UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

DIGESTIVE PHYSIOLOGY AND DIETARY OVERLAP OF AQUATIC INVERTEBRATES IN THE UPPER MISSISSIPPI RIVER BASIN

A Research Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of Master of Science in Cellular and Molecular Biology

Blake William Sauey

College of Science and Health
Cellular and Molecular Biology

August, 2013

DIGESTIVE PHYSIOLOGY AND DIETARY OVERLAP OF AQUATIC INVERTEBRATES IN THE UPPER MISSISSIPPI RIVER BASIN

By Blake W. Sauey

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Cellular and Molecular Biology.

The candidate has completed the oral defense of the thesis.

Roger Haro, Ph.D.
Thesis Committee Chairperson — Aug. 21, 2013 — Date

Jon Amberg Ph.D.
Thesis Committee Member — 21 Aug 2013 — Date

Mark Gaikowski, M.A.
Thesis Committee Member — 21 Aug 2013 — Date

Scott Cooper, Ph.D.
Thesis Committee Member — Aug. 21, 2013 — Date

Sandra Grunwald, Ph.D.
Thesis Committee Member — Aug. 21, 2013 — Date

Thesis Accepted

Steven Simpson, Ph.D.
Graduate Studies Director — 9/25/13 — Date

ABSTRACT

Sauey, B.W. Digestive physiology and dietary overlap of aquatic invertebrates in the upper mississippi river basin. MS in Cellular and Molecular Biology, August 2013, 82pp. (R. Haro)

Zebra mussels *Dreissena polymorpha* are invasive species in North America posing significant threats to native ecosystems and organisms. Costs to waste-water treatment and electrical power plants are in the hundreds of millions of dollars. Resource managers lack control tools that selectively target zebra mussels. One potential avenue to control zebra mussels is their digestive physiology, of which little information is known. If specific enzyme(s) can be identified as unique to zebra mussels, a species-specific biocide potentially can be formulated. The api®ZYM kit was modified to screen 19 enzymes in the digestive tract of zebra mussels to identify potential differences between zebra mussels and native invertebrates. Digestive enzymes from zebra mussels, threeridge mussels *Amblema plicata*, and a caddisfly *Hydropsyche orris* were screened over four months at three sites along the Mississippi River to observe relative enzyme profiles. Results suggest sample location and time did not affect digestive enzyme profiles; however species were different ($p < 0.01$). Four enzymes (alkaline phosphatase, acid phosphatase, leucine arylamidase, and N-acteyl-β-glucosaminidase) were quantified using specific enzyme kits. Zebra mussel acid phosphatase did not respond to temperature decrease. Acid phosphatase may be exploitable to develop a selectively targeted control tool.

ACKNOWLEDGEMENTS

I thank Dr. Jon Amberg, Mark Gaikowski, Dr. Roger Haro, Dr. Scott Cooper, and Dr. Sandra Grunwald for their support and guidance with every aspect of my thesis. I thank Nathan Jensen, Kristin Shuda, Sunnie McCalla, Lauren Turinetti, Patricia Ries, Ashley Hunt and Alissa Ganser for their help with field collections and lab work. I thank Shelby Storsveen for her work with the validation of the api®ZYM test kit. I thank Robert Kratt and JC Nelson for their time making illustrations and maps. I thank Nathan De Jager for his assistance in data analysis. I also thank the US EPA GLRI and USGS Science Support Program for their funding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Zebra mussels (*Dreissena polymorpha*) originated from the Azov, Black and Caspian seas (Mackie and Schloesser, 1996) and spread across North America in ballast water (Mackie, 1991) and by recreational boating (Johnson et al. 2001). Since their introduction in the Laurentian Great Lakes in the late 1980's, zebra mussels have spread across the U.S. causing significant economic damage. Zebra mussels have been known to clog water intake pipes for water treatment plants and power generation facilities costing these industries $267 million from 1989 to 2004 in the United States (Connelly et al. 2007; Strayer, 2009). Other industries, such as fisheries, shipping, and tourism, have also been impacted by zebra mussels. For example, it has been estimated that in the New York State Canal and the Hudson River systems, dreissenid mussels cost approximately $12.5 million per year and that for the Great Lakes Basin it was estimated dreissenid mussels cost approximately $500 million per year to the same industries (Pimentel et al. 2005).

Besides economic impacts, zebra mussels affect aquatic systems where they have become established. Zebra mussels have been found to decrease dissolved oxygen (Effler et al. 1996; Caraco et al. 2000; Effler et al. 2004; Strayer, 2009), reduce phytoplankton biomass (Holland, 1993; Effler et al. 1996; Caraco et al. 1997; Effler et al. 2004; Strayer, 2009), allow light to penetrate deeper in the water (Holland, 1993; Effler et al. 1996; Caraco et al. 1997; Effler et al. 2004; Barbiero et al., 2006; Zhu et al., 2006; Strayer, 2009), increase soluble reactive phosphorous and total ammonia concentrations (Effler et al. 1996; Caraco et al. 1997; Effler et al. 2004; Strayer 2009), lower pH of water systems

(Effler et al. 1996; Effler et al. 2004; Strayer 2009), decrease the alkalinity of water systems via reduction in calcium concentrations (Barbiero et al., 2006; Strayer, 2009) and can stimulate toxic cyanobacterial blooms (Knoll et al. 2008; Vanderploeg et al 2001; Caraco et al. 2006; Raikow et al. 2004; Strayer, 2009). Zebra mussels have also been shown to affect the flow of energy and nutrients from the pelagic to benthic areas (Gergs et al., 2009; Miehls et al., 2009; Ozersky et al., 2012).

Zebra mussels are also known to affect the species assemblage of a lentic or a lotic habitat. With increased light penetration due to zebra mussels removing phytoplankton, macrophytes can colonize deeper waters than in systems without zebra mussels (Zhu et al., 2006; Strayer, 2009). Following zebra mussel infestation, the abundance of pelagic fishes decreases, while the abundance of littoral fishes increases (Strayer et al., 2004; Strayer, 2009). Zebra mussels can also impact other macroinvertebrates. Zebra mussel shells provided habitat for other benthic macroinvertebrates (Stewart et al., 1998; Ward and Ricciardi, 2007; Strayer, 2009), allowing their populations to increase. However, in areas lacking zebra mussels or zebra mussel shells, macroinvertebrate populations are decreased (Nalepa et al., 1998; Lozano et al., 2001; Strayer, 2009).

One family that has been significantly affected by zebra mussels has been the Unionidae. Unionid mussels are one of the most diverse taxa worldwide and are also one of the most endangered groups in North America (Williams et al. 1993). Two hundred and thirteen species (71.7%) of North American unionid mussel are endangered, threatened, or of special concern (Williams et al. 1993). The decline in unionid populations has been attributed to the destruction of habitat, over-harvesting and the

presence of zebra mussels (Strayer et al. 2004). Zebra mussels colonize on the hard shell of native unionid mussels using a byssal thread for attachment (Schloesser et al. 1996; Vanderploeg et al., 2002). More than 20 zebra mussels can colonize on a single native unionid mussel (Schloesser et al. 1996). Not only do zebra mussels colonize on native unionids, but these colonies are thought to steal food resources from the native unionid (Baker and Levinton, 2003; Hörmann and Maier, 2006; Schloesser et al. 1996). It has been reported that glycogen concentrations in native mussels infested with zebra mussels was less than in those from un-infested native mussels (Sousa et al., 2011; Haag et al., 1993). Baker and Hornbach (2000) found that total carbohydrate and protein levels were lower in *Amblema plicata* infested with zebra mussels than those not infested. It has been hypothesized that these infested unionids were actually starving (Baker and Hornbach, 2000).

Zebra mussels are typically thought to be much more efficient filter feeders than unionids (Vanderploeg et al., 1995; Luoma et al., unpublished data). Filter-feeding is based upon physical occlusion of particles. Species-specificity is determined by how the particles are filtered and particle size; however, both zebra mussels and native mussels filter particles the same way and can filter particles as small as 1 µm (Vanderploeg et al., 1995, 2001, 2002).

Unionid mussels are not the only native filter-feeding organisms in freshwater environments that compete with zebra mussels. Many orders of Hexapoda, such as Trichoptera, depend on filter-feeding for nutrient uptake (Wallace and Merritt, 1980). Most of the filter-feeding Trichoptera belong to Hydropsychoidea, and use their silk glands to make net for filters (Wallace and Merritt, 1980). Net mesh size is dependent

upon species and upon larval instar stage, although some overlap of mesh size between species is possible (Wallace and Merritt, 1980). It has been previously shown that zebra mussels have a negative impact upon net-spinning caddisflies (Ricciardi et al., 1997; Ward and Ricciardi, 2007). In the Upper Mississippi River region, *Hydropsyche orris* is a net-spinning caddisfly which cohabitates with mussels, and is suspected of filtering and consuming similar food sources as native unionids and zebra mussels.

Typically, three methods have been used to determine dietary overlap among these invertebrates. The first, gut content analysis involves removing the contents of the stomach or digestive system of the organism. These contents are then identified and counted. Using this method, it has been shown that mussels consume many things including phytoplankton, zooplankton, rotifers, and detritus (Vaughn et al., 2008). However there are several limitations to using gut content analysis. First, the small size of the food makes identification sometimes very difficult (Strayer et al., 2004). This method also assumes the contents of the gut are completely digested and assimilated (Strayer et al., 2004; Vaughn et al., 2008), which is not always the case.

A second and very powerful method is the use of stable isotopes. Stable isotope analysis has indicated that native mussels digest and assimilate phytoplankton and algae (Thorp et al. 1998; Herwig et al., 2007; Vaughn et al., 2008). Nichols and Garling (2000) have reported that bacteria and other phytoplankton are also incorporated into the native mussel's diet. Since stable isotopes rely upon assimilation of dietary nitrogen and carbon, analysis of stable isotopes is dependent on the metabolic rate, which can vary among species, temperatures, and tissues (Bosley et al., 2002). For example in native mussels, energy reserves are stored as glycogen in the foot ((Haag et al., 1993; Naimo et al., 1998;

Naimo and Monroe, 1999; Raikow and Hamilton, 2001; Newton et al., in press). When studies have used whole mussels for stable isotope analysis to compare among species, the relative contribution from the foot to the sample may differ and bias interpretation (Naimo and Monroe, 1999).

The third and indirect method of assessing diet is analyzing the activity of digestive enzymes. These enzymes are primarily used within the lumen secreted by cells associated with the digestive system. In the presence of the specific substrate, digestive enzymes will breakdown complex nutrients into their constituent parts, reducing their size and complexity and allowing uptake by the organism. So far most invertebrates appear to have similar digestive capabilities, but which enzymes an invertebrate is using at a particular time is dependent upon diet (López-Rodríguez et al., 2012). Comparisons of the activities of digestive enzymes among species, may allow for an indirect inference of what the animal is consuming at the time the animal was collected.

Besides providing information about what an animal may be consuming, evaluating digestive enzymes can provide information about what an animal can digest. This type of information is important in the development of diets and/or controls for invertebrates. Currently, resource managers lack tools that can be used to control zebra mussels without significantly affecting native filter-feeders. New controls are currently being developed using micro-particle technologies, which require the presence of specific digestive enzymes to release a control agent.

The primary focus of this research was to provide valuable information about the digestive capabilities of zebra mussels compared with those of some commonly found native filter-feeding invertebrates. Therefore analyzed the digestive enzymes of zebra

mussels, threeridge mussels (*Amblema plicata*), and a caddisfly (*H. orris*). Threeridge mussels were chosen as a representative Unionidae since it frequently occurs in similar locations with zebra mussels at sampling sites. *Hydropsyche orris* was chosen as a representative non-mussel species because it frequently resides in areas similar to those inhabited by zebra mussels. Very little is known about the digestive enzymes of caddisflies. As a result, this study will be among the first to characterize digestive enzymes of caddisflies, and to compare the digestive enzymes used by native and invasive freshwater mussels.

GOAL AND OBJECTIVES

The goal of this study was to identify a digestive enzyme that is more active in zebra mussels than in native filter-feeding invertebrates, and thus be incorporated into the development of a novel tool to control zebra mussels

Specific objectives of this study are as follows:

Objective 1: Develop an assay to assess several digestive enzymes simultaneously from mussel tissues.

This will be accomplished by using a commercially available colormetric kit that is typically used in medicine to measure the activities of 19 digestive enzymes.

Hypothesis 1.1: If there is a color change in the visual spectrum in the presence of a specific enzyme, then there will be greater absorbance at a specific wavelength of light that can easily be measured using a spectrophotometer.

Hypothesis 1.2: If there is a brighter color observed when greater amounts of an enzyme is present, then there should be a linear response in the absorbance of that particular wavelength of light in the presence of greater amounts of that enzyme.

Objective 2: Determine if relative activities of digestive enzymes are consistent between sampling times.

This will be accomplished using methods developed during objective 1. Activities of digestive enzymes will be compared in zebra mussels and threeridge mussels collected from the same site on the Mississippi River near Winona, Minnesota during September and October of 2010.

Hypothesis 2.1: If an organism is feeding on the same substrates, then the activity of those particular digestive enzymes will be similar between sample times.

Hypothesis 2.2: If zebra mussels are feeding on the same substrates as threeridge mussels, then the activity of those particular digestive enzymes will be similar between species.

Objective 3: Compare spatial and temporal changes in relative activities of digestive enzymes in zebra mussels, threeridge mussels and caddisflies.

This will be accomplished using methods developed during objective 1. Activities of digestive enzymes will be compared among zebra mussels, threeridge mussels and caddisflies that have been collected from three sites along the Upper Mississippi River at 4 times between July and October of 2012.

Hypothesis 3.1: If an organism is feeding on the same substrates over time, then the presence of and the activity of those digestive enzymes will be similar between sample times.

Hypothesis 3.2: If an organism feeds on the same substrates regardless of its location, then the activity of those particular digestive enzymes will be similar among sample locations at a sampling time.

Hypothesis 3.3: If zebra mussels, threeridge mussels and caddisflies feed on the same substrates, then the presence and activities of digestive enzymes will be similar among species.

Objective 4: Compare activities of digestive enzymes that have been identified as more active in zebra mussels than in threeridge mussels and caddisflies.

This will be accomplished using commercially available enzyme kits following the manufacturers' instructions, except all assays will be run at two temperatures that are within the temperatures commonly observed in the Mississippi River.

Hypothesis 4.1: If the activity of an enzyme is associated with more enzyme being present, then the quantity of the enzyme will be greater.

Hypothesis 4.2: If the activity of an enzyme is associated with greater efficiency, then the rate at which the enzyme converts substrate to product will be greater.

Hypothesis 4.3: If temperature affects the activity of an enzyme, then the rate at which the enzyme converts substrate to product will differ between temperatures.

CHAPTER I

api®ZYM KIT VALIDATION

Introduction

Since their introduction in the late 1980s, zebra mussels (*Dreissena polymorpha*, Pallas 1769) have spread across the USA and have negatively impacted native unionid populations (Strayer and Malcom, 2007). Zebra mussels have been reported to colonize and, ultimately, smother many species of unionids (Schloesser et al. 1996), but they also may compete with native mussels for food resources (Baker and Levinton 2003). This dietary overlap can have significant impacts of the health, growth and fitness of native mussels. Unfortunately understanding this overlap in diets remains incomplete. Completing our understanding of the digestive physiology of native mussels and zebra mussels will help in predicting the impacts of zebra mussels on native mussels and potentially identifying unique ecological and physiological characteristics of zebra mussels for the development of new targeted controls.

Many studies on dietary preferences in mussels have focused on gut-contents (Yeager et al., 1994; Parker et al., 1998; Beck et al., 2003; Mandal et al., 2007; Kamiyama, 2011). This analysis of gut contents does not differentiate between particles that are ingested and passed and those ingested, digested, and assimilated in mussels. This method provides information on the items consumed by the mussels, but not which items are important for the functioning of the animal. Results from gut content analysis do not provide conclusive evidence of dietary overlap. Some researchers have used stable

isotopes to overcome this problem. This method depends on the digestion of particular items and the assimilation of nitrogen and carbon isotopes related to those items into tissues. Still, researchers do not agree that there is dietary overlap. For example, Nichols and Garling (2000) reported that unionid mussels primarily feed on bacteria according to their isotopic signatures, while Baker and Levinton (2003) suggest they feed on algae. These differences in isotopic signatures could be explained by replacement of isotopes upon a shift in diet and nutrient turnover. This turnover occurs at variable rates depending upon metabolism of the animal (Hawkins 1985, Bosley et al. 2002, Gamboa-Delgado and Le Vay 2009). Therefore, stable isotopes provide insight into the feeding habits of animals prior to capture, but not at the time they were captured.

An alternative method to assess digestion of foods in an animal is monitoring digestive enzymes. These enzymes are dependent on the types and chemical composition of the substrates present within the digestive tract (Deren et al., 1967; Rosensweig and Herman, 1969; Nitsan et al., 1974; Kelly et al., 1991). Nutrient composition of zooplankton has been found to be highly variable (Raymont et al. 1971, Jagadeesan et al. 2010) and in algae, the protein content ranged from 6% - 71%, and lipid content from 2% - 22% (Becker 2007). It is generally thought that these differences in nutrient contents are dependent upon species (Becker 2007). Therefore, if zebra mussels and native mussels consume the same foods, presence and activity levels of digestive enzymes would be expected to be similar. At date, most studies have focused on verifying the presence and activities of only a few enzymes in bivalves (Kuz'mina 1999, Areekijseree et al. 2002, Supannapong et al. 2008, Khrueanet et al. 2009, Palais et al. 2010, Golovanova 2011). Analysis of a single digestive enzyme provides little information on the diet, but

analyzing several digestive enzymes at the same time, may allow inference about the diet and feeding status of an animal at the time of capture.

The goal of this study was to develop an assay that can be used to assess several digestive enzymes simultaneously from mussel tissues. Specific objectives of this study include: 1) validate the use of a commercially available assay to measure digestive enzymes, 2) identify digestive enzymes unique to each species, and 3) describe changes in the activities of specific enzymes of a species between two sampling times. To our knowledge, this is the first study to simultaneously measure a large number of digestive enzymes that include phosphatases, proteases, lipases and glycolytic enzymes in multiple species of freshwater mussels.

Materials and Methods

Any use of trade, product, or company name is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Animals and Collection

Ten threeridge, three plain pocketbook, and seven zebra mussels were captured by wading August 9, 2010 and then 10 individuals of each species were captured by snorkeling September 10, 2010 in Pool 6 of the Mississippi River, near Winona, Minnesota. Water temperatures (°C) were obtained from each site on both sampling days. Following capture, digestive glands were excised from each threeridge and plain pocketbook. Individual digestive glands were immediately frozen with dry ice and stored at -80°C until further processing. Whole zebra mussels were also immediately frozen in the field and transported to the laboratory since magnification was required to extract

their digestive glands. Upon thawing, zebra mussel digestive glands were removed and processed identical to those from the threeridge and plain pocketbook.

pH and Processing

Immediately following thawing, pH of each digestive gland was determined with an AB15 pH meter (Accumet®, Fisher Scientific, Fairhaven, NJ) and a Micro pH electrode (Accumet®, Fisher Scientific, Fairhaven, NJ). Then each digestive gland was thoroughly homogenized using a Microtube Pestle (USA Scientific, Inc., Ocala, FL). Samples were then centrifuged at 23 x g for 20 minutes and supernatants retained for all subsequent analyses. In all samples, de-ionized (DI) water was added to increase volumes and dilute each to measurable concentrations of total protein. Total protein concentration for each sample was quantified using a micro-BCA assay (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions on a Biotek Synergy 2 spectrophotometer (Biotek, Winooski, VT). All samples were then diluted with DI water to 1.0 µg protein μL^{-1}.

Enzyme Analysis

Nineteen digestive enzymes (Table 1) were assayed for each sample using api®ZYM test kits (bioMérieux, Inc. Durham, NC) according to the manufacturer's instructions. August samples required 2.5 µg total protein loaded into each well of the strip to provide adequate color production, while September samples required 5.0 µg total protein to produce similar color change. Directly following the final step of the api®ZYM assay, content of each well, including the blank, were transferred to a corresponding well in a 96-well plate. Absorbance of each well was measured at 400, 450, 500, 500 and 650 nm with a Synergy 2 Multi-Mode Microplate Reader (BioTek®,

Winooski, VT). Apparent enzyme activity (AEA) was determined at each wavelength using the following equation:

$$AEA_x = (AbsX_{sample}/Ptn_{sample}) - (AbsX_{blank}/Ptn_{blank})$$

where AEA_x is apparent activity of enzyme determined by absorbance at wavelength X, AbsX is absorbance of the sample/well at wavelength X and Ptn is the amount of protein loaded into the well.

The appropriate wavelengths used to analyze each enzyme were determined using a standard curve and identifying the wavelength that produced a linear response curve. The standard curve was comprised of supernatant of the homogenate of a single threeridge digestive gland.

Validation of Enzyme Kit (Relative Quantification)

Each well of the api®ZYM strip was loaded with 12 µg, 9 µg, 6 µg, 3 µg, 1.5 µg, or 0 µg total protein from a single digestive gland homogenate. Each strip was processed as mentioned above and absorbencies measured at wavelength of 400, 450, 500, 550 and 600 nm. Standard curves were developed for each wavelength and those wavelengths that provided the best fit model ($R^2 \geq 0.90$) were used for further analysis. These standards curves were used to calculate arbitrary units from the absorbance of each mussel. Those enzymes that did not provide an appropriate model were only considered to be present or absent.

Table 1. Enzymes assayed from the api®ZYM kit, their absorbance (nm), standard curve trend line equations and the R^2 values.

Enzyme	Well number	Absorbance measured (nm)	Trendline equation	R^2
Blank	1	---	---	---
Alkaline phosphatase*	2	550	y = 0.04533x - 0.0135	0.973
Esterase	3	---	---	---
Esterase lipase	4	---	---	---
Lipase	5	---	---	---
Leucine arylamidase*	6	550	y = 0.0441x - 0.0124	0.950
Valine Arylamidase	7	---	---	---
Cystine Arylamidase	8	---	---	---
Trypsin	9	---	---	---
α-chymotrypsin	10	---	---	---
Acid phosphatase*	11	550	y = 0.0432x – 0.0882	0.964
Naphthol-AS-BI-phosphohydrolase*	12	600	y = 0.0137x + 0.0122	0.910
α-galactosidase	13	---	---	---
β-galactosidase*	14	550	y = 0.0774x – 0.1402	0.959
β-glucuronidase	15	---	---	---
α-glucosidase	16	---	---	---
β-glucosidase	17	---	---	---
N-acetyl-β-glucosaminidase*	18	550	y = 0.1008x – 0.0087	0.980
Mannosidase	19	---	---	---
Fucosidase*	20	550	y = 0.0907x – 0.103	0.993

*Enzymes able to be analyzed beyond a presence/absence basis.

Validation of Enzyme Kit (Absolute Quantification)

Using reagent-grade enzymes, we evaluated if enzyme activity determined with the api®ZYM kit could be quantified. Due to availability, only alkaline phosphatase, acid phosphatase, leucine arylamidase and N-acteyl-β-glucosaminidase (NAGase) were analyzed. Each enzyme was serially diluted: alkaline phosphatase: 1, 2, 3 mU/well; acid phosphatase: 0.25, 0.5, 0.75 mU/well; leucine arylamidase: 0.75, 1, 2 mU/well; NAGase: 3, 4, 5 mU/well. All enzymes were purchased from the same vendor (Sigma-Aldrich, St.

Louis, MO, USA). Absorbances at the targeted wavelengths were measured for the contents of the well that corresponded to the targeted enzymes.

Analysis

Shell lengths (mm) of mussels are expressed as means (± SD). A T-test was used to determine if size and mass of mussels, within a species, differed between sampling times. Enzyme concentrations were compared among species by ANOVA and Tukey's Post-Hoc test from samples only collected in September. T-tests were used to identify temporal changes in enzyme concentrations by comparing August and September using only data from threeridge and zebra mussels. Plain pocketbooks were excluded from this temporal analysis due to limited captures. Enzymes that were not quantified are simply expressed as present or absent. Digestive enzyme comparison statistical analyses were performed using SYSTAT® 11.0 (Systat Software, Inc., San Jose, CA USA) with a significance level of $P \leq 0.02$. This significance level was used due to limited sample sizes and minimized potential of Type II error. The statistical analyses for the api®ZYM kit compared to the specific enzymatic kits were performed using R version 2.11.1 with a significance level of $p < 0.05$.

Results

Body Size and pH

Within each species, no differences in shell lengths were observed between sample times. The mean (SD) shell length for threeridge captured during August was 82.0 (14.0) mm and 75.1 (5.7) mm in September. Plain pocketbooks were 123.8 (6.5) mm and 108.6 (5.9) mm when captured in August and September, respectively. In August, zebra mussels were 20.4 ± 2.0 mm and 20.8 ± 2.6 mm long in September.

Mean pH (SE) of digestive glands were generally considered circumneutral and did not differ between months for each species. Digestive glands of threeridge mussels had a pH of 6.80 (0.21) and zebra mussels had a pH of 6.93 (0.19), while plain pocketbooks had a pH of 7.10 (0.29). Differences were found among the species. The pH of digestive glands from zebra mussels did not differ from those in threeridge or plain pocketbook ($p = 0.20$), but digestive gland pH was significantly lower in threeridge than that of plain pocketbook ($p = 0.01$).

Validation of Enzyme Kit

Only seven enzymes were found to produce an upward-sloping linear trend in one of the wavelengths measured in this study (Table 1). Six enzymes were quantifiable using absorbance at 550 nm and naphthol-AS-BI-phosphohydrolase was quantified at 600 nm. Enzymes that were semi-quantifiable were phospholytic (alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase), glyocolytic (β-galactosidase, N-acetyl-β-glucosaminidase, fucosidase) or proteolytic (leucine arylamidase). No lipolytic enzymes (lipase, esterase and esterase lipase) could be quantified. Twelve enzymes could be classified as present or absent.

Validation of Enzyme Kit (Absolute Quantification)

Of the seven enzymes found to have a positive linear response, reagent grade enzymes were used to generate a standard curve of the absorbance vs. enzyme concentration. A comparison of the resulting api®ZYM generated concentrations and the concentrations generated via the specific enzyme kits showed that of the four enzymes tested, only one resulted in a linear correlation (NAGase, $p < 0.05$; Figure 1). These results suggest that this kit should not be used to quantify enzymatic activity, but could be

a valuable tool to compare relative activities among samples and be used as an initial screening tool to identify specific enzymes for further analysis.

Digestive Enzyme Activity (Species Comparison)

Threeridge had a greater quantity of alkaline phosphatase (Figure 2) than plain pocketbook, 0.826 (0.129) units / µg total protein and 0.334 (0.075) units / µg total protein ($p = 0.02$), respectively. No differences were determined in alkaline phosphatase (Figure 2) quantity between zebra mussels and threeridge or plain pocketbook. In zebra mussels, 0.623 (0.046) units / µg total protein of leucine arylamidase (Figure 4) was greater than that in either threeridge or plain pocketbook ($p \leq 0.01$). Also, acid phosphatase (Figure 2) concentrations in zebra mussels (1.525 [0.125] units / µg total protein) were greater than that determined in threeridge (0.960 [0.091] units / µg total protein) or plain pocketbook (0.769 [0.050] units / µg total protein; $p < 0.01$). All other semi-quantifiable enzymes (Figures 2, 3, and 4) were determined to be similar among species. Those enzymes classified as absent or present were all identified in zebra mussels, threeridge and plain pocketbook.

Digestive Enzyme Activity (Monthly Comparison)

Water temperatures decreased from 28.6°C in August to 19.4°C in September. Concentrations of phospholytic enzymes were less in threeridge captured in September than those captured in August, but no differences were determined for these enzymes in zebra mussels (Figure 2). Concentrations of naphthol-AS-BI-phosphohydrolase in threeridge decreased from 12.990 (2.170) units / µg total protein in August to 0.934 (0.621) units / µg total protein in September ($p < 0.01$). Acid phosphatase decreased from 3.820 (0.659) to 0.960 (0.289) units / µg total protein and alkaline phosphatase decreased

from 2.937 (1.254) to 0.825 (0.407) units / µg total protein from August to September, respectively ($p < 0.01$). The glycolytic enzyme N-acetyl-β-glucosaminidase (Figure 3) did not change in concentration between August and September in either threeridge or zebra mussels ($p = 0.04$). Both fucosidase and β-galactosidase (Figure 3) decreased in concentrations from August to September in threeridge and zebra mussels ($p < 0.01$). The concentration of leucine arylamidase (Figure 4) was similar in threeridge captured in August and those captured in September ($p = 0.07$). Leucine arylamidase increased from 0.206 (0.097) to 0.623 (0.144) units / µgtotal protein from zebra mussels captured in August and September ($p < 0.01$).

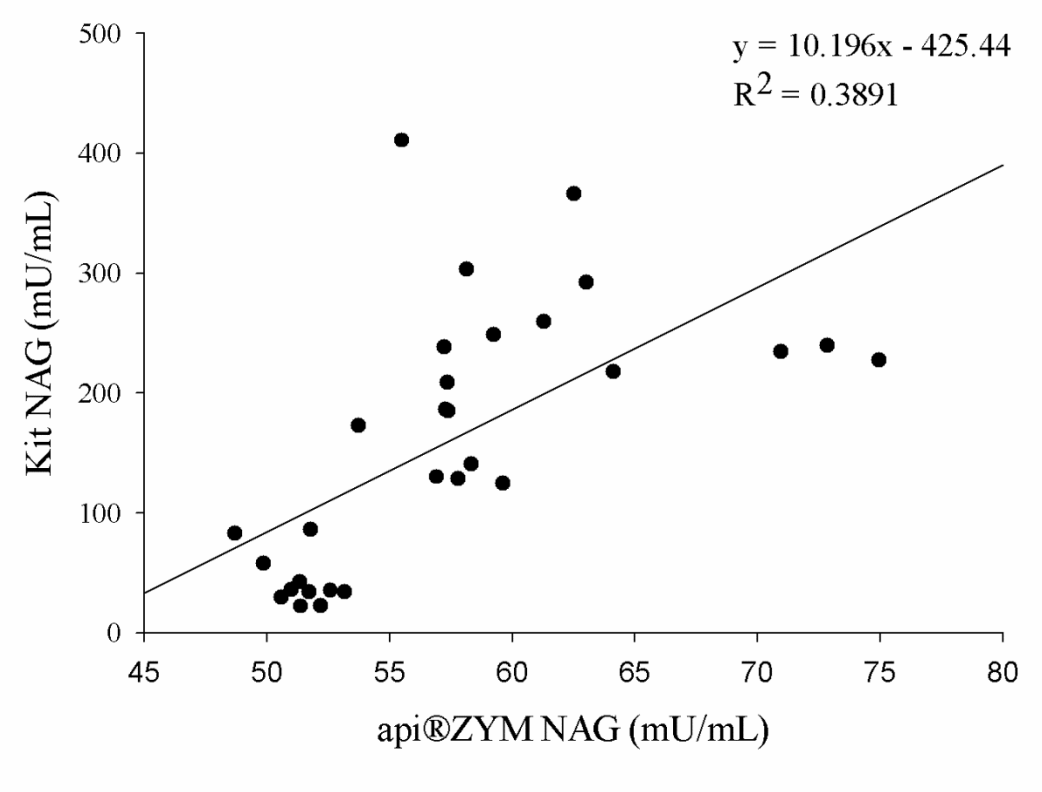

Figure 1. Concentration correlation between the api®ZYM kit and the NAGase kit.

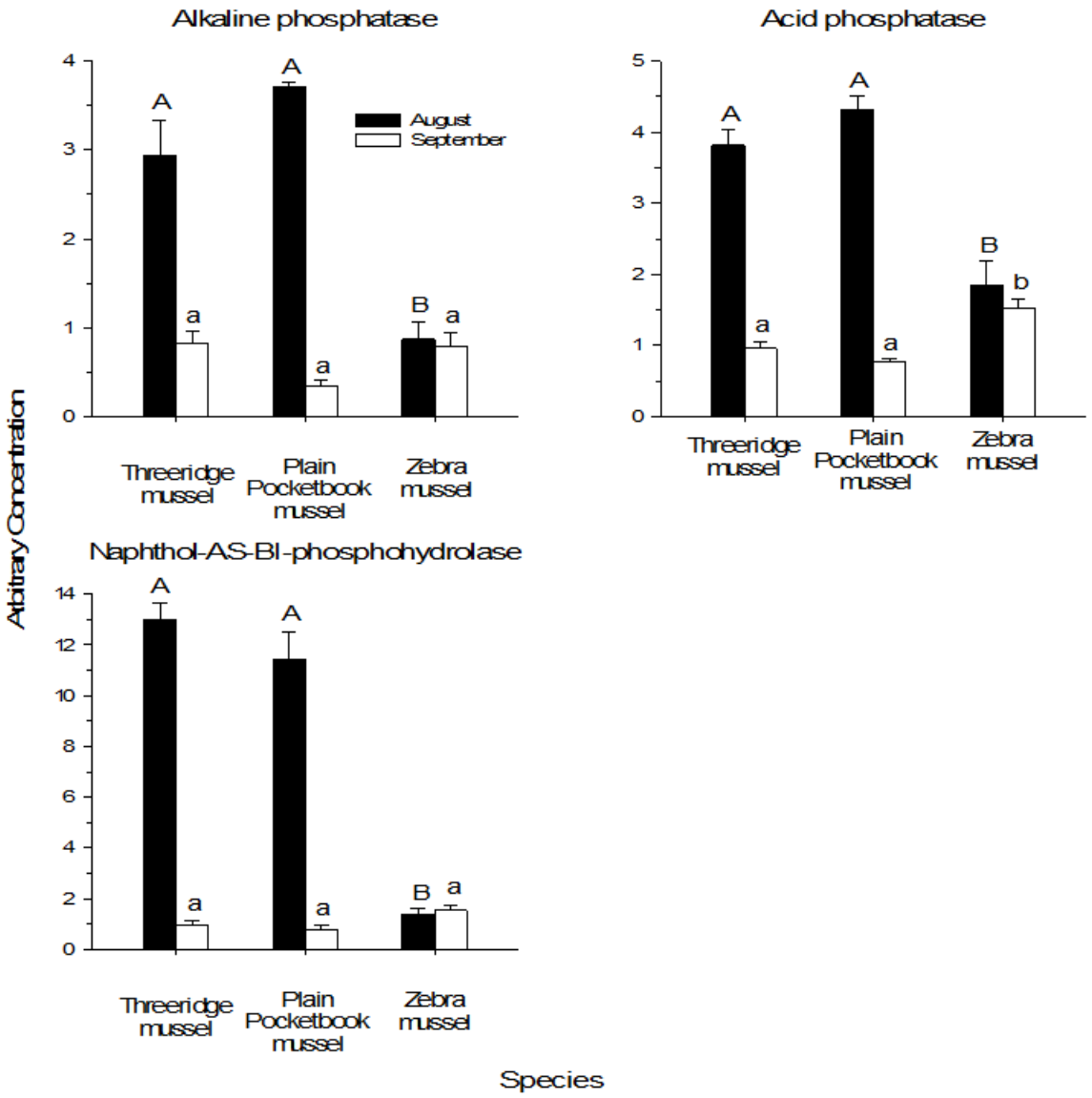

Figure 2. Comparison of arbitrary concentrations of individual phospholytic enzymes analyzed over the sampling points for threeridge, plain pocketbook, and zebra mussels. Letters above error bars represent statistical significance ($P<0.02$) only when compared between species over an individual month.

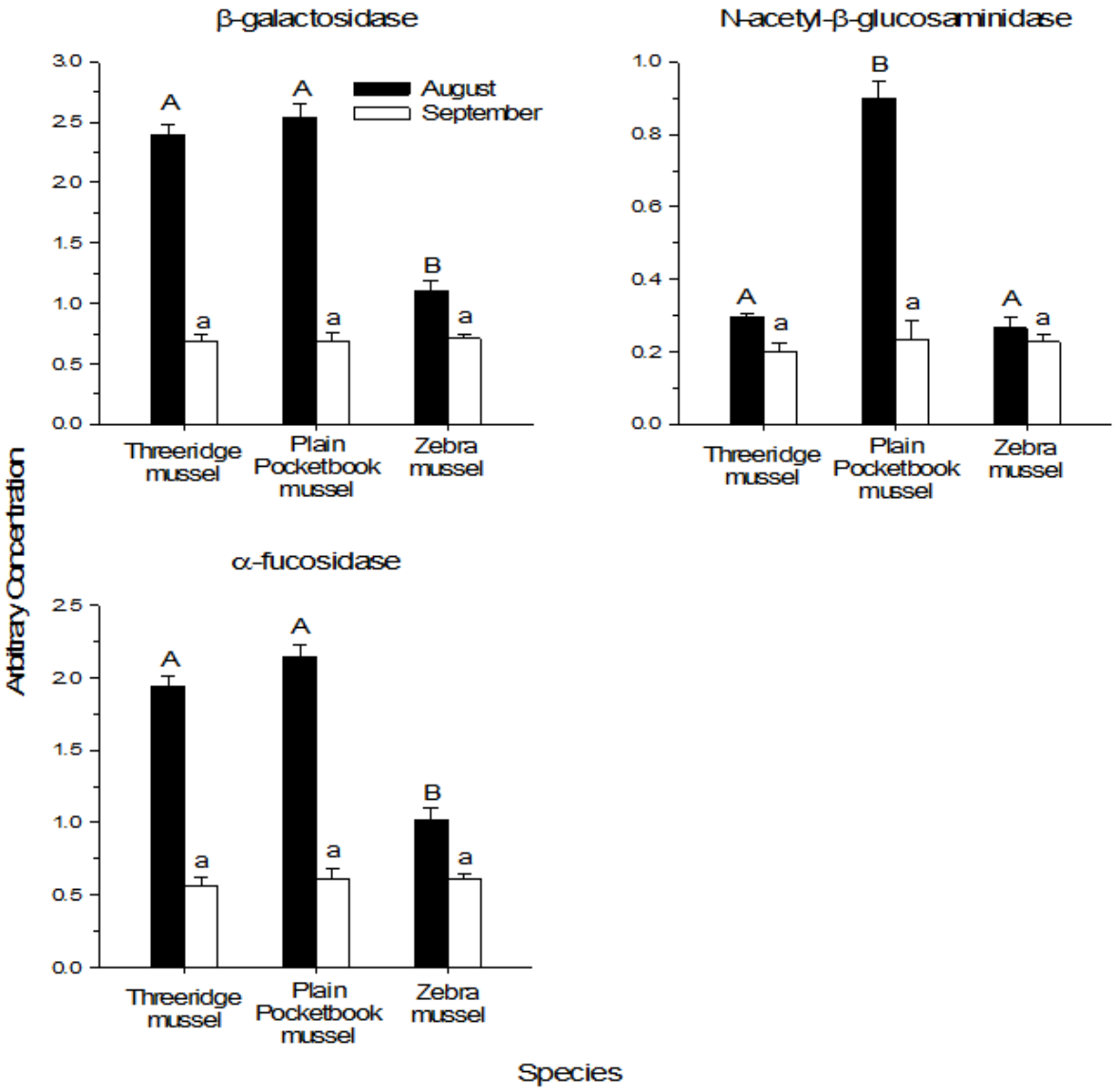

Figure 3. Comparison of arbitrary concentrations of individual glycolytic enzymes analyzed over the sampling points for threeridge, plain pocketbook, and zebra mussels. Letters above error bars represent statistical significance ($P<0.02$) only when compared between species over an individual month.

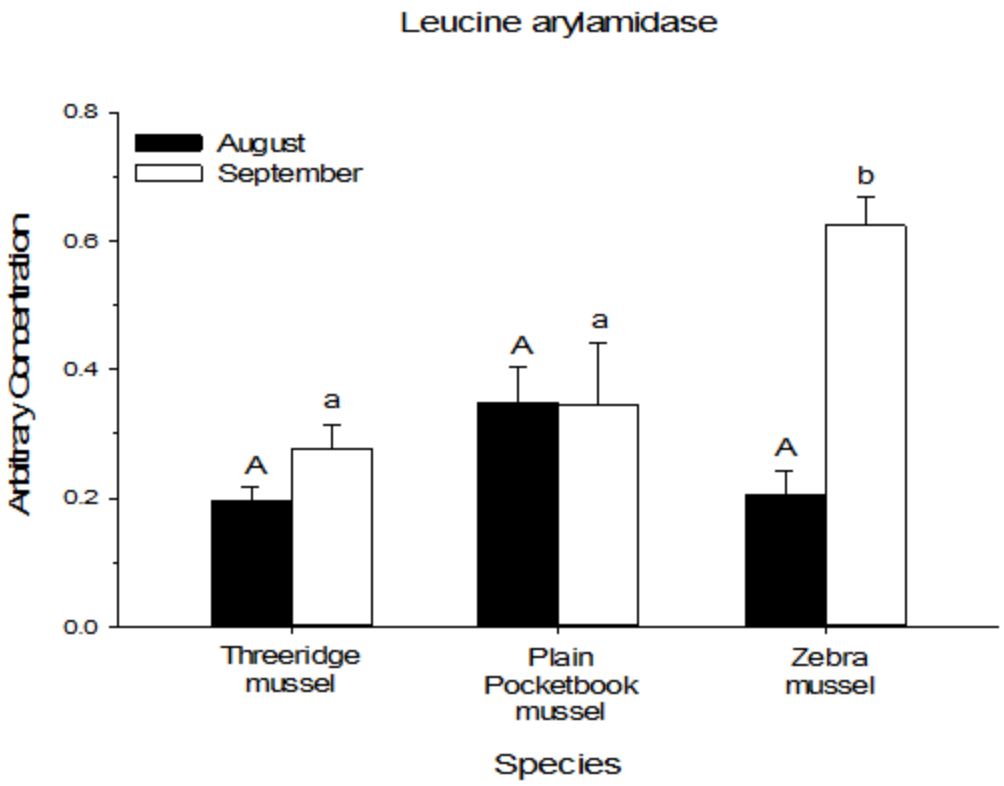

Figure 4. Comparison of arbitrary concentrations of leucine arylamidase analyzed over the sampling points for threeridge, plain pocketbook, and zebra mussels. Letters above error bars represent statistical significance (P<0.02) only when compared between species over an individual month.

Table 2. Percent change in enzyme concentration between September and August collections.

Enzyme	Threeridge Δ (P)	Plain pocketbook Δ (P)	Zebra mussel Δ (P)
Alkaline phosphatase	-71.9 (<0.001)	-91.0 (<0.001)	-8.7 (0.818)
Leucine arylamidase	41.6 (0.286)	-0.65 (0.984)	203 (<0.001)
Acid phosphatase	-74.9 (<0.001)	-82.1 (<0.001)	-17.1 (0.216)
Naphthol-AS-BI-phosphohydrolase	-92.8 (<0.001)	-92.9 (<0.001)	11.5 (0.788)
β-galactosidase	-71.4 (<0.001)	-73.3 (<0.001)	-35.5 (<0.001)
N-acetyl-β-glucosaminidase	-32.2 (0.029)	-74.1 (<0.001)	-14.1 (0.426)
Fucosidase	-70.9 (<0.001)	-71.8 (<0.001)	-39.9 (<0.001)

Discussion

The study of digestive enzymes provides great insight into the diet and physiology of an animal. A commercially available enzyme test kit (i.e. api®ZYM enzyme test kit) was found to be a useful tool in the determination of the presence of 19 digestive enzymes. Our study was the first to describe the presence of these 19 digestive enzymes in zebra mussels, threeridge, and plain pocketbook, but only 7 enzymes produced a high correlation with the amount of digestive enzyme and thus could be relatively quantified. These 7 enzymes can be useful to compare relative activity of the enzyme studies where quantification is not necessary. The remaining 12 enzymes can be used for absence/presence detection.

Despite that the colorimetric method used to analyze enzymes had a positive correlation with amount of enzymes present, the api®ZYM kit cannot determine an absolute amount of enzyme present for all of the enzymes tested. Only NAGase had a linear relationship, albeit weak, when analyzed with both the digestive gland homogenate and the reagent grade enzyme. Other enzymes had linear responses with the reagent-grade enzymes; however, the assay was saturated at very low amounts of the enzyme. Our results suggest that this kit should not be used to quantify enzymatic activity, but could be a valuable tool to compare relative activities among samples and identify trends.

Even though all of the enzymes analyzed were present in each of the species, the activities of analyzed enzymes in native unionids appeared to be greater than those in zebra mussels during August. This may suggest that native mussels could be feeding upon a different food source than zebra mussels at this time. As mentioned earlier, previous studies using gut contents and stable isotopes have suggested these organisms

are feeding upon similar food sources. However, these methods are limited in determining the actual food source, and stable isotope methods are limited in fractionating bacteria, picoalgae, and fungi from each other. Studies utilizing stable isotopes may have determined the trophic level of both invasive and native mussels, however, they could be utilizing two different food sources that occupy a similar niche, but have very different compositions (% protein and % lipid). If food sources have different compositions, one would expect the consumers to have differing compositions. A comparative study conducted on Atlantic salmon (*Salmo salar*) showed that body composition changes slightly between fish living in river and lake habitats (Dempson et al., 2004).

The activity of digestive enzymes in threeridge and pocketbook mussels decreased between August and September (Table 2). During this time, water temperatures also decreased from 28.6°C to 19.4°C. Ribbed mussels *Geukensia demissa* have been reported to diet shifts between summer/fall and winter/spring (Kreeger and Newell 2001). Other species of ectothermal animal have been shown to have seasonal differences in digestive enzymes (Aragón-Axomulco et al., 2012; Sánchez-Muros et al., 2013). Also, threeridge and pocketbooks could be responding to changes in temperature, seston concentration, and flow and altering the amount of digestive enzymes present so that the animal can meet its energetic needs (Newell and Langdon, 1996). In ectothermal animals, energetic needs are directly dependent upon temperature; the lower the temperature the lower the energy needed for maintenance. This suggests that there may be seasonal differences in enzymatic activity among freshwater mussels.

Of the 19 enzymes, only leucine arylamidase did not change between months in native mussels, but did change in zebra mussels. Leucine arylamidase has been linked with nitrogen excretion in blue mussel *Mytilus edulis*. Blue mussels with the Lap^{94} allele had excreted more nitrogen than blue mussels lacking the Lap^{94} allele (Hilbish and Koehn 1985). It has been shown that zebra mussels are highly metabolically active in fall (Sprung, 1995), which is different when compared to native mussels, which are more metabolically active in spring (Baker and Hornbach, 2001). This difference in metabolic activity may account for increase in enzyme activity in zebra mussels and the decrease in enzyme activity in native mussels.

The increased enzyme activity in zebra mussels, however, should be interpreted with caution. Zebra mussels were dissected differently than native mussels, and as a result the concentration of enzymes used in the analysis could be lower in zebra mussels due to dilution by other cellular components and tissues. The majority of enzymes analyzed by the api®ZYM enzyme test kit are primarily located in tissues associated with digestive function (BRENDA enzyme portal; accessed 7/31/13; www.brenda-enzymes.org). If all mussels were dissected using the same method, the concentration of the enzymes should increase in zebra mussel samples, which may cause even greater differences between mussel species.

In conclusion, we found the api®ZYM enzyme test kit to be a valuable tool in determining trends and comparing relative digestive enzyme activities among species. For absolute enzyme quantification, a specific enzymatic kit must be used. Our results suggest that zebra mussels and native mussels may feed on different food sources even if they occupy similar trophic positions. Our results also suggest that there are seasonal

differences in enzyme activity in mussels. These results are important information for the growing knowledge pool about mussel digestive physiology, and require further validation through more stringent methods.

CHAPTER II

DIGESTIVE PHYSIOLOGY COMPARISONS

Introduction

Studies on digestive enzymes in mussels have focused on select enzymes (amylase, protease, and lipase, Khrueanet et al, 2009; amylase and proteinase, Supannapong et al, 2008; cellulose and lipase, Areekijseree et al, 2002; amylase and proteinase, Areekijseree et al, 2004; amylase and cellulose, Palais et al, 2010). These enzymes were primarily investigated as a means to understand mussel digestive physiology for culture purposes. Because these studies did not focus on the ecology of these organisms, this information is of little use to infer the true diet of a mussel. An increase in the number of enzymes being investigated at one time may allow for greater inference and comparison into what these animals are feeding upon and how they digest those food sources.

Two important factors are known to influence food sources: season and location. Food sources for many animals change seasonally (Kreeger and Newell 2001; Taylor and Batzer, 2010; Bergamino et al., 2011; Kamiyama 2011), and as a result, the enzymes the animals use change seasonally (Aragón-Axomulco et al., 2012; Sánchez-Muros et al., 2013). One portion of the River Continuum Concept is that food resources shift as the river progresses downstream (Vannote et al., 1980; Rosi-Marshall and Wallace, 2002; Woodward and Hildrew, 2002). Many studies have shown that food resources of consumers change with location (Zerba and Collins, 1992; Rosi-Marshall and Wallace,

2002; Kanaya et al., 2008; Taylor and Batzer, 2010; Bergamino et al., 2011), and because of this, digestive enzymes also can change with changes in location (Buchholz and Saborowski, 1996). The fluctuation of these enzymes in native and invasive macroinvertebrates may be important ecologically, and as such requires further study.

Therefore, the goal of this study was to identify a digestive enzyme that is more active in zebra mussels than in both threeridge mussels and caddisflies. The caddisfly species, *Hydropsyche orris*, was used as an out-group to represent filter-feeding insects. The specific objective of this study was to identify both spatial and temporal changes in digestive enzyme profiles of these invertebrates. To do this we used the api®ZYM kit and relative quantification (Chapter 1) for comparisons among the three species, three sites and five months.

Materials and Methods

Site Location

To study spatial and temporal differences in digestive enzyme profiles, we analyzed zebra mussels, threeridge mussels and caddisflies at two sites, Winona, MN and Wyalusing, WI, along the Mississippi River (Figure 5). Due to the lack of zebra mussels, only threeridge mussels and caddisflies were collected at the third site, Hastings, MN (Figure 5). Animals were collected monthly from each site between July and October (Table 3). Animals were not collected in July at Wyalusing due to high water, but were collected at all other times. Hastings, MN (Pool 3) was chosen because this area was previously heavily infested with zebra mussels. All sites were chosen because they were approximately 100 river miles apart (Table 3).

Table 3. Site location and collection dates.

Site	Coordinates	River Mile	Collection Date 1	Collection Date 2	Collection Date 3	Collection Date 4
Hastings, MN	44.751488, -92.857089	814.5	7/17/2012	8/14/12	9/20/12	10/16/12
Winona, MN	44.064924, -91.652799	726.3	7/18/2012	8/15/12	9/21/12	10/17/12
Wyalusing, WI	42.949622, -91.143737	627.8	NC	8/13/12	9/18/12	10/15/12

NC=Not collected for this month.

Figure 5. Map depicting study site locations. Study sites are denoted by black crosses.

Animal Collection and Identification

Mussels were collected by hand or with D-nets while wading. All mussels were immediately placed on dry ice and transported to the Upper Midwest Environmental Sciences Center in La Crosse, WI. There, animals were stored at -80°C until processed further. Upon processing, mussels were thawed at room temperature and lengths of shells were measured with a micrometer (Tables 4&5). All tissues were then removed from the

shell for each individual. Tissue samples from threeridge mussels were individually transplanted into 50 mL conical vial, while tissues from zebra mussels were place into 15 mL conical vials. All samples were then re-frozen and stored at -80°C until processed further.

Table 4. Average shell lengths of Amblema plicata analyzed for digestive enzymes. Values are represented in millimeters (±SD). Number in parenthesis is sample size.

Site	July	August	September	October
Hastings, MN	75.07±8.31 (7)	82.22±7.06 (10)	82.25±8.38 (10)	60.61±3.96 (10)
Winona, MN	79.10±13.19 (10)	69.66±7.78 (10)	80.78±7.10 (10)	73.50±10.73 (10)
Wyalusing, WI	NC	74.72±6.52 (10)	82.82±3.21 (10)	84.00±5.38 (10)

NC=Not collected for this month.

Table 5. Average shell lengths of Dreissena polymorpha analyzed for digestive enzymes. Values are represented in millimeters (±SD). Number in parenthesis is sample size.

Site	July	August	September	October
Hastings, MN	10.83±3.84 (3)	16.95±1.16 (10)	NC	NC
Winona, MN	19.40±0.77 (10)	18.43±1.85 (10)	16.73±1.64 (10)	15.74±2.79 (10)
Wyalusing, WI	NC	10.41±0.57 (10)	13.72±1.03 (10)	13.67±0.97 (10)

NC=Not collected for this month.

Caddisfly larvae were collected using Hester-Dendy samplers (HD; Figure 6; Wildco, Yulee, FL, USA). Hester-Dendy samplers were placed near mussel collection areas, in water at an adequate depth to allow for proper flow alignment, anchored with cinderblocks, and marked with buoys.

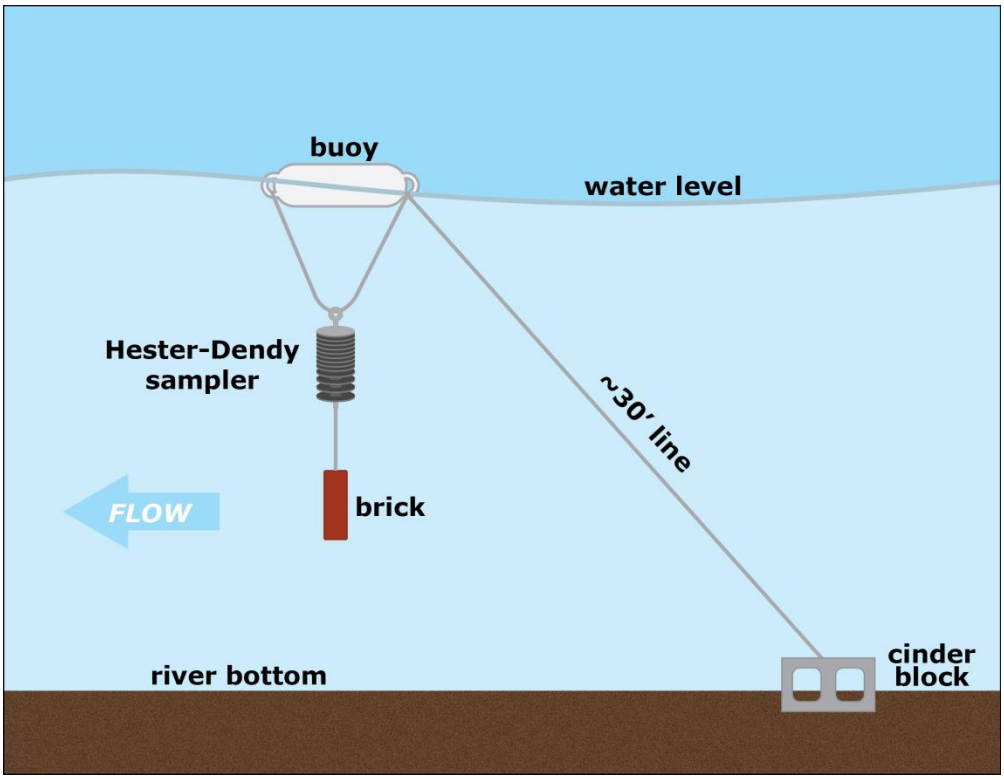

Figure 6. Hester-Dendy sampler set up used to collect caddisfly larvae.

HDs were placed at least 4 weeks prior to capture of invertebrates. At each sampling time, the HD sampler was removed from the water and caddisfly larvae were captured from the plates and immediately placed in a Ziploc® bag filled with river water. The bags were then placed in a cooler with dry ice to keep cool. All bags were transported to the University of Wisconsin-La Crosse, La Crosse, WI for sorting. During sorting, caddisfly larvae were kept at 4°C. Caddisfly larvae were identified based on morphological characteristics (Wiggins, 1996). Only *Hydropsyche orris* was used for subsequent analysis. Larvae were then photographed. The dry weight of each larva was estimated from the photos using a body area to dry weight regression model (Haro, unpublished; Table 6). Images were analyzed with Image-Pro® Plus 5.1.1.38 (Media Cybernetics Inc., Rockville, MD, USA). The mass of the caddisfly larve was determined

with the equation: $Mass_{mg} = 1.563(Log(body\ area_{mm}))-1.542$. Due to the small sizes of the caddisflies, 2 and 4 larvae, dependent upon size, were pooled for each sample for enzymatic analysis.

Table 6. Hydropsyche orris dry weight determined by area. Averaged values are in milligrams (±SD). Number in parenthesis is sample size.

Site	July	August	September	October
Hastings, MN	2.390±0.690 (16)	3.936±1.880 (16)	4.059±1.361 (20)	4.222±0.914 (51)
Winona, MN	2.500±0.778 (16)	4.846±1.472 (16)	3.546±1.942 (19)	4.116±0.918 (60)
Wyalusing, WI	NC	2.528±0.792 (16)	5.818±0.933 (20)	5.062±1.154 (55)

NC=Not collected for this month.

Homogenization of Samples

Each sample was homogenized using a Geno/Grinder® (SPEX Sample Prep, Metuchen, NJ) and steel shot. Prior to homogenization, small amounts of deionized (DI) water were added to each sample to prevent drying and minimize the sticking of tissue to the tube walls during homogenization. The amount of water was dependent on the amount of tissue: two milliliters for zebra mussels, one milliliter for caddisfly larvae and no water was added to threeridge. Following homogenization, each sample was centrifuged at more than 6,800 x g for 20 minutes. Threeridge mussel supernatants were then diluted with an equal volume of DI water. Diluted supernatant was then used for further analysis. Amount of tissue used was not weighed.

Protein Concentration

Total protein concentration of each homogenate/water mixture was determined according to manufacturer's instructions using a Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). All samples were diluted to a concentration of 0.0385

µg total protein per µL homogenate. These diluted homogenates were used in all subsequent analysis.

api®ZYM Kit

Each well of the api®ZYM strip was loaded with 2.5 µg total protein. One strip was used for each sample. All api®ZYM strips were processed as according to manufacturer's instructions. Contents from each well of the api®ZYM strips were loaded into separate wells of a 384 well plate. The absorbencies at wavelengths in 50 nm increments between 400 and 600 were measured on a Multiskan Spectrum plate reader (Thermo Scientific, Rockford, IL). Using the appropriate wavelength, determined in Chapter 1, the apparent enzyme activity was determined using the following equation: $AEA_x = (AbsX_{sample}/PTN_{sample}) - (AbsX_{blank}/PTN_{blank})$ where AEA_x is apparent activity of enzyme determined by absorbance at wavelength X, AbsX is absorbance of the sample/well at wavelength X and PTN is the amount of protein loaded into the well.

To determine presence/absence of enzymes in the wells that could not have apparent enzyme activity determined, a threshold of 0.010 absorbance units was used for enzymes at the same wavelength as the enzymes that could be analyzed for apparent enzyme activity that produced a similar colored product. Averaged values of enzyme absorbance units were compared to the threshold value. Eleven enzymes (esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, α-glucosidase, β-glucosidase, and α-mannosidase) were analyzed at 550 nm; only β-glucuronidase was analyzed at 600 nm.

Water Quality

At each sampling time, water quality data was taken to determine if water quality parameters change over time and may play a role in the expression of digestive enzymes of mussels and caddisflies. Dissolved oxygen (DO) and temperature (°C) were measured with a Handheld Dissolved Oxygen Meter (model 550A-12; YSI, Yellow Springs, Ohio, USA). pH was measured with a model ϕ410 pH meter (Beckman Coulter, Brea, CA, USA). Conductivity (µS/cm) was measured, from a half-liter of river water kept cool, with a Portable Waterproof Conductivity Meter, model AP75, (Accumet, Thermo Fisher Scientific Inc., Pittsburg, PA, USA). Total dissolved solid (ppm) amounts were calculated by multiplying conductivity measurements by 0.67 (www.stevenswater.com; accessed 03/27/2012).

Chlorophyll *a* was measured by filtration of river water (1/2 L kept cool until filtration) through a glass fiber filter (934-AH, Whatman, Piscataway, NJ, USA). Analysis was conducted as according to US EPA Method 150.1 except for the following modification. Absorbance of uncorrected chlorophyll *a* was measured in 96-well plate format by measuring the absorbance of 300 µL of extract with a Multiskan Spectrum plate reader (Thermo Scientific, Rockford, IL).

Data Analysis

Apparent enzyme activity data were analyzed using nonparametric multidimensional scaling (NMDS). Each variable was standardized by the maximum before constructing a Bray-Curtis similarity matrix. Analysis of similarity (ANOSIM; $p < 0.05$), was conducted to determine if species, location, time, or their various interactions were significant predictors of multivariate enzyme activity. Similarity percentages

(SIMPER) were also used to determine which enzymes contributed the most similarity within groupings, and which contributed to the most dissimilarity between groups. ANOSIM and SIMPER were conducted using PRIMER version 6.1.6.

Results

Water Quality Parameters

No major anomalies in seasonal averages were detected for any water quality parameters. Water temperatures decreased from 29°C to 10°C over the course of the study (Table 7). Dissolved oxygen concentrations increased with decreasing temperature. pH, conductivity, total dissolved solids, and uncorrected chlorophyll *a* values remained stable throughout the course of the study. Discharge rates decreased as time progressed during a drought period.

Enzyme Presence/Absence

Esterase (C4), esterase lipase (C8), lipase (C14), and valine arylamidase were detected in all samples analyzed. Cystine arylamidase was detected in all but four samples (Table 8). β-glucuronidase was detected in all but one of threeridge mussel samples and in all zebra mussel samples from Hastings, MN; however it was not detected in any *H. orris* samples. Trypsin was detected in only three samples for each mussel species; it was not detected in any *H. orris* samples. α-galactosidase, α-chymotrypsin, and β-glucosidase were detected in only one zebra mussel sample. α-glucosidase was present in all *H. orris* samples and only one threeridge mussel sample. α-mannosidase was present in most mussel samples, but it was only present in only one *H. orris* sample.

Table 7. Water quality parameters analyzed at sampling times at the three study sites.

Site	Month	Dissolved Oxygen (mg/L; % saturation)	Temperature (°C)	pH	Conductivity (µS/cm)	Total Dissolved Solids (ppm)	Discharge (cfs)*	Uncorrected chlorophyll *a* (µg/L)
Hastings, MN	July	7.63 (95%)	29.2	7.77	304	203.68	26450	77.65±1.07
	August	7.87 (92.2%)	23.8	8.23	422	282.74	18050	59.95±2.30
	September	8.02 (82.7%)	16.8	8.38	670	448.9	18300	87.17±6.78
	October	9.67 (87.2%)	10.6	8.39	552	369.84	17650	93.77±0.65
Winona, MN	July	7.51 (98.3%)	29.6	8.23	374	250.58	33950	39.37±0.65
	August	7.62 (89.4%)	24.2	8.29	303	203.01	17050	47.59±1.93
	September	9.09 (93.7%)	17.1	8.63	323	216.41	11440	74.59±9.86
	October	11.98 (105%)	10.3	8.78	310	207.7	13300	46.99±1.16
Wyalusing, MN	August	7.62 (89.6%)	23.1	8.56	335	224.45	20300	72.47±12.35
	September	9.22 (97.5)	18.4	8.97	301	201.67	13600	73.63±1.71
	October	13.78 (122%)	10.3	8.78	259	173.53	18500	61.35±1.57

*Discharge data from USGS Water Resources (waterdata.usgs.gov; accessed 11/6/2012)

Table 8. Enzymes detected on a presence/absence basis with the api®ZYM kit using and averaged 0.01 absorbance unit threshold to detect for presence/absence (n = 10).

Species	Enzyme*	Hastings, MN				Winona, MN				Wyalusing, WI		
		July	August	September	October	July	August	September	October	August	September	October
Threeridge mussel	CYS	-	+	+	+	+	+	+	+	+	+	+
	TRY	-	-	-	-	-	-	-	-	-	+	+
	CHY	-	-	-	-	-	-	-	-	-	-	-
	GAL	-	-	-	-	-	-	-	-	-	-	-
	GLR	+	+	+	+	-	+	+	+	+	+	+
	A-GLU	-	-	-	-	-	+	-	-	-	-	-
	B-GLU	-	-	-	-	-	-	-	-	-	-	-
	MAN	-	+	+	+	-	+	+	+	+	+	+
Zebra mussel	CYS	-	+	+	+	+	-	+	+	+	+	+
	TRY	-	-	-	-	-	-	+	-	-	+	-
	CHY	-	-	-	-	-	-	+	-	-	-	-
	GAL	-	-	-	-	-	-	+	-	-	-	-
	GLR	+	+	+	+	-	-	+	-	-	-	-
	A-GLU	-	-	-	-	-	-	-	-	-	-	-
	B-GLU	-	-	-	-	-	-	+	-	-	-	-
	MAN	-	+	+	+	-	+	+	+	+	+	+
Hydropsyche orris	CYS	+	+	-	+	+	+	+	+	+	+	+
	TRY	-	-	-	-	-	-	-	-	-	-	-
	CHY	-	-	-	-	-	-	-	-	-	-	-
	GAL	-	-	-	-	-	-	-	-	-	-	-
	GLR	-	-	-	-	-	-	-	-	-	-	-
	A-GLU	+	+	+	+	+	+	+	+	+	+	+
	B-GLU	-	-	-	-	-	-	-	-	-	-	-
	MAN	-	+	-	-	-	-	-	-	-	-	-

* Enzyme names are as follows. CYS: cystine arylamidase. TRY: trypsin. CHY: α-chymotrypsin. GAL: α-galactosidase. GLR: β-glucuronidase. A-GLU: α-glucosidase. B-GLU: β-glucosidase. MAN: α-mannosidase

api®ZYM Kit Evaluation of Field Samples

Neither location ($p = 0.907$) nor time ($p = 0.986$) affected activities of the digestive enzymes. The activities of the digestive enzymes within a species were similar to those of the same species from different locations and sampling times (threeridge mussel: 79.5%; zebra mussel: 70.4%; *H. orris*: 80%; Table 9). The similarities among threeridge mussel (79.5%) were primarily due to acid phosphatase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase (Table 9). The similarities among zebra mussel (70.4%) were due to the activities of naphthol-AS-BI-phosphohydrolase, acid phosphatase, and leucine arylamidase; and naphthol-AS-BI-phosphohydrolase, alkaline phosphatase, and leucine arylamidase were important for *Hydropsyche orris* (80.0%; Table 9).

Activities of enzymes differed among species ($p = 0.001$). Approximately 70% of the differences between groups was due to naphthol-AS-BI-phosphohydrolase, acid phosphatase, and alkaline phosphatase (Table 10). The dissimilarity in naphthol-AS-BI-phosphohydrolase activity explained an average of 28.44% between native species and zebra mussels; this enzyme only accounted for 21.93% of the dissimilarity between native species (Table 10). Acid phosphatase activity dissimilarities were able to account for an average of 24.14% between mussel species and *H. orris*; this enzyme accounted for only 20.07% of the dissimilarity between mussel species. Alkaline phosphatase activity was dissimilar between all groups with an average dissimilarity of 20.82% (Table 10). Between threeridge mussel and *H. orris*, β-galactosidase accounted for 10.33% of the dissimilarity between these two groups (Table 10).

Table 9. Species average similarity and similarity percentages within a species based upon SIMPER analysis of api®ZYM enzyme activity: acid phosphatase: ACP; alkaline phosphatase: ALP; leucine arylamidase: LAP; naphthol-AS-BI-phosphohydrolase: NAP; β-galactosidase: BGAL; NAGase: NAG; fucosidase: FUC. Species compared in the analysis were threeridge mussel (TR), zebra mussel (ZM), and *Hydropsyche orris* (HO).

	Threeridge mussel			Zebra mussel			*Hydropsyche orris*		
Enzyme	Average Activity	Average Similarity	Contributing %	Average Activity	Average Similarity	Contributing %	Average Activity	Average Similarity	Contributing %
NAP	3.01	12.12	15.23	6.05	18.68	26.52	5.41	27.67	34.60
ACP	4.88	21.92	27.54	4.73	16.01	22.73	1.84	10.27	12.85
ALP	2.34	10.09	12.67	4.08	11.48	16.29	4.68	21.12	26.41
LAP	3.00	14.08	17.69	3.15	11.63	16.52	2.68	13.70	17.13
BGAL	1.99	9.25	11.62	1.43	5.18	7.36	0.69	4.20	5.26
FUC	1.29	6.22	7.82	1.38	4.68	6.64	0.26	1.30	1.62
NAG	1.28	5.92	7.43	0.80	2.77	3.93	0.35	1.71	2.14

Table 10. Species average dissimilarity and dissimilarity percentages between species based upon SIMPER analysis of api®ZYM enzyme activity: acid phosphatase: ACP; alkaline phosphatase: ALP; leucine arylamidase: LAP; naphthol-AS-BI-phosphohydrolase: NAP; β-galactosidase: BGAL; NAGase: NAG; fucosidase: FUC. Species compared in the analysis were threeridge mussel (TR), zebra mussel (ZM), and *Hydropsyche orris* (HO).

Enzyme	TR:ZM Average Dissimilarity	TR:ZM Contributing %	TR:HO Average Dissimilarity	TR:HO Contributing %	ZM: HO Average Dissimilarity	ZM:HO Contributing %
NAP	8.85	31.61	8.07	21.93	8.17	25.27
ACP	5.62	20.07	8.97	24.36	7.73	23.92
ALP	5.59	19.99	7.24	19.66	7.37	22.80
LAP	2.76	9.85	2.93	7.96	2.99	9.25
BGAL	2.14	7.65	3.80	10.33	1.92	5.96
NAG	1.59	5.68	2.76	7.50	1.30	4.02
FUC	1.44	5.14	3.04	8.25	2.84	8.78

Discussion

This study was designed to determine temporal and spatial effects on the activities of seven digestive enzymes in threeridge mussels, zebra mussels and caddisfly. An ANOSIM analysis was able to show that season and location did not have an effect on the digestive enzymes of the three species of aquatic invertebrates in this study, the only factor that played a part in digestive enzymes was taxonomic. One study has shown that digestive enzymes shift with location (Buchholz and Saborowski, 1996), however this study was conducted in the waters off of the Antarctic Peninsula, and does not represent a river system. The sites chosen in this study are all approximately 100 river miles equidistant. The Mississippi River along this stretch is 7 or 8 on the Strahler stream order scale, and because the stream order does not change, this may explain some of the lack of change in the digestive enzyme activities spatially. It may be important to test enzyme activities over a larger spatial scale to determine if the River Continuum Concept can help to explain potential differences. All three species tested here are located over a broad range in the United States, and may be able to be used to test these enzymes over a larger spatial scale.

Because species, rather than environmental influences of season or location, was the sole factor influencing enzyme composition, there are two possible explanations for this phenomenon: the animals are feeding upon different resources or there are differences in the regulation of the production of the enzymes. The regulation of these enzymes may be different among these species, with one species producing high amounts of a low activity enzyme and another species producing low amounts of a high activity

enzyme or any such combination. The influence of enzyme regulation differences may be further supported by considering the taxonomic similarities and differences between groups of mussels versus caddisflies. The similarity of digestive enzyme composition between threeridge mussel and zebra mussel corresponded to their taxonomic convergence (Thorp and Covich, 2010), while differences noted between mussels and caddisflies corresponded to their taxonomic divergence (Thorp and Covich, 2010). Besides digestive enzymes, the efficiency of nutrient assimilation differs between the groups. Assimilation efficiency may be dependent on the level of enzyme activity in the digestive tract of an animal, and may also play some part in distinguishing the digestive physiology of these animals. Assimilation efficiency in zebra mussels has been found to range from 37 to 54% (Wong et al., 2003); two other species of mussel have assimilation efficiencies that range from 37 to 73% (Wong et al., 2003a). *Hydropsyche occidentalis* (Banks) was found to have an assimilation efficiency of 50% (McCullough et al., 1979; Pandian and Marian, 1986). It does appear that mussels have the capacity to assimilate more nutrients than other invertebrates. Comparisons between the values should be made with caution due to the influence of food type, temperature, and larval stage on assimilation efficiency.

The enzymatic differences found in our study may allow some inference of what an animal is feeding upon or its metabolic needs. For example, threeridge mussels have higher activities of acid phosphatase, β-galactosidase, leucine arylamidase, NAGase, and fucosidase. From this, one may inferred that these threeridge mussels are feeding on food sources high in phosphates, galactosides, proteins, N-acteylglucosamines, and fucose. These dietary components are high in planktonic crustaceans and algae (Ventura 2006;

Wen et al., 2006; Garcia-Rios et al., 2012). N-acteylglucosamines make up the core structure of chitin (Kurita 2006), and fucose is an important molecule in fucoidans, which are important structural polysaccharides in algae (Kloareg and Quatrano, 1988; Garcia-Rios et al., 2012). A diet made up of planktonic algae and crustaceans is in concurrence with what is known about native mussel diets (Strayer et al., 2004; Vaughn et al., 2008).

Activities of enzymes in zebra mussels were similar to those of threeridge mussels, except that activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase were greater in zebra mussels. This suggests that zebra mussels are also feeding on similar resources as native mussels, which has been previously shown (Mitchell et al., 1996; Parker et al., 1998; Baker and Levinton, 2003). Feeding upon similar resources is inherently linked to competition for food between zebra mussels and native species, which has been shown previously (Baker and Levinton, 2003; Garton et al., 2005).

H. orris had greater activity of two phosphatases, alkaline phosphatase and naphthol-AS-BI-phosphohydrolase, and one protease, leucine arylamidase. Interestingly, it has been reported that caddisflies are phosphorous limited (Evans-White et al., 2005). This would suggest that greater activities of these two phosphatases would allow the caddisflies to compensate for living in an environment that is limited in phosphorus, like most freshwater systems. The activity of leucine arylamidase may be important for the digestive physiology of Hydropsychid caddisflies because it has been shown previously that up to 64% of production in *Hydropsyche rossi* was due to animal material and are facultative carnivores (Benke and Wallace, 1997). This enzyme would be crucial for

digestion if this high of an amount of animal/protein material is consumed in *Hydropsyche orris*.

The presence/absence analysis of the other twelve enzymes from the api®ZYM kit further adds to the growing data pool of mussel and caddisfly digestive enzymes. Because 4 enzymes (esterase (C4), esterase lipase (C8), lipase (C14), and valine arylamidase) were detected in all samples tested, it can be said that these enzymes are important for the digestive process in these animals. It has been shown previously that zooplankton have a lipid content that can range from 11-20% (Jagadeesan et al., 2010), and algae lipid content can range from 2% - 22% (Becker 2007). If nearly a fifth of the composition of a mussels food sources are comprised of lipids, enzymes that break down these molecules become extremely important in the digestive process.

Many of the other enzymes that were only analyzed on a presence/absence basis did not show changes on a temporal or a spatial basis. However, this lack of apparent change could be due to the lack of ability to analyze the enzyme data further. If these enzymes were able to be analyzed further it may provide a clearer understanding of how mussels and caddisflies digest their food sources and what those food sources might be.

CHAPTER III

ENZYME ACTIVITES

Introduction

Aquatic invasive species pose a significant threat to native organisms and ecosystems, especially ones that are already critically endangered or threatened due to man's influence. Zebra mussels are of special concern not only because of their effects on native ecosystems (Holland, 1993; Effler et al. 1996; Caraco et al. 1997; Effler et al. 2004; Vanderploeg et al 2001; Raikow et al. 2004; Barbiero et al., 2006; Caraco et al. 2006; Zhu et al., 2006; Knoll et al. 2008; Strayer, 2009) and native organisms (Nalepa et al., 1998; Stewart et al., 1998; Lozano et al., 2001; Strayer et al., 2004; Ward and Ricciardi, 2007; Strayer, 2009), but also because they exert an influence on human health and economics (Pimentel et al. 2005; Connelly et al. 2007; Strayer, 2009). The population densities and effects of zebra mussels have become so great that a new management tool is needed. The control tools resource managers possess at present time are not able to discriminate between target and non-target species, and as a result will not aid in protecting native organisms in areas of concern due to high zebra mussel density. A new control tool is needed that will preferentially target zebra mussels and minimize impacts on native organisms, in particular the Unionidae, a critically endangered or threatened group of organisms (Williams et al. 1993).

An effective way to increase specificity of a toxicant is to examine life history traits of the target organism. Zebra mussels are filter-feeders, and so an orally delivered

toxicant may prove useful in reducing their numbers. However, because zebra mussels are not the only filter-feeding organism in an aquatic system care must be taken to ensure that the toxicant is released under specified conditions. Because the zebra mussels will be ingesting the toxicant, it was necessary to examine their digestive physiology and more specifically their digestive enzymes. A commercially available kit, api®ZYM, was used to screen for which enzymes may be of interest in designing a control tool against zebra mussels. The api®ZYM kit tests for the presence/absence of 19 enzymes. We previously validated the use of the api®ZYM kit and found that seven enzymes could be analyzed on more than a presence/absence basis; however, due to lack of information or the inability to purchase, only four of the enzymes were further examined: alkaline phosphatase, acid phosphatase, leucine arylamidase, and N-acteyl-β-glucosaminidase.

Enzymes have many effectors, one of which includes temperature. The effect of temperature on an animal's digestive enzymes is an important aspect of determining the application time of a toxicant. Previous studies on digestive enzymes in mussels have shown a significant decrease in enzyme activity when the enzyme is analyzed at a low temperature (Brock et al., 1986; Khrueanet et al, 2009; Supannapong et al, 2008; Areekijseree et al, 2002; Areekijseree et al, 2004). In specifically zebra mussels, temperature has been shown to decrease amylase activity by 28% (Golovanova, 2011). A study conducted on cellulase in zebra mussels showed that this enzyme has low thermal sensitivity which was similar to other species cellulase thermal sensitivity (Payne et al., 1972; Palais et al, 2010). If other enzymes in zebra mussels have a similar lack of thermal sensitivity, these enzymes could be exploited to digest a toxicant when water temperatures are low, and minimize impacts to native animals.

Because the api®ZYM kit does not discriminate between activity and concentration of enzyme present, amount of enzyme present and Q_{10} values were determined by utilizing a specific kit for each enzyme screened for by the api®ZYM kit. Q_{10} values are a measure of the effects of temperature on the rate of change of a chemical reaction when the temperature is changed by 10°C, and may give insight into the activity level of an enzyme at a given temperature. Relative enzyme efficiency was measured by the amount of substrate converted to product over a given period of time at a given temperature compared among species. Relative enzyme efficiency was used as a surrogate for K_{cat}/K_M due to the length and cost of studies needed to generate such data.

The main objective of this study was to determine if there are any detectable differences in enzyme amount or activity among the three species of filter-feeder studied: zebra mussels, threeridge mussels, and *H. orris*. The secondary objective was to determine what effects temperature has on these enzymes in these species.

Materials and Methods

Sample Collection and Processing

Ten threeridge mussels and 10 zebra mussels were collected via wading from the Mississippi River near Winona, MN (44.064924,-91.652799) on two dates (8/15/12 and 9/21/12). A species of caddisfly (*Hydropsyche orris*) was also collected and used to represent other filter-feeding invertebrates. Caddisfly larvae were collected using Hester-Dendy samplers placed near mussel collection areas (HD; Wildco, Yulee, FL, USA). Caddisfly larvae were identified based on morphological characteristics (Wiggins, 1996). Only *Hydropsyche orris* was used for subsequent analysis. Due to the small sizes of the

caddisflies, 2 and 4 larvae, dependent upon size, were pooled for each sample for enzymatic analysis.

Each sample was homogenized using a Geno/Grinder® (SPEX Sample Prep, Metuchen, NJ) and steel shot. Prior to homogenization, small amounts of deionized (DI) water were added to each sample to prevent drying and minimize the sticking of tissue to the tube walls during homogenization. The amount of water was dependent on the amount of tissue: two milliliters for zebra mussels, one milliliter for caddisfly larvae and no water was added to threeridge. Following homogenization, each sample was centrifuged at more than 6,800 x g for 20 minutes. Threeridge mussel supernatants were then diluted with an equal volume of DI water. Diluted supernatant was then used for further analysis. Amount of tissue used was not weighed.

β-galactosidase Assay

β-galactosidase activity assay was performed on September Winona samples by adding 2.85 mL assay buffer (60 mM Na_3PO_4, 10 mM KCl, 1 mM $MgSO_4$, 1 mM Dithiothreitol, pH 7.0), 140 µL of 4 mg/mL o-nitrophenyl-β-galactoside (ONPG) with 10 µL of sample. The absorbance was measured for 30 sec at 420 nm with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Rockford, IL). Activity was measured in Units/ml with one unit of β-galactosidase defined to produce 1.0 µmol ONPG produced per min. *E. coli* strain 3.300 CGSC 808 cell extract was used as a positive control. *E. coli* strain 3.300 CGSC 808 containing a *lac122* mutation was obtained from the Yale *E. coli* genetics stock center. *E. coli* cells were grown in LB medium for 24 hours.

Phosphatase Assays

Alkaline and acid phosphatases in August Winona samples were assayed following the protocol for a Phosphatase Assay Kit (G-Biosciences, St. Louis, MO, USA) according to the manufacturer's instructions, except no Stop Solution was added. All alkaline phosphatase assays were diluted and run in a buffer of 0.1 M Tris-HCl (pH 8.6) and 10 mM $MgCl_2$. All acid phosphatase assays were diluted and run in a buffer of 0.1 M sodium acetate (pH 5.5) and 10 mM $MgCl_2$. Fifty microliters of a 1:8 dilution of homogenate:buffer was used to quantify the amount of enzyme in a 96 well plate. After an incubation of 10 minutes at RT, the absorbance of the wells of the plate was measured with a Multiskan Spectrum plate reader (Thermo Scientific, Rockford, IL) at 405 nm. A purified alkaline or acid phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was used in known amounts (alkaline phosphatase: 0.3125 µU/µL to 2.5 µU/µL; acid phosphatase: 1.563 mU/µL to 6.25 mU/µL) to generate a standard linear curve to quantify the amount of enzyme.

After quantification of amount of enzyme present in each sample, an aliquot of each sample was used to dilute the amount of each enzyme to an equal amount (Alkaline phosphatase: 1 mU/µL; Acid phosphatase: 5 mU/µL). Relative enzyme efficiency was then assayed in the three species at two different temperatures (Alkaline phosphatase: 26.4 and 11°C; Acid phosphatase: 25.3 and 11°C) for 1 hour each with absorbance readings every 1 minute using the same spectrophotometer.

Leucine Ayrlamidase assays

Leucine arylamidase, in September Winona samples, was assayed using a Leucine arylamidase kit (Randox Laboratories Limited, Crumlin, UK). The kit protocol was

followed with the following exceptions. The reaction size was scaled down to a 96-well plate format from a cuvette format. The ratio of sample:working reagent was kept the same using 18 µL of sample and 282 µL of working reagent. The working reagent was prepared similarly to keep the ratio of substrate:buffer the same, using 9.40 µL of substrate and 272.6 µL of buffer. The plate was incubated at 25°C for 4 minutes, and the absorbances readings were read every 1 minute at 405 nm with a Multiskan Spectrum plate reader (Thermo Scientific, Rockford, IL). Following quantification of amount of enzyme, an aliquot of sample was used to dilute the amount of leucine arylamidase to an equal amount (5 mU/mL), and the relative enzyme efficiency was assayed at two temperatures (24 and 10°C) for 1 hour with absorbance readings at 405 nm every 1 minute.

N-acetyl-β-glucosaminidase Assays

N-acetyl-β-glucosaminidase (NAGase) concentration was assayed on August Winona samples using a β-N-Acetylglucosaminidase kit (Sigma-Aldrich, St. Louis, MO, USA). Diluted homogenate (5 µL) was used to quantify the amount of enzyme present. The assays were incubated in a 96-well plate for 5 minutes at 37°C, and the absorbance of each well was measured at 405 nm with a Multiskan Spectrum plate reader (Thermo Scientific, Rockford, IL). An aliquot was used to dilute all samples to an equal amount of enzyme (5 mU/mL). Relative efficiency was determined by extending the kit assay to 30 minutes and by taking absorbance readings every 1, 5, 15, and 30 minutes with 4 samples of each species. Efficiency assay temperature was also varied at either 24°C or 10°C.

Data Analysis

Enzyme Q_{10} data, enzyme quantification data, and relative enzyme efficiency data were analyzed by a 1-way ANOVA and Tukey's Post-Hoc test with species as the independent variable. Alkaline phosphatase and acid phosphatase were only analyzed to the 10 minute time-point because of non-linearity issues with zebra mussel enzymes after this point. Enzyme efficiency data was analyzed only at the higher temperature for each enzyme, which we defined relative efficiency as the amount of substrate converted to product over a period of time at a given temperature. The statistical analyses were performed using R version 3.0.1 with a significance level of $p < 0.05$.

Results

Enzyme Quantification

Enzyme concentrations were significantly higher in threeridge mussels compared to both zebra mussels and *H. orris* for all enzymes quantified (Table 11). Zebra mussels were significantly higher in leucine arylamidase and NAGase concentrations compared to *H. orris* (Table 1). *Hydropsyche orris* had significantly more alkaline phosphatase than zebra mussels (Table 11).

Table 11. Enzyme concentrations from all three species evaluated with specific enzyme kits. Values represented average concentrations ±SD (n = 10 for all except for NAGase values where n = 4). Letters indicate significant differences between groups ($p \leq 0.05$).

Species	Alkaline phosphatase (µU/µL)	Acid phosphatase (mU/µL)	Leucine arylamidase (U/L)	NAGase (mU/mL)
Threeridge mussel	20.12±6.59[a]	22.63±3.83[a]	122.29±48.13[a]	274.63±61.81[a]
Zebra mussel	5.53±1.76[b]	15.84±4.79[b]	69.39±40.15[b]	165.13±55.29[b]
Hydropsyche orris	14.03±4.84[c]	16.65±6.11[b]	27.21±21.86[b]	40.09±18.19[c]

β-galactosidase Assay

The assay for β-galactosidase resulted in no enzyme activity in the September Winona samples when compared to the positive control (data not shown), and as a result the amount of β-galactosidase was below detectable limits for all species tested.

Relative Enzyme Efficiency

For alkaline phosphatase, differences were found between the Q_{10} values for zebra mussel and *H. orris* (p=0.003; Figure 7; Table 12). There was no difference between threeridge mussel and the other species (versus zebra mussel p = 0.069; versus *H. orris* p = 0.359; Figure 7; Table 12). No differences were detected in relative enzyme efficiency among species (p = 0.168).

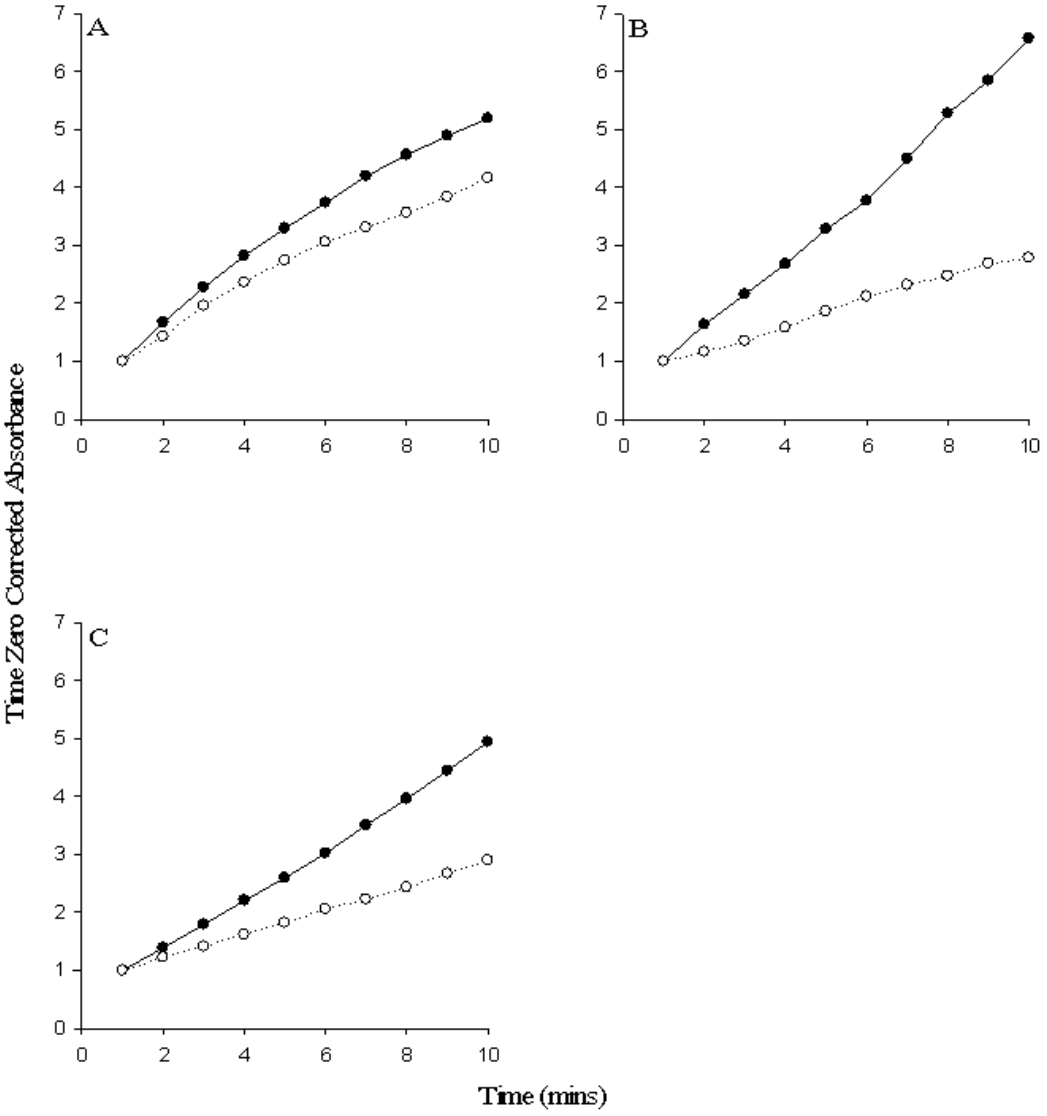

Figure 7. Alkaline phosphatase relative efficiency compared between temperatures and among species at 1 mU/µL. Black circled line corresponds to 26.4°C; white circled line corresponds to 11°C. Panel A: zebra mussel; B: *Hydropsyche orris*; C: threeridge mussel.

Table 12. Q10 values for enzymes tested. Values represented average concentrations ±SD (n = 10 for all except for NAGase values where n = 4). Letters indicate significant differences between groups ($p \leq 0.05$).

Species	Alkaline phosphatase	Acid phosphatase	Leucine arylamidase	NAGase
Threeridge mussel	1.63 ± 0.49^{ab}	1.32 ± 0.20^{a}	1.68 ± 0.20^{a}	1.52 ± 0.18^{a}
Zebra mussel	1.18 ± 0.23^{b}	1.02 ± 0.09^{b}	1.77 ± 0.27^{a}	1.18 ± 0.40^{a}
Hydropsyche orris	1.89 ± 0.47^{a}	1.30 ± 0.27^{a}	1.26 ± 0.17^{b}	1.27 ± 0.15^{a}

The next enzyme, leucine arylamidase, had significant differences between the Q_{10} values of *H. orris* compared to both species of mussel (both $p < 0.001$; Figure 8; Table 12), but no difference was found between Q_{10} values of mussel species ($p = 0.631$). Differences were found in relative enzyme efficiency among species ($p = 0.008$). The difference between relative enzyme efficiencies was between threeridge mussel and *H. orris* ($p = 0.008$), with a higher relative enzyme efficiency in *H. orris*. The difference between threeridge mussel and zebra mussel acid phosphatase was not statistically significant ($p = 0.052$), but may be biologically significant.

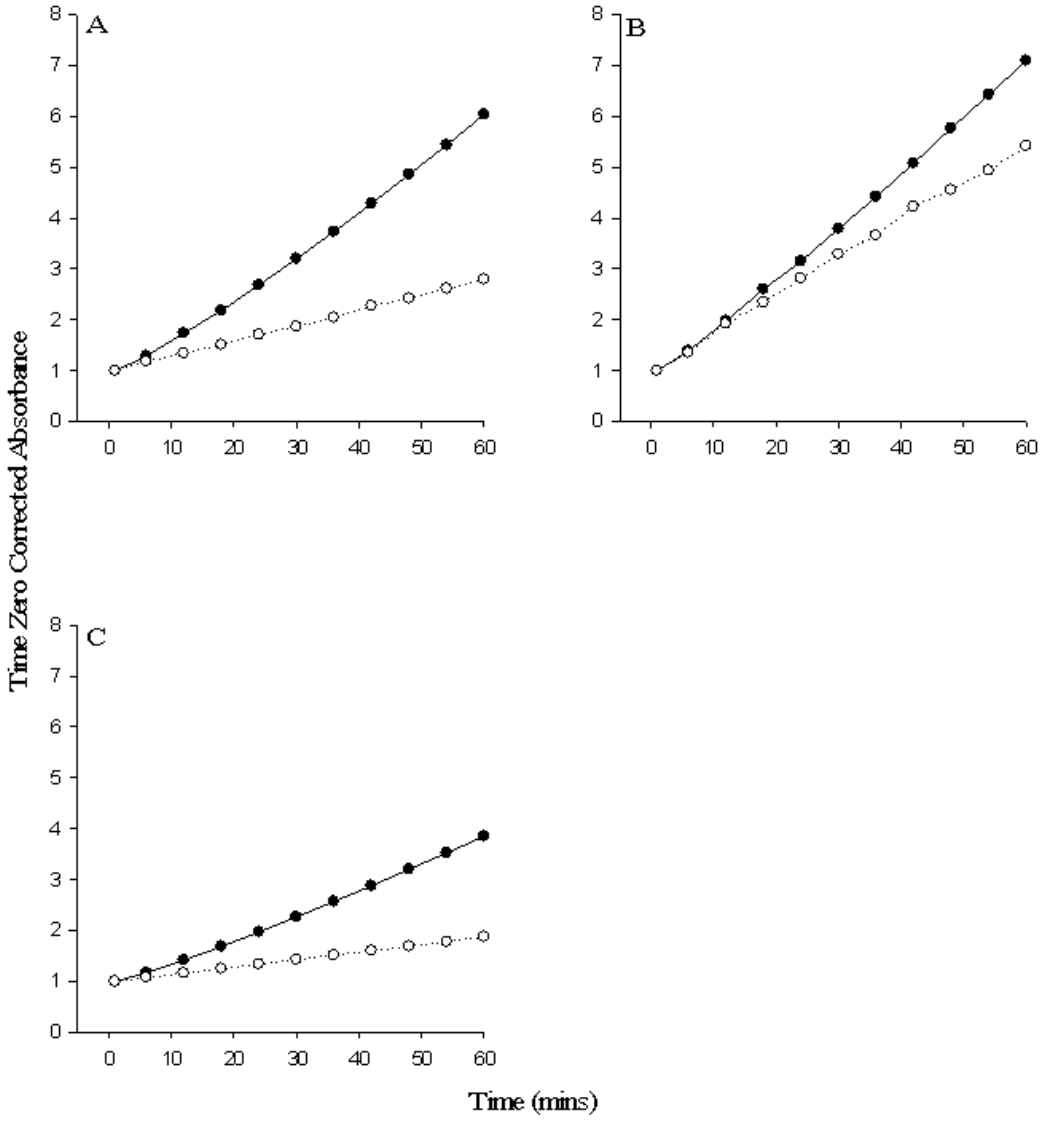

Figure 8. Leucine arylamidase relative efficiency compared between temperatures and among species at 5 mU/mL. Black circled line corresponds to 24°C; white circled line corresponds to 10°C. Panel A: zebra mussel; B: *Hydropsyche orris*; C: threeridge mussel.

The third enzyme, NAGase, had no significant differences among species Q10 values ($p = 0.257$; Figure 9; Table 12). No differences were detected in relative enzyme efficiency among species ($p = 0.237$).

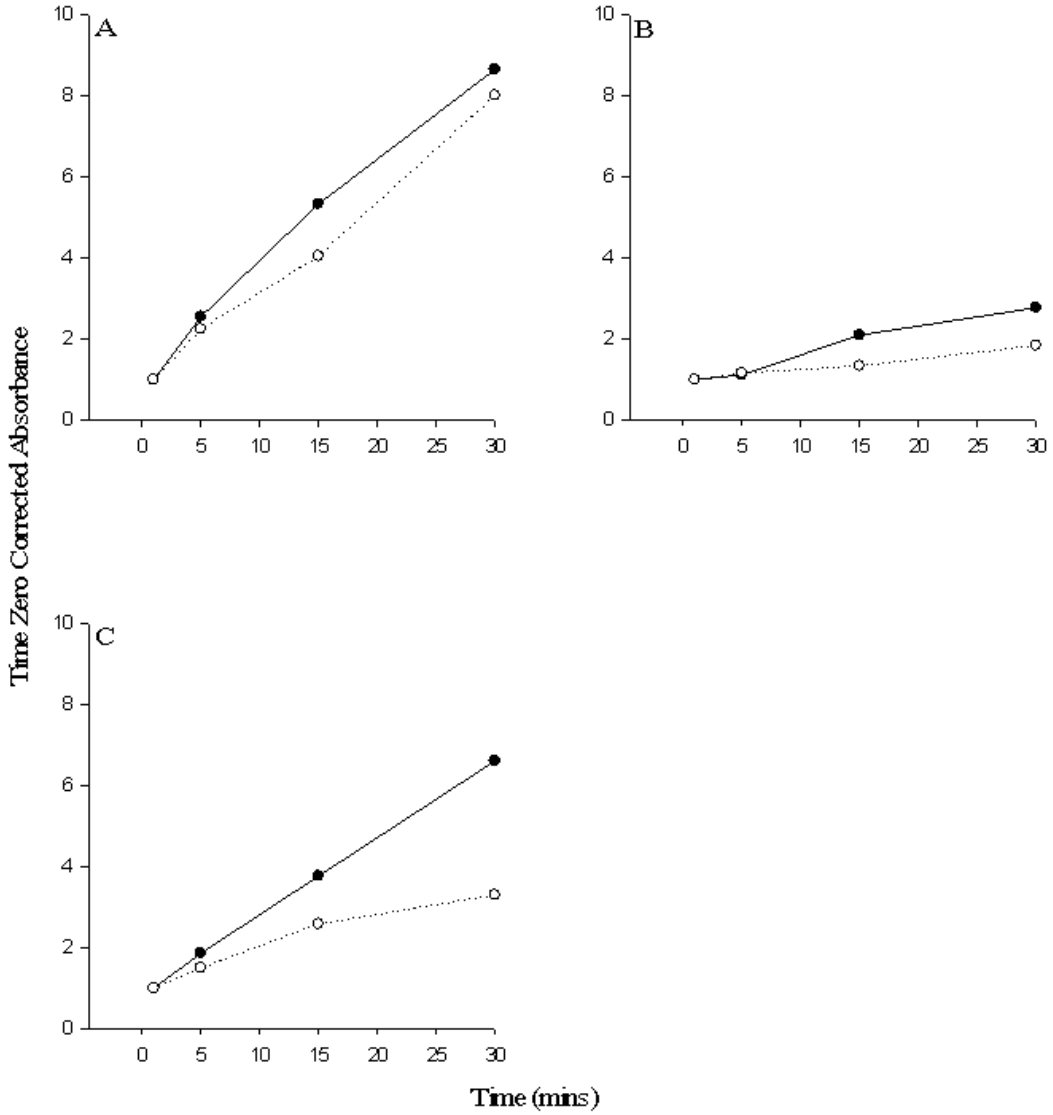

Figure 9. NAGase relative efficiency compared between temperatures and among species at 5 mU/µL. Black circled line corresponds to 24°C; white circled line corresponds to 10°C. Panel A: zebra mussel; B: *Hydropsyche orris*; C: threeridge mussel.

Acid phosphatase showed differences, in Q10 values, between zebra mussel and the other two species (versus threeridge mussel $p = 0.008$; versus *H. orris* $p = 0.014$), but not between threeridge mussel and *H. orris* ($p = 0.969$; Figure 10; Table 12). No differences were detected in relative enzyme efficiency among species ($p = 0.303$).

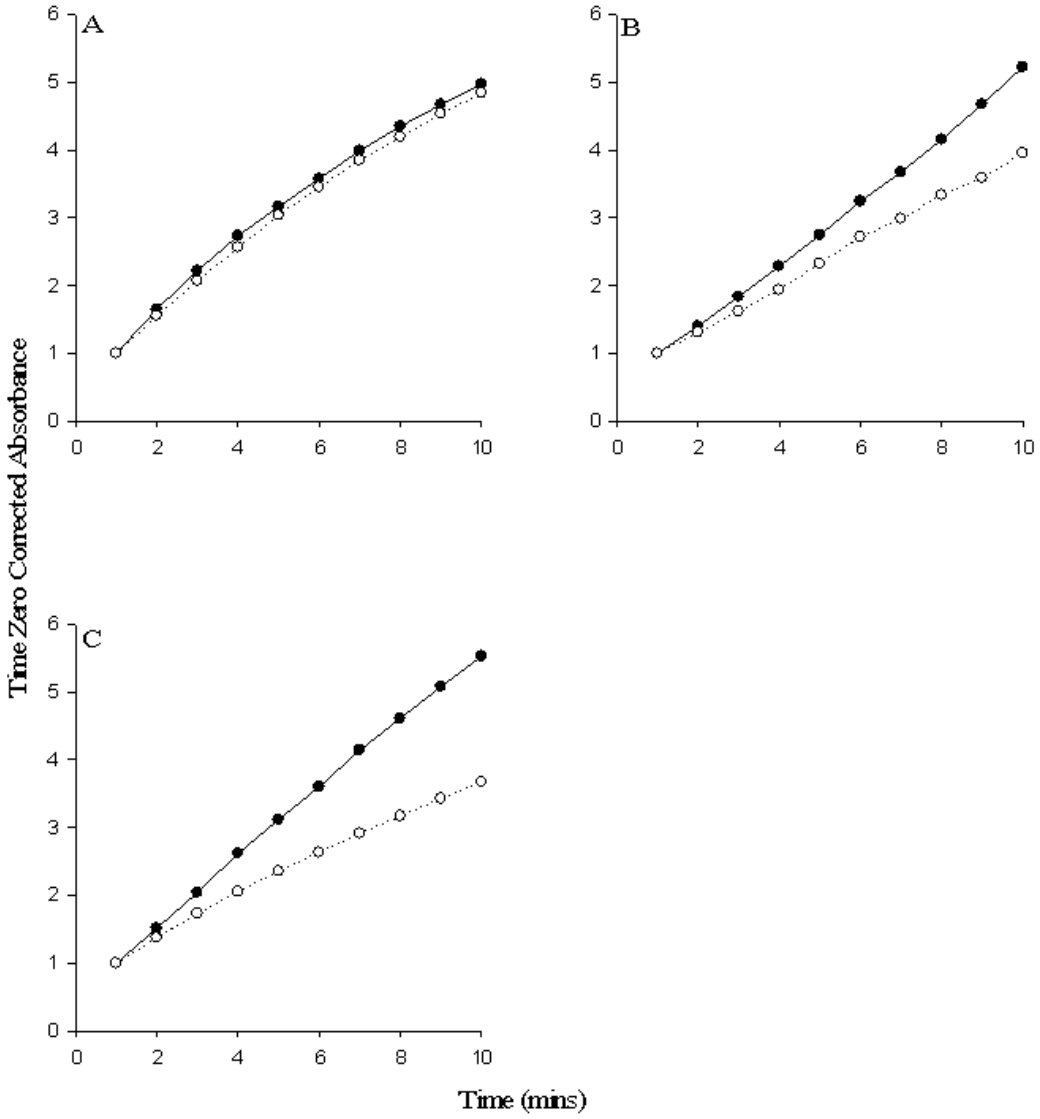

Figure 10. Acid phosphatase relative efficiency compared between temperatures and among species at 5 mU/µL. Black circled line corresponds to 25.3°C; white circled line corresponds to 11°C. Panel A: zebra mussel; B: *Hydropsyche orris*; C: threeridge mussel.

Discussion

β-galactosidase activity was not detected in threeridge, any zebra mussels, threeridge mussels or caddisflies in our study. To our knowledge, this was the first study that has attempted to measure β-galactosidase activity in mussels and caddisflies.

Because no enzyme activity was detected, the diets of these animals must not have contained any galactose residues at the time of sampling, or else the enzyme would have been present. It may also be present as a zymogen, but this information is unknown for these or similar species.

The Q10 values presented here are similar to other Q_{10} values calculated for bivalves (Payne et al., 1972; Doucet-Beaupre et al., 2010; Palais et al, 2010). To our knowledge ours is the first study to characterize the Q_{10} values for digestive enzymes from a species of caddisfly.

Alkaline phosphatase Q_{10} values differed between zebra mussel and *H. orris,* but no differences were found between Q_{10} values for threeridge mussel and either other species. Alkaline phosphatase in zebra mussels may not be as thermally sensitive as it is in native species. Because there were no detectable differences in relative enzyme efficiency, native animals may be producing more alkaline phosphatase to compensate for the fall in activity due to thermal sensitivity. It has been shown previously, in common carp *Cyprinus carpio,* that enzyme expression can be induced by low temperatures to compensate for the fall in enzyme activity (Metz et al., 2003).

The Q_{10} value for *H. orris* leucine arylamidase differed significantly from either mussel species, but the relative enzyme efficiency of *H. orris* leucine arylamidase only differed from that of threeridge mussel. Leucine arylamidase from this species of caddisfly is thermally insensitive, which suggests enzymatic activity during times of low water temperatures. As seen with alkaline phosphatase, threeridge mussels and zebra mussels compensate for thermal sensitivity by producing a greater amount of enzyme. It may still be biologically significant that caddisfly leucine arylamidase activity was higher

than that of zebra mussel. To our knowledge, this is the first study to characterize leucine arylamidase from these types of animals and no other information is available.

The third enzyme, NAGase, did not show any differences in Q_{10} values among the species. This result may be due to a lack of power due to the low number of samples used in the analysis (n = 4 for each species). If the number of samples could be increased, statistical differences would likely be found. The only differences between species were found to be in the amount of enzyme present in the samples (Table 7). This difference in amount among species is likely because this enzyme is likely evolutionarily conserved between mussel species due to its importance in digesting chitin, which is an important structural molecule for planktonic crustaceans (Kurita 2006), which may be an important part of the mussel diet (Strayer et al., 2004; Vaughn et al., 2008).

The final enzyme, acid phosphatase, had a higher Q_{10} value in zebra mussels compared to the other two species; zebra mussel acid phosphatase concentration was similar to that of *H. orris*, and only slightly less compared to threeridge mussel. Other studies have looked at acid phosphatase in zebra mussels with a focus on contaminant/stress effects (Giamberini and Pihan, 1997; Liu et al. 2004; Giamberini and Cajaraville, 2005; Chen et al., 2007; Izagirre et al., 2009), however, the effect of temperature, in regards to acid phosphatase's use as a digestive enzyme, has not been studied in mussels or Trichoptera until now to our knowledge. Because the relative efficiency of the enzyme was similar in all species, and that this enzyme was thermally insensitive in zebra mussels, this enzyme may be useful for the formulation of a control agent to protect native species.

CONCLUSIONS

From chapter 1, we were able to validate the use of the api®ZYM kit to measure digestive enzymes. Seven of the 19 enzyme activities were able to be relatively quantified on more than a presence/absence basis; however, only one enzyme had a linear relationship between amount of enzyme and the absorbance produced. This lack of linear relationships for the other enzymes suggests that the api®ZYM kit can only be used to identify trends and relative activities of the enzymes. We were able to find that enzyme activities were elevated in native mussels over those in zebra mussels in August. In September, the same enzyme activities decreased in native mussels; the enzyme activities stayed relatively the same or increased in zebra mussels.

Using the api®ZYM kit approach, we compared the activities of digestive enzymes in threeridge mussels, zebra mussels and caddisflies from three locations over five months to investigate species, temporal, and spatial effects on digestive enzyme activity. Our results suggest that temporal and spatial effects do not play a role in the activities of the seven digestive enzymes analyzed. Species was the only factor that influenced digestive enzyme activity, and as a result inferences about the diet based on inferences from the digestive enzymes can be generalized for the species tested.

The enzymes that were tested with the api®ZYM kit had their concentrations, relative efficiencies, and Q_{10} values analyzed with specific enzymatic kits. Our results showed the enzyme efficiencies were almost all similar between species; however the concentrations of the enzymes differed between species. The Q10 values differed

between species and showed that certain species have enzymes that have lower thermal sensitivities or have a high activity even at a low temperature. These results suggest that the species with enzyme efficiencies produce more of the enzyme to compensate for the lack of enzyme activity.

Even though all of the enzymes tested were found in all three species, differences were found in the amount of enzyme, how the enzymes respond to temperature, and how efficient the enzymes are. These enzymes require further investigation if one of them is to be the target for a control agent against the invasive zebra mussel. Acid phosphatase may present an exploitable way to reduce zebra mussel populations where they are a major problem because of the thermal insensitivity of this enzyme in zebra mussels. Two relatively simple enzyme assays cannot provide the information to confirm this, and more exhaustive methods are required to ensure that if acid phosphatase is used effects on non-target species are minimized.

Another strategy for targeting enzymes that may need to be considered are enzymes that are more active in native animals than in zebra mussels. If the control agent is designed in such a way that it is inactivated in the presence of an enzyme, this may serve to protect native animals if they have either a higher amount or a higher activity of the enzyme. This approach would still allow for a control agent to be designed and implemented even if native animal enzymes are expressed in higher amounts or the native animal enzymes are more active.

REFERENCES

Aragon-Axomulco H., Chiappa-Carrara X., Soto L., Cuzon G., Arena L., Maldonado C., Cardenas R. & Gaxiola G. (2012) Seasonal Variability in trypsin and alpha-amylase activities caused by the molting cycle and feeding habits of juvenile pink shrimp *Farfantepenaeus duorarum* (Burkenroad, 1939). *Journal of Crustacean Biology,* **32,** 89-99.

Areekijseree M., Engkagul A., Kovitvadhi U., Thongpan A., Mingmuang M. & Kovitvadhi S. (2002) Activity Profiles at Different pH and Temperature of Cellulases and Lipases in Freshwater Pearl Mussel: *Hyriopsis* (*Hyriopsis*) *bialatus*, Simpson 1900. *Kasetsart Journal: Natural Science,* **36,** 8.

Areekijseree M., Engkagul A., Kovitvadhi U., Thongpan A., Mingmuang M., Pakkong P. & Rungruangsak-Torrissen K. (2004) Temperature and pH characteristics of amylase and proteinase of adult freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus* Simpson 1900. *Aquaculture,* **234,** 575-587.

Baker S. & Levinton J. (2003) Selective feeding by three native North American freshwater mussels implies food competition with zebra mussels. *Hydrobiologia,* **505,** 97-105.

Baker S.M. & Hornbach D.J. (2000) Physiological status and biochemical composition of a natural population of unionid mussels (*Amblema plicata*) infested by zebra mussels (*Dreissena polymorpha*). *American Midland Naturalist,* **143,** 443-452.

Baker S.M. & Hornbach D.J. (2001) Seasonal metabolism and biochemical composition of two unionid mussels, *Actinonaias ligamentina* and *Amblema plicata*. *Journal of Molluscan Studies,* **67,** 407-416.

Barbiero R.P., Tuchman M.L. & Millard E.S. (2006) Post-dreissenid increases in transparency during summer stratification in the offshore waters of Lake Ontario: Is a reduction in whiting events the cause? *Journal of Great Lakes Research,* **32,** 131-141.

Beck K. & Neves R.J. (2003) An evaluation of selective feeding by three age-groups of the rainbow mussel *Villosa iris*. *North American Journal of Aquaculture,* **65,** 203-209.

Becker E.W. (2007) Micro-algae as a source of protein. *Biotechnology Advances,* **25,** 207-210.

Benke A.C. & Wallace J.B. (1997) Trophic basis of production among riverine caddisflies: Implications for food web analysis. *Ecology,* **78,** 1132-1145.

Bergamino L., Lercari D. & Defeo O. (2011) Food web structure of sandy beaches: Temporal and spatial variation using stable isotope analysis. *Estuarine Coastal and Shelf Science,* **91,** 536-543.

Bosley K., Witting D., Chambers R. & Wainright S. (2002) Estimating turnover rates of carbon and nitrogen in recently metamorphosed winter flounder *Pseudopleuronectes americanus* with stable isotopes. *Marine Ecology-Progress Series,* **236,** 233-240.

Brock V., Kennedy V.S. & Brock A. (1986) Temperature dependency of carbohydrase activity in the hepatopancreas of 13 estuarine and coastal bivalve species from the North-American east-coast. *Journal of Experimental Marine Biology and Ecology,* **103,** 87-101.

Buchholz F. & Saborowski R. (1996) A field study on the physiology of digestion in the Antarctic krill, *Euphausia superba*, with special regard to chitinolytic enzymes. *Journal of Plankton Research,* **18,** 895-906.

Caraco N.F., Cole J.J., Findlay S.E.G., Fischer D.T., Lampman G.G., Pace M.L. & Strayer D.L. (2000) Dissolved oxygen declines in the Hudson River associated with the invasion of the zebra mussel (*Dreissena polymorpha*). *Environmental Science & Technology,* **34,** 1204-1210.

Caraco N.F., Cole J.J., Raymond P.A., Strayer D.L., Pace M.L., Findlay S.E.G. & Fischer D.T. (1997) Zebra mussel invasion in a large, turbid river: Phytoplankton response to increased grazing. *Ecology,* **78,** 588-602.

Caraco N.F., Cole J.J. & Strayer D.L. (2006) Top-down control from the bottom: Regulation of eutrophication in a large river by benthic grazing. *Limnology and Oceanography,* **51,** 664-670.

Chen M.Y., Yang H.S., Delaporte M. & Zhao S.J. (2007) Immune condition of *Chlamys farreri* in response to acute temperature challenge. *Aquaculture,* **271,** 479-487.

Connelly N.A., O'neill C.R., Knuth B.A. & Brown T.L. (2007) Economic impacts of zebra mussels on drinking water treatment and electric power generation facilities. *Environmental Management,* **40,** 105-112.

Dempson J.B., Schwarz C.J., Shears M. & Furey G. (2004) Comparative proximate body composition of Atlantic salmon with emphasis on parr from fluvial and lacustrine habitats. *Journal of Fish Biology,* **64,** 1257-1271.

Deren J.J., Broitman S.A. & Zamcheck N. (1967) Effect of Diet upon Intestinal Disaccharidases and Disaccharide Absorption. *Journal of Clinical Investigation,* **46,** 186-195.

Doucet-Beaupre H., Dube C., Breton S., Poertner H.O. & Blier P.U. (2010) Thermal sensitivity of metabolic enzymes in subarctic and temperate freshwater mussels (Bivalvia: Unionoida). *Journal of Thermal Biology,* **35,** 11-20.

Effler S.W., Brooks C.M., Whitehead K., Wagner B., Doerr S.M., Perkins M., Siegfried C.A., Walrath L. & Canale R.P. (1996) Impact of zebra mussel invasion on river water quality. *Water Environment Research,* **68,** 205-214.

Effler S.W., Matthews D.A., Brooks-Matthews C.M., Perkins M.G., Siegfried C.A. & Hassett J.M. (2004) Water quality impacts and indicators of metabolic activity of the zebra mussel invasion of the Seneca River. *Journal of the American Water Resources Association,* **40,** 737-754.

Evans-White M.A., Stelzer R.S. & Lamberti G.A. (2005) Taxonomic and regional patterns in benthic macroinvertebrate elemental composition in streams. *Freshwater Biology,* **50,** 1786-1799.

Gamboa-Delgado J. & Le Vay L. (2009) Natural stable isotopes as indicators of the relative contribution of soy protein and fish meal to tissue growth in Pacific white shrimp (*Litopenaeus vannamei*) fed compound diets. *Aquaculture,* **291,** 115-123.

Garcia-Rios V., Rios-Leal E., Robledo D. & Freile-Pelegrin Y. (2012) Polysaccharides composition from tropical brown seaweeds. *Phycological Research,* **60,** 305-315.

Garton D.W., Payne C.D. & Montoya J.P. (2005) Flexible diet and trophic position of dreissenid mussels as inferred from stable isotopes of carbon and nitrogen. *Canadian Journal of Fisheries and Aquatic Sciences,* **62,** 1119-1129.

Gergs R., Rinke K. & Rothhaupt K.O. (2009) Zebra mussels mediate benthic-pelagic coupling by biodeposition and changing detrital stoichiometry. *Freshwater Biology,* **54,** 1379-1391.

Giamberini L. & Cajaraville M.P. (2005) Lysosomal responses in the digestive gland of the freshwater mussel, *Dreissena polymorpha*, experimentally exposed to cadmium. *Environmental Research,* **98,** 210-214.

Giamberini L. & Pihan J.C. (1997) Lysosomal changes in the hemocytes of the freshwater mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. *Diseases of Aquatic Organisms,* **28,** 221-227.

Golovanova I. (2011) Effects of abiotic factors (temperature, pH, heavy metals) on activities of glycosidases in invertebrate animals. *Journal of Evolutionary Biochemistry and Physiology,* **47,** 15-20.

Haag W.R., Berg D.J., Garton D.W. & Farris J.L. (1993) Reduced survival and fitness in native bivalves in response to fouling by the introduced zebra mussel (*Dreissena polymorpha*) in western Lake Erie. *Canadian Journal of Fisheries and Aquatic Sciences,* **50,** 13-19.

Hawkins A. (1985) Relationships between the synthesis and breakdown of protein, dietary absorption and turnovers of nitrogen and carbon in the blue mussel, *Mytilus-edulis*-L. *Oecologia,* **66,** 42-49.

Herwig B.R., Wahl D.H., Dettmers J.M. & Soluk D.A. (2007) Spatial and temporal patterns in the food web structure of a large floodplain river assessed using stable isotopes. *Canadian Journal of Fisheries and Aquatic Sciences,* **64,** 495-508.

Hilbish T.J. & Koehn R.K. (1985) The physiological-basis of natural-selection at the *Lap* locus. *Evolution,* **39,** 1302-1317.

Holland R.E. (1993) Changes in planktonic diatoms and water transparency in Hatchery Bay, Bass-Island area, western Lake Erie since the establishment of the zebra mussel. *Journal of Great Lakes Research,* **19,** 617-624.

Hormann L. & Maier G. (2006) Do zebra mussels grow faster on live unionids than on inanimate substrate? A study with field enclosures. *International Review of Hydrobiology,* **91,** 113-121.

Izagirre U., Ruiz P. & Marigomez I. (2009) Time-course study of the early lysosomal responses to pollutants in mussel digestive cells using acid phosphatase as lysosomal marker enzyme. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology,* **149,** 587-597.

Jagadeesan L., Arivuselvan N., Thirumaran G., Anantharaman P. & Balasubramanian T. (2010) Biomass and biochemical composition of zooplankton along the Arabian Sea, west coast of India. *Advance Journal of Food Science and Technology,* **2,** 96-99.

Johnson L.E., Ricciardi A. & Carlton J.T. (2001) Overland dispersal of aquatic invasive species: A risk assessment of transient recreational boating. *Ecological Applications,* **11,** 1789-1799.

Kamiyama T. (2011) Microzooplankton as a food source for the Pacific oyster *Crassostrea gigas*: seasonal variation in gut contents and food availability. *Fisheries Science,* **77,** 961-974.

Kanaya G., Takagi S. & Kikuchi E. (2008) Spatial dietary variations in *Laternula marilina* (Bivalva) and *Hediste* spp. (Polychaeta) along environmental gradients in two brackish lagoons. *Marine Ecology Progress Series,* **359,** 133-144.

Kelly D., Smyth J.A. & Mccracken K.J. (1991) Digestive development of the early-weaned pig .1. Effect of continuous nutrient supply on the development of the digestive-tract and on changes in digestive enzyme-activity during the 1st week post-weaning. *British Journal of Nutrition,* **65,** 169-180.

Khrueanet W., Kovitvadhi U., Engkagul A., Kovitvadhi S. & Rungruangsak-Torrissen K. (2009) Characterization of Digestive Enzymes from Adult Freshwater pearl Mussel *Chamberlainia hainesiana* (Lea, 1856). *KKU Science Journal,* **37,** 10.

Kloareg B. & Quatrano R.S. (1988) Structure of the cell-walls of marine-algae and ecophysiological functions of the matrix polysaccharides. *Oceanography and Marine Biology,* **26,** 259-315.

Knoll L.B., Sarnelle O., Hamilton S.K., Kissman C.E.H., Wilson A.E., Rose J.B. & Morgan M.R. (2008) Invasive zebra mussels (*Dreissena polymorpha*) increase cyanobacterial toxin concentrations in low-nutrient lakes. *Canadian Journal of Fisheries and Aquatic Sciences,* **65,** 448-455.

Kreeger D.A. & Newell R.I.E. (2001) Seasonal utilization of different seston carbon sources by the ribbed mussel, *Geukensia demissa* (Dillwyn) in a mid-Atlantic salt marsh. *Journal of Experimental Marine Biology and Ecology,* **260,** 71-91.

Kurita K. (2006) Chitin and chitosan: Functional biopolymers from marine crustaceans. *Marine Biotechnology,* **8,** 203-226.

Kuz'mina V. (1999) Effect of temperature on activities of some hydrolases in aquatic invertebrate animals. *Journal of Evolutionary Biochemistry and Physiology,* **35,** 19-25.

Liu S.L., Jiang X.L., Hu X.K., Gong J., Hwang H. & Mai K. (2004) Effects of temperature on non-specific immune parameters in two scallop species: *Argopecten irradians* (Lamarck 1819) and *Chlamys farreri* (Jones & Preston 1904). *Aquaculture Research,* **35,** 678-682.

Lopez-Rodriguez M.J., Trenzado C.E., De Figueroa J.M.T. & Sanz A. (2012) Digestive enzyme activity and trophic behavior in two predator aquatic insects (Plecoptera, Perlidae). A comparative study. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* **162,** 31-35.

Lozano S.J., Scharold J.V. & Nalepa T.F. (2001) Recent declines in benthic macroinvertebrate densities in Lake Ontario. *Canadian Journal of Fisheries and Aquatic Sciences,* **58,** 518-529.

Mackie G.L. (1991) Biology of the exotic zebra mussel, *Dreissena polymorpha*, in relation to native bivalves and its potential impact in Lake St. Clair. *Hydrobiologia,* **219,** 251-268.

Mackie G.L. & Schloesser D.W. (1996) Comparative biology of zebra mussels in Europe and North America: An overview. *American Zoologist,* **36,** 244-258.

Mandal R.N., Kumar K., Mohanty U.L. & Meher P.K. (2007) Estimation of gut contents of freshwater mussel, *Lamellidens marginalis L*. *Aquaculture Research,* **38,** 1364-1369.

Mccullough D.A., Minshall G.W. & Cushing C.E. (1979) Bioenergetics of Lotic Filter-Feeding Insects *Simulium* spp. (Diptera) and *Hydropsyche occidentalis* (Trichoptera) and their Function in Controlling Organic Transport in Streams. *Ecology,* **60,** 585-596.

Metz J.R., Van Den Burg E.H., Bonga S.E.W. & Flik G. (2003) Regulation of branchial Na+/K+-ATPase in common carp *Cyptinus carpio L*. acclimated to different temperatures. *Journal of Experimental Biology,* **206,** 2273-2280.

Miehls A.L.J., Mason D.M., Frank K.A., Krause A.E., Peacor S.D. & Taylor W.W. (2009) Invasive species impacts on ecosystem structure and function: A comparison of Oneida Lake, New York, USA, before and after zebra mussel invasion. *Ecological Modelling,* **220,** 3194-3209.

Mitchell M.J., Mills E.L., Idrisi N. & Michener R. (1996) Stable isotopes of nitrogen and carbon in an aquatic food web recently invaded by *Dreissena polymorpha* (Pallas). *Canadian Journal of Fisheries and Aquatic Sciences,* **53,** 1445-1450.

Naimo T.J., Damschen E.D., Rada R.G. & Monroe E.M. (1998) Nonlethal evaluation of the physiological health of unionid mussels: methods for biopsy and glycogen analysis. *Journal of the North American Benthological Society,* **17,** 121-128.

Naimo T.J. & Monroe E.M. (1999) Variation in glycogen concentrations within mantle and foot tissue in *Amblema plicata plicata*: Implications for tissue biopsy sampling. *American Malacological Bulletin,* **15,** 51-56.

Nalepa T.F., Hartson D.J., Fanslow D.L., Lang G.A. & Lozano S.J. (1998) Declines in benthic macroinvertebrate populations in southern Lake Michigan, 1980-1993. *Canadian Journal of Fisheries and Aquatic Sciences,* **55,** 2402-2413.

Newell R.I.E. & Langdon C.J. (1996) Mechanisms and Physiology of Larval and Adult Feeding. In: *The Eastern Oyster: Crassostrea virginica.* (Ed^(Eds V.S. Kennedy & R.I.E. Newell & A.F. Eble), p. 734. University of Maryland Sea Grant Publications, College Park, MD.

Newton T.J., Vaughn C.C., Spooner D.E., Nichols S.J. & Arts M.T. (2013) Profiles of biochemical tracers in unionid mussels across a broad geographical range. *Journal of Shellfish Research,* **32,** 1-11.

Nichols S. & Garling D. (2000) Food-web dynamics and trophic-level interactions in a multispecies community of freshwater unionids. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* **78,** 871-882.

Nitsan Z., Dror Y., Nir I. & Shapira N. (1974) The effects of force-feeding on enzymes of the liver, kidney, pancreas, and digestive tract of chicks. *British Journal of Nutrition,* **32,** 241-247.

Ozersky T., Evans D.O. & Barton D.R. (2012) Invasive Mussels Alter the Littoral Food Web of a Large Lake: Stable Isotopes Reveal Drastic Shifts in Sources and Flow of Energy. *Plos One,* **7,** 11.

Palais F., Jubeaux G., Dedourge-Geffard O., Biagianti-Risbourg S. & Geffard A. (2010) Amylolytic and cellulolytic activities in the crystalline style and the digestive diverticulae of the freshwater bivalve *Dreissena polymorpha* (Pallas, 1771). *Molluscan Research,* **30,** 29-36.

Pandian T.J. & Marian M.P. (1986) An indirect procedure for the estimation of assimilation efficiency of aquatic insects. *Freshwater Biology,* **16,** 93-98.

Parker B.C., Patterson M.A. & Neves R.J. (1998) Feeding interactions between native freshwater mussels (Bivalvia : Unionidae) and zebra mussels (*Dreissena polymorpha*) in the Ohio River. *American Malacological Bulletin,* **14,** 173-179.

Payne D.W., Thorpe N.A. & Donaldson E.M. (1972) Cellulolytic activity and study of the bacterial population in the digestive tract of *Scrobicularia plana* (Da Costa). *Journal of Molluscan Studies,* **40,** 147-160.

Pimentel D. (2005) Aquatic nuisance species in the New York State Canal and Hudson River systems and the Great Lakes Basin: An economic and environmental assessment. *Environmental Management,* **35,** 692-701.

Raikow D.F. & Hamilton S.K. (2001) Bivalve diets in a midwestern US stream: A stable isotope enrichment study. *Limnology and Oceanography,* **46,** 514-522.

Raikow D.F., Sarnelle O., Wilson A.E. & Hamilton S.K. (2004) Dominance of the noxious cyanobacterium *Microcystis aeruginosa* in low-nutrient lakes is associated with exotic zebra mussels. *Limnology and Oceanography,* **49,** 482-487.

Raymont J.E.G., Srinivasagam R.T. & Raymont J.K.B. (1971) Biochemical studies on marine zooplankton-VIII: Further Investigations on *Meganyctiphanes norvegica* (M. Sars). *Deep-Sea Research,* **18,** 1167-1178.

Ricciardi A., Whoriskey F.G. & Rasmussen J.B. (1997) The role of the zebra mussel (*Dreissena polymorpha*) in structuring macroinvertebrate communities on hard substrata. *Canadian Journal of Fisheries and Aquatic Sciences,* **54,** 2596-2608.

Rosensweig N.S. & Herman R.H. (1969) Diet and Disaccharidases. *The American Journal of Clinical Nutrition,* **22,** 99-102.

Rosi-Marshall E.J. & Wallace J.B. (2002) Invertebrate food webs along a stream resource gradient. *Freshwater Biology,* **47,** 129-141.

Sanchez-Muros M.J., Gomez-Milan E., Barroso F.G. & Manzano-Agugliaro F. (2013) Daily and Annual Variation in Digestive Enzymes Amylase and Basic and Acid Proteases in Gilt-head Sea Bream, *Sparus aurata*. *Journal of the World Aquaculture Society,* **44,** 105-114.

Schloesser D., Nalepa T. & Mackie G. (1996) Zebra mussel infestation of unionid bivalves (Unionidae) in North America. *American Zoologist,* **36,** 300-310.

Sousa R., Pilotto F. & Aldridge D.C. (2011) Fouling of European freshwater bivalves (Unionidae) by the invasive zebra mussel (*Dreissena polymorpha*). *Freshwater Biology,* **56,** 867-876.

Sprung M. (1995) Physiological energetics of the zebra mussel *Dreissena polymorpha* in lakes .3. Metabolism and net growth efficiency. *Hydrobiologia,* **304,** 147-158.

Stewart T.W., Miner J.G. & Lowe R.L. (1998) Quantifying mechanisms for zebra mussel effects on benthic macroinvertebrates: organic matter production and shell-generated habitat. *Journal of the North American Benthological Society,* **17,** 81-94.

Strayer D.L. (2009) Twenty years of zebra mussels: lessons from the mollusk that made headlines. *Frontiers in Ecology and the Environment,* **7,** 135-141.

Strayer D.L., Downing J.A., Haag W.R., King T.L., Layzer J.B., Newton T.J. & Nichols S.J. (2004) Changing perspectives on pearly mussels, North America's most imperiled animals. *Bioscience,* **54,** 429-439.

Strayer D.L., Hattala K.A. & Kahnle A.W. (2004) Effects of an invasive bivalve (*Dreissena polymorpha*) on fish in the Hudson River estuary. *Canadian Journal of Fisheries and Aquatic Sciences,* **61,** 924-941.

Strayer D.L. & Malcom H.M. (2007) Effects of zebra mussels (*Dreissena polymorpha*) on native bivalves: the beginning of the end or the end of the beginning? *Journal of the North American Benthological Society,* **26,** 111-122.

Supannapong P., Pimsalee T., A-Komol T., Engkagul A., Kovitvadhi U., Kovitvadhi S. & Rungruangsak-Torrissen K. (2008) Digestive enzymes and in-vitro digestibility of different species of phytoplankton for culture of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus*. *Aquaculture International,* **16,** 437-453.

Taylor A.N. & Batzer D.P. (2010) Spatial and temporal variation in invertebrate consumer diets in forested and herbaceous wetlands. *Hydrobiologia,* **651,** 145-159.

Thorp J.H. & Covich A.P. (2010) *Ecology and Classification of North American Freshwater Invertebrates,* Academic Press, San Diego, CA.

Thorp J.H., Delong M.D., Greenwood K.S. & Casper A.F. (1998) Isotopic analysis of three food web theories in constricted and floodplain regions of a large river. *Oecologia,* **117,** 551-563.

Vanderploeg H.A., Liebig J.R. & Nalepa T.F. (1995) From picoplankton to microplankton - temperature-driven filtration by the unionid bivalve *Lampsilis radiata siliquoidea* in Lake St. Clair. *Canadian Journal of Fisheries and Aquatic Sciences,* **52,** 63-74.

Vanderploeg H.A., Nalepa T.F., Jude D.J., Mills E.L., Holeck K.T., Liebig J.R., Grigorovich I.A. & Ojaveer H. (2002) Dispersal and emerging ecological impacts of Ponto-Caspian species in the Laurentian Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences,* **59,** 1209-1228.

Vannote R.L., Minshall G.W., Cummins K.W., Sedell J.R. & Cushing C.E. (1980) The River Continuum Concept. *Canadian Journal of Fisheries and Aquatic Sciences,* **37,** 130-137.

Vaughn C.C., Nichols S.J. & Spooner D.E. (2008) Community and foodweb ecology of freshwater mussels. *Journal of the North American Benthological Society,* **27,** 409-423.

Ventura M. (2006) Linking biochemical and elemental composition in freshwater and marine crustacean zooplankton. *Marine Ecology Progress Series,* **327,** 233-246.

Wallace J. & Merritt R. (1980) Filter-Feeding Ecology of Aquatic Insects. *Annual Review of Entomology,* **25,** 29.

Ward J.M. & Ricciardi A. (2007) Impacts of *Dreissena* invasions on benthic macroinvertebrate communities: a meta-analysis. *Diversity and Distributions,* **13,** 155-165.

Wen X., Peng C.L., Zhou H.C., Lin Z.F., Lin G.Z., Chen S.W. & Li P. (2006) Nutritional composition and assessment of *Gracilaria lemaneiformis* Bory. *Journal of Integrative Plant Biology*, **48,** 1047-1053.

Williams J.D., Warren M.L., Cummings K.S., Harris J.L. & Neves R.J. (1993) Conservation status of fresh-water mussels of the United States and Canada. *Fisheries*, **18,** 6-22.

Winterbourn M.J., Rounick J.S. & Cowie B. (1981) Are New Zealand steam ecosystems really different? *New Zealand Journal of Marine and Freshwater Research*, **15,** 321-328.

Wong W.H., Levinton J.S., Twining B.S. & Fisher N. (2003a) Assimilation of micro- and mesozooplankton by zebra mussels: A demonstration of the food web link between zooplankton and benthic suspension feeders. *Limnology and Oceanography*, **48,** 308-312.

Wong W.H., Levinton J.S., Twining B.S., Fisher N.S., Kelaher B.P. & Alt A.K. (2003b) Assimilation of carbon from a rotifer by the mussels Mytilus edulis and Perna viridis: a potential food-web link. *Marine Ecology Progress Series*, **253,** 175-182.

Woodward G. & Hildrew A.G. (2002) Food web structure in riverine landscapes. *Freshwater Biology*, **47,** 777-798.

Yeager, M. M., D. S. Cherry & R. J. Neves. 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, V*illosa iris* (Bivalvia, Unionidae). Journal of the North American Benthological Society 13:217-222.

Zerba K.E. & Collins J.P. (1992) Spatial heterogeneity and individual variation in diet of an aquatic top predator. *Ecology*, **73,** 268-279.

Zhu B., Fitzgerald D.G., Mayer C.M., Rudstam L.G. & Mills E.L. (2006) Alteration of ecosystem function by zebra mussels in Oneida Lake: Impacts on submerged macrophytes. *Ecosystems*, **9,** 1017-1028.