring experiment. The X indicates when each parameter was included in the model. The best two models with each number of parameters are presented. Higher adjusted-R² values indicate better models. glu/mg=glucose/mg tissue; gly/mg=glycogen/mg tissue; dry/wet=mantle dry weight/wet weight; glu/gly=(glucose/mg)/(glycogen/mg); eff chng=change in effluent time of CMC solution after incubation with visceral homogenate; rel/wt=sugar released in CMC solution after incubation with visceral homogenate; F16bP=fructose-1,6-bisphosphatase activity; PFK=phosphofructokinase activity; AChE=acetylcholinesterase activity; F16/PFK=fructose-1,6-bisphosphatase activity / phosphofructokinase activity; F16*PFK= fructose-1,6-bisphosphatase activity X phosphofructokinase activity; exen10⁵=exocellulase activity X endocellulase activity X 10⁵; ex/en-4= exocellulase activity / endocellulase activity X 10⁴; ratio/w=ratio of glucose to glycogen / tissue weight; H2Ocont=percent water content of foot tissue; RNA:DNA=ratio of RNA to DNA.

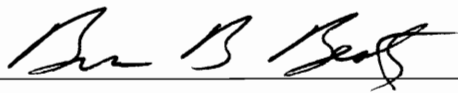
Vars	R-sq	Adj. R-sq	C-p	s	g	g	e	r	(F	P	A	e
					/	/	y	A	g	t	t	n	0
1	26.5	21.6	7.0	1.4427				X					
1	17.1	11.6	9.5	1.5320									X
2	41.4	33.0	4.9	1.3334	X	X							
2	35.8	26.6	6.4	1.3955		X						X	
3	57.1	47.1	2.7	1.1845	X	X	X						
3	54.6	44.1	3.3	1.2180	X	X	X						
4	60.9	47.8	3.6	1.1768	X	X	X						X
4	59.2	45.7	4.1	1.2010	X	X	X					X	
5	67.5	52.7	3.8	1.1208	X	X	X	X					X
5	66.1	50.7	4.2	1.1436	X	X	X	X					X
6	72.5	56.0	4.5	1.0805	X	X	X		X	X			X
6	72.1	55.3	4.6	1.0895	X	X	X		X	X			X
7	73.4	52.8	6.2	1.1196	X	X	X	X	X	X			X
7	73.3	52.6	6.2	1.1215	X	X	X	X		X			X
8	79.7	59.4	6.5	1.0376	X	X	X	X		X	X		X
8	76.9	53.9	7.3	1.1064	X	X	X	X		X			X
9	80.8	56.1	8.2	1.0794	X	X	X	X	X	X			X
9	80.5	55.4	8.3	1.0883	X	X	X	X	X	X			X
10	81.6	50.9	10.0	1.1417	X	X	X	X	X	X	X		X
10	81.3	50.2	10.1	1.1495	X	X	X	X	X	X	X		X
11	82.9	45.4	11.6	1.2043	X	X	X	X	X	X	X	X	X
11	82.7	44.7	11.7	1.2119	X	X	X	X	X	X	X	X	X
12	85.5	42.2	12.9	1.2392	X	X	X	X	X	X	X	X	X
12	85.3	41.2	13.0	1.2494	X	X	X	X	X	X	X	X	X
13	94.2	69.2	12.6	0.90401		X	X	X	X	X	X	X	X
13	89.8	45.4	13.8	1.2035	X	X	X	X	X	X	X	X	X
14	95.9	67.1	14.1	0.93493	X	X	X	X	X	X	X	X	X
14	94.6	56.5	14.5	1.0747	X	X	X	X	X	X	X	X	X
15	96.3	41.1	16.0	1.2500	X	X	X	X	X	X	X	X	X

Minitab output of best subsets regressions for *Corbicula fluminea* during the summer experiment. The X indicates when each parameter was included in the model. The best two models with each number of parameters are presented. Higher adjusted-R² values indicate better models. glu/mg=glucose/mg tissue; gly/mg=glycogen/mg tissue; dry/wet=mantle dry weight/wet weight; glu/gly=(glucose/mg)/(glycogen/mg); eff chng=change in effluent time of CMC solution after incubation with visceral homogenate; rel/wt=sugar released in CMC solution after incubation with visceral homogenate; F16bP=fructose-1,6-bisphosphatase activity; PFK=phosphofructokinase activity; AChE=acetylcholinesterase activity; F16/PFK=fructose-1,6-bisphosphatase activity / phosphofructokinase activity; F16*PFK= fructose-1,6-bisphosphatase activity X phosphofructokinase activity; exen10⁵=exocellulase activity X endocellulase activity X 10⁵; ex/en-4= exocellulase activity / endocellulase activity X 10⁴; ratio/w=ratio of glucose to glycogen / tissue weight; H2Ocont=percent water content of foot tissue; RNA:DNA=ratio of RNA to DNA.

Vars	R-sq	Adj. R-sq	C-p	s	g	g	e	r	(F	P	A	e
					l	l	g	R	f	a	H	1	F
					u	y	l	N	f	r	t	2	6
					((u	A	e	i	O	b	(
					u	u	/	:	c	l	o	c	P
					g	g	g	D	h	/	/	o	U
))	l	N	n	w	w	n	U
					/	/	y	A	g	t	t	t	n
					X								
1	23.2	21.3	27.2	2.0041									
1	22.4	20.5	27.8	2.0140	X								
2	39.8	36.7	15.1	1.7967	X	X							
2	36.0	32.7	18.3	1.8522	X					X			
3	41.8	37.2	15.3	1.7891	X	X	X			X			
3	41.4	36.8	15.7	1.7954	X	X	X	X					
4	54.5	49.6	6.6	1.6038	X	X	X					X	
4	53.8	48.8	7.2	1.6163	X	X	X	X					
5	57.3	51.4	6.2	1.5753	X	X	X	X				X	
5	56.7	50.6	6.8	1.5869	X	X	X			X	X		
6	57.5	50.2	8.0	1.5931	X	X	X			X	X		X
6	57.5	50.2	8.1	1.5944	X	X	X	X	X				
7	59.5	51.2	8.3	1.5779	X	X	X			X	X	X	X
7	58.4	49.9	9.3	1.5993	X	X	X	X		X	X		X
8	61.8	52.6	8.4	1.5555	X	X	X			X	X	X	X
8	61.2	51.8	8.9	1.5678	X	X	X	X		X	X	X	X
9	62.9	52.4	9.5	1.5576	X	X	X			X	X	X	X
9	62.5	52.0	9.8	1.5649	X	X	X			X	X	X	X
10	64.1	52.5	10.5	1.5573	X	X	X	X		X	X	X	X
10	63.9	52.3	10.6	1.5596	X	X	X			X	X	X	X
11	65.1	52.3	11.6	1.5592	X	X	X			X	X	X	X
11	64.9	52.1	11.7	1.5632	X	X	X	X		X	X	X	X
12	66.6	52.7	12.4	1.5530	X	X	X			X	X	X	X
12	66.2	52.2	12.7	1.5616	X	X	X	X	X	X	X	X	X
13	68.1	53.3	13.0	1.5430	X	X	X	X	X	X	X	X	X
13	67.4	52.3	13.6	1.5598	X	X	X	X	X	X	X	X	X
14	69.1	53.0	14.2	1.5481	X	X	X	X	X	X	X	X	X
14	68.4	52.1	14.8	1.5636	X	X	X	X	X	X	X	X	X
15	69.3	51.7	16.0	1.5703	X	X	X	X	X	X	X	X	X

VITA

Braven Brock Beaty was born in Culver City, California in 1962 to Margaret B. and R. Carroll Beaty. He was raised mostly in Raleigh, North Carolina, where he went to high school at W. G. Enloe High School. Braven went on to study Biomedical Engineering at Duke University, and graduated with BS in Engineering in May, 1984. He worked in a research lab for the next several years investigating the viscoelastic properties of cell membranes when deciding he wanted to further his education. Braven enrolled part-time as a master's student in the Duke University Nicholas School of the Environment and studied ecotoxicology in fish under Dr. Richard DiGiulio. He finished his MS degree in August, 1992 and began working on his doctorate at Virginia Tech under the advising of Dr. Richard Neves. Braven's interests are now in the areas of stream ecology, anthropogenic impacts on aquatic fauna, the biology and ecology of freshwater mussels, and conservation biology. He is currently working as a Stewardship Ecologist for The Nature Conservancy in the Clinch Valley Program.



Braven B. Beaty