

ABSTRACT

HURLEY-SANDERS, JENNIFER LEE. Environmental Metabolomics as a Novel Tool for Assessment of Freshwater Bivalve Health. (Under the direction of Jay F. Levine, DVM and Stacy A. C. Nelson, PhD).

Freshwater mussels inhabit limnic ecosystems world-wide. As burrowing suspension feeders, they are potentially subject to chemical, physical and biotic changes within the benthos and overlying waters, making them unique sentinels for the entire freshwater ecosystem. One major barrier to the use of freshwater mussels as sentinels of ecosystem health is our ability to assess freshwater mussel health in its own right. Major strides have occurred in the non-lethal assessment of freshwater mussel health through hemolymph chemistries and magnetic resonance imaging. However, we need further information on basic freshwater mussel physiology and their subtle responses to their environment to improve our diagnostic capabilities.

The sister fields of metabolomics and metabonomics make use of a variety of technologies to study physiology at the level of metabolism. Through the measurement of the intermediate and end products of metabolism, physiologic status at a baseline or perturbed state can be assessed in a functional and dynamic manner. These techniques can be applied in a number of overlapping sub-fields such as environmental metabolomics studying an organism's response to stressors, or nutritional metabolomics assessing the impact of diet on the organism's metabolic profile.

In this dissertation we apply proton nuclear magnetic resonance (NMR) spectroscopic techniques in the metabolomic investigation of the freshwater mussel *Elliptio complanata*. First, we look at three extraction solutions and two incubation times to create a tissue

processing protocol for freshwater mussel tissues. We determined that Ringer's solution was adequate for metabolite extraction, with additional benefits of cost and safety. Second, the Ringer's protocol was applied to seven tissues from *E. complanata*, describing differences in metabolome based on tissue type. Third, the impact of seven diet treatments on the metabolome were assessed highlighting the use of the carbohydrates glucose and glycogen, as well as the polyamines putrescine and cadaverine as possible markers of nutritional status.

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Environmental Metabolomics as a Novel Tool for Assessment of Freshwater Bivalve Health

by
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DEDICATION

The time, effort, and resources that go into the development and completion of a dissertation are more than one person could ever imagine to create on one's own. To that end, I dedicate this work to the many who allowed me to draw on their patience, flexibility, emotional support, financial support, and free (and not-so-free) time in the creation of this dissertation. This includes my committee, my lab mates, my parents, my sister, my friends, my co-workers and most especially my husband and son, Daniel and Desmond Sanders who have borne the daily brunt of Mama's crazy obsession with getting ANOTHER degree.

BIOGRAPHY

A North Carolina ‘native’ by way of Mississippi, Hawaii and Virginia, Jennifer Lee Hurley-Sanders grew up in and around Raleigh, NC. And despite serious expectations of worldly travel, she somehow managed to move no more than 25 miles away from her family home in the time it took her to complete her Bachelors of Science in Biology (College of Agriculture and Life Sciences), her Doctorate of Veterinary Medicine (College of Veterinary Medicine) and her Doctorate of Philosophy in Fisheries, Wildlife and Conservation Medicine (College of Natural Resources), all from North Carolina State University. She has spent her time post-veterinary degree establishing herself as a small animal and exotic pet practitioner and acting as a relief vet for the Duke University Lemur Center. Jennifer returned for graduate school to further her interest in the fields of wildlife medicine and One Health and is still figuring out where that will lead her next.

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CHAPTER 1 – Introduction and Background

Freshwater mussels (Mollusca: Bivalvia: Unionida: Unionoidae) have captured the attention and imagination of researchers from a wide range of fields including ecology, biology, toxicology and veterinary medicine. This interest is partly due to the urgency of their imperiled status as approximately 70% of freshwater mussel species are endangered, threatened or at risk (Williams et al., 1993; Bogan, 1993; Master et al., 2000), but also because of their particular role in aquatic freshwater ecosystems (Vaughn et al., 2004; Grabarkiewicz and Davis 2008). Building a body of knowledge related to these cryptic animals furthers our understanding of their health status as individuals and provides information about the health of the environments they inhabit.

Freshwater Mussels as Indicators of Ecosystem Health

The health status of freshwater ecosystems is based on a dynamically changing combination of biotic and abiotic factors (Rapport et al., 1998; Burkhard et al., 2008). As with other aquatic ecosystems, freshwater streams are subject to the influences of atmospheric, climactic and riparian phenomena that affect stream habitat, water chemistry, microbial communities as well as plant and animal populations. Measuring physical parameters, such as temperature and chemical composition, provides a picture of abiotic factors that characterizes a freshwater stream's potential ability to support resident biota. Assessments of aquatic biota can give an indication of the combined influence of these abiotic changes and biotic interactions and potentially help identify factors that can negatively affect stream health. To understand the implications of these assessments it is critical to study key species that are broadly integrated within the ecosystem. Effective use of these species as indicators is dependent on our understanding of their niche in the stream community and baseline physiology (Hines et al., 2007a,b).

Freshwater mussels provide a number of ecosystem services. Mussels enhance the quality of water available for human and non-human consumers by removing contaminants

and decreasing turbidity through filtration (Vaughn and Hakenkamp, 2001; Cummings and Bogan, 2006). Deposited suspended solids and nutrients improve sediment quality for the benthic community. Mussel burrowing behaviors further improves nutrient, water and oxygen availability (Vaughn et al., 2004; Vaughn and Hakenkamp, 2001). Live and spent shells stabilize sediments and provide habitat for epifaunal communities and other benthic organisms (Vaughn and Hakenkamp, 2001). Immature stages serve as a food resource for aquatic vertebrates and juvenile and adult mussels support the diets of riparian vertebrates such as raccoons and muskrats (Grabarkiewicz and Davis, 2008).

The life history of unionid freshwater mussels makes them unique biological indicators of freshwater ecosystem health (Grabarkiewicz and Davis, 2008). The approximately 800 species of these mussels are distributed world-wide, inhabiting the benthos of rivers, streams, and lakes on all continents except Antarctica (Bogan, 2008). Individually, their home range is quite small, making freshwater mussels arguably easier to tag and monitor using established survey and sampling techniques (Grabarkiewicz and Davis, 2008) than more mobile species with large home ranges. Many freshwater mussel species have life spans that extend decades allowing repeated sampling for long term assessment (Cummings and Graf, 2010). Reliant on microbiota for food and on fish hosts for reproduction, the status of freshwater mussel populations can reflect ecosystem impacts across several trophic levels (Cummings and Graf, 2010; Vaughn et al., 2008; Atkinson et al., 2013). And as burrowing suspension feeders, freshwater mussels are exposed to contaminants in the water column and benthic sediments making them useful for pollution monitoring.

Traditional health assessments of freshwater mussels have challenges, and there is a need for expanding our diagnostic tool box. For example, toxicological testing with freshwater mussels routinely uses death as an endpoint to determine critical concentrations or measures chemical residues in tissues as evidence of exposure (ASTM 2006). Lethal concentration testing provides a dichotomous outcome (dead or alive), and a single end-point

value provides little to no information on the individual or population effects of low-level chronic exposures. Tissue detection methods for bioaccumulated toxicants result in presence/absence data, but presence alone does not speak to the effects on mussel health. To address some of these issues, alternative techniques used in veterinary medicine have been adapted to study more subtle markers of freshwater mussel health through hemolymph analysis (Gustafson et al., 2005a,b; Burkhard et al., 2009), advanced imaging techniques (Holliman et al., 2008) and behavioral responses (Bucci et al., 2008). These procedures facilitate nonlethal examination and allow for repeated sampling of the same individual.

To date, little information exists in the literature related to baseline freshwater mussel physiology and physiological responses to stressors. This impacts our ability to understand what affects the persistence of mussel populations, to manage them in captivity for propagation efforts, and to use them as biomonitors. However, to fully understand the relationship of metabolic health as an indicator of ecosystem health, a basic knowledge of metabolic status and biochemical composition must be determined for the organism of interest and degree of variation expected in a non-exposed population (Gibb et al., 1997). The application of a growing field, environmental metabonomics, may aid in answering some of these basic questions of mussel health and disease and enhance our understanding of freshwater mussel health as a proxy for aquatic ecosystem health (Miller, 2007; Viant et al., 2003; Lankadurai et al., 2013; Leonard et al., 2014).

Environmental Metabonomics

The sister fields of metabolomics and metabonomics are not new, but the scope of their application has broadened remarkably in the recent decade to include environmental investigations due to demand for environmental monitoring, need for novel diagnostic techniques and baseline information on model organisms (Dunn and Ellis, 2005; Grivet and Delort, 2009). Through the study of endogenous, low molecular weight (<1 kDa) organic metabolites as they occur within a cell, tissue, whole organism or biofluid (Viant, 2007; Bundy et al., 2009), metabolomics and metabonomics have been applied to research in

pharmacology, nutrition, toxicology, microbiology and other fields (Lankadurai et al., 2013, Leonard et al., 2014).

Metabolomics has been defined as the analysis of a biological system by identification and quantification of metabolites whereas metabonomics is the measurement of metabolic response to perturbation such as chemical exposure, physical change, etc. (Nicholson et al., 1999). In reality, the two fields overlap significantly and the terms are sometimes used interchangeably.

Through spectrographic methods techniques, tens to thousands of metabolites in small quantities are measured as a snapshot of physiologic status for an organism (Viant, 2007). The resulting measurements can then be mechanistically related to the organism's phenotype through metabolic pathways (Tikunov et al., 2010). The data yielded in a single metabolomic profile, or metabolome, yields a robust image of health status, in contrast to the small picture we obtain when measuring a single biomarker (Miller, 2007). That being said, sometimes identification of a single biomarker is useful, and the whole-picture view of metabolomics can identify those biomarkers (Miller, 2007).

Metabolomic investigations can be performed in a hypothesis-driven manner through target analysis, as certain disease conditions have predictable impacts on metabolic pathways and end products (Villas-Boas et al., 2005). However, one of the benefits of metabolomic techniques is that prior knowledge of metabolic influence does not have to be known. The detection of many metabolites at once allows metabolomic profiling, which can yield unexpected relationships and responses, driving further investigation and understanding (Bundy et al., 2009). This exploratory nature of metabolomics lends itself readily to environmental investigations and allows it to be easily transferrable to new organismal models.

Environmental metabonomics, defined as the measurement of metabolic response to external stimuli, characterizes the interaction between the organism and its environment by measuring physiological changes at a molecular level (Morrison, et al., 2007; Miller, 2007; Bundy et al., 2009; Viant, 2007; Lankadurai et al., 2013). Through metabolomic techniques, an organism's function and health at a molecular level can be evaluated as it responds directly and indirectly to abiotic and biotic stimuli using a systems-based approach (Bundy et al., 2009; Viant, 2007). Metabolic pathways are relatively conserved phylogenetically and the detection of individual metabolites is not species-specific, minimizing the need for validation of techniques in novel species. Through in-vivo and in-vitro investigations, it is possible to determine the relationship between cellular response measured by presence and level of metabolites to chemical, nutritional, disease states or other stressors (Miller, 2007). Potentially, patterns of metabolites could suggest responses to particular stimuli, information about exposure to particular toxicants, nutritional status, or presence of infectious disease. Investigations in environmental metabolomics have detected withering disease in abalone (*Haliotis rufescens*) and evaluated the effect of treatment (Viant et al., 2003, Rosenblum et al., 2006), described heat stress and recovery in *Drosophila melanogaster* (Malmendal et al., 2006), distinguished between earthworms (*Lumbricus rubellus*, *L. terrestris* and *Eisenia andrei*) from a metal contaminated and non-contaminated site (Bundy et al., 2004), and characterized the effects of heavy metal toxicity in Manila clams (*Ruditapes philippinarum*) (Zhang et al., 2011). Data from environmental metabolomic studies can inform a wide range of ecological and environmental studies (Miller, 2007; Bundy et al., 2009), and elucidate important life history questions (Tikunov et al., 2010; Hines et al., 2007b; Dove et al., 2012; Leonard et al., 2014).

Metabolomic techniques

No one bioanalytical technique is capable of detecting all metabolites (Viant, 2007). A metabolome is comprised of fatty and amino acids, vitamins, lipids, carbohydrates and all other intermediate and end products of an organism's general metabolic processes; each with distinct chemical characteristics and behaviors (Dunn and Ellis, 2005).

The two primary analytical platforms to detect metabolites as part of metabolomics are nuclear magnetic resonance (NMR) and mass (MS) spectroscopy. Mass spectroscopy in combination with chromatographic techniques such as gas or liquid chromatography is currently the most widely applied method. A complex platform, the strength of MS in metabolomics lies in the rapid, highly sensitive detection of metabolites for particular pathways or chemical classes while also allowing molecule identification (Dunn and Ellis 2005, Villas-Boas et al., 2005; Miller, 2007). The addition of chromatographic techniques broadens the analytical capabilities of MS, improving identification and quantification of metabolites. Mass spectroscopy requires solubilization of biomolecules and the sample is not retrievable after analysis. Nuclear magnetic resonance spectroscopy is also rapid and provides a quantitative, non-selective, method with minimal sample preparation (Dunn and Ellis, 2005; Pelczer, 2005; Holmes and Antti, 2002) contributing to its growing popularity in the metabolomics field, especially with the improved sensitivity associated with improvements in electromagnet technology (Grivet and Delort, 2009; Molinski, 2010). Its non-destructive nature makes analysis highly repeatable and allows secondary analysis of the same sample with MS or other platforms if needed (Brown et al., 2008). Nuclear magnetic resonance spectroscopy can be applied *in vitro* to biofluids, excised tissues or tissue extracts or *in vivo* to live organisms (Fan et al., 1986; Morrison et al., 2007).

For metabolome analysis, NMR samples are subject to strong magnetic field and radio frequency pulses (Dunn and Ellis, 2005; Fan and Lane, 2008). The nucleus of atoms with an odd atomic number (^1H) or odd mass number (^{13}C) develop spin in the presence of

the magnetic field and are promoted to a high-energy spin state by radio frequency pulses (Lin et al., 2006). Between pulses, the nuclei relax into a low-energy spin state and the emitted radiation is measured (Dunn and Ellis, 2005). The radio frequency at which the change in energy state occurs is characteristic for the paramagnetic nucleus and its relation to other paramagnetic nuclei on the molecule (Dunn and Ellis, 2005). In this way, NMR can be used to determine chemical structure and identify metabolites. By recording the frequency at which the state transitions occur a resonance spectrum is produced with predictable peaks related to chemical moiety (Fan, 1996; Fan and Lane, 2008). Intensity of peaks on an NMR spectrum is directly related to the number of paramagnetic nuclei with that resonance frequency allowing quantification of metabolites. Hydrogen-NMR is most commonly performed for metabolomic investigations as the majority of known metabolites contain hydrogen, yielding a non-biased profile of virtually all metabolites (Villas-Boas et al., 2005; Miller, 2007) without addition of an isotopic compound. Addition of isotopic compounds is useful for tracing carbons through metabolic pathways and for labeling food items to determine incorporation into body tissues. Through the use of pattern matching algorithms, metabolic perturbations can be detected making ^1H -NMR particularly useful in defining biomarkers (Miller, 2007).

Statistical analysis

Interpretation of the large amount of data resulting from a metabolomics investigation can be a challenge as only a portion of the information yielded may be useful and data sets with a large number of variables have inherent problems (Eriksson et al., 2004). Through the use of multivariate projection techniques, such as principal components analysis (PCA) and partial least-squares (PLS) regression, very large data sets can be represented in a way that can be understood by a general audience (Eriksson et al., 2004; Griffin, 2003). Principal components analysis collapses the multidimensional variable space into two or three hyperplanes based upon the maximum variance between response and independent variables whereas PLS finds a linear regression model by projecting predicted and observed variables

into a new space (Viant, 2007; Eriksson et al., 2004). Both techniques allow identification of correlation and can lead to the identification of subsets of data which correspond to real changes in the metabolic profile. Principal components analysis is applied to a metabolomic data set to investigate the innate variation within a data set, whereas PLS is more useful when particular questions are posed (Griffin, 2003). Once important subsets of the data are identified, further post-hoc tests can be applied to determine statistical significance (Schock et al., 2012; Schock et al., 2013).

The application of environmental metabonomics to freshwater mussels

The following chapters describe the first steps toward the routine application of metabolomic and metabonomic techniques as a tool in the investigation of freshwater mussel physiology. The freshwater mussel *Elliptio complanata*, is used as a model. The Eastern Elliptio (*Elliptio complanata* (Lightfoot, 1786)) is a widely distributed habitat generalist in eastern NA that is relatively tolerant to disturbance (Nedeau, 2008) and is abundant and stable throughout its range (Cummings and Cordiero, 2011) The Eastern Elliptio has been extensively used to biomonitor pollutants (Campbell and Evans, 1991; Renaud et al., 1995; Beckvar et al., 2000; Gagne et al., 2001; Mierzykowski and Carr, 2001; Gewurtz et al., 2002; Gewurtz et al., 2003; Martel et al., 2003) and to better understand bioavailability, bioaccumulation and biotransformation of pollutants (Day et al., 1990; Metcalfe-Smith, 1994; Tessier et al., 1994; Gewurtz et al., 2002; O'Rourke et al., 2004, Thorsen et al., 2004). This species has also been used as a model to develop diagnostic techniques and investigated for use as a proxy model for sympatric species (Gustafson et al., 2005a,b; Chittick et al., 2001)

Chapter 2 presents the results of a comparison between three extraction solvents with the objective of determining an uncomplicated, reliable, environmentally safe, and inexpensive method of obtaining a robust metabolic profile. An assessment of intra-organism variation is included. Chapter 3 describes the variation in metabolic profile by tissue type and by geographic location as the first steps in determining a characteristic biochemical profile

for *Elliptio complanata*. Chapter 4 builds on the results of the previous chapters through the application of metabonomic techniques to investigate the use of laboratory diets and evaluate the metabolic effects of fasting on *E. complanata*. The final chapter presents a summary of the dissertation work, including research challenges and future directions.

References

- American Society for Testing and Materials (ASTM). 2006. Standard guide for conducting laboratory toxicity tests with freshwater mussels. E2455-06. In *Annual Book of ASTM Standards*, Vol. 11.06. Philadelphia, PA, pp 1393–1444.
- Atkinson CL, CC Vaughn, KJ Forshay, and JT Cooper. 2013. Aggregated filter-feeding consumers alter nutrient limitation – Consequences for ecosystem and community dynamics. *Ecology* 94: 1359-1369.
- Beckvar N, S Salazar, M Salazar, and K Finkelstein. 2000. An in-situ assessment of mercury contamination in the Sudbury River, Massachusetts, using transplanted freshwater mussels (*Elliptio complanata*). *Canadian Journal of Fisheries and Aquatic Sciences* 57:1103-1112.
- Bogan AE. 1993. Freshwater Bivalve Extinctions (Mollusca: Unionoida): A search for causes. *American Zoologist* 33:599-609.
- Bogan AE. 2008. Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. *Hydrobiologia* 595: 139-147.
- Brown SAE, AJ Simpson, and MJ Simpson. 2008. Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with *Eisenia fetida*. *Environmental Toxicology and Chemistry* 27: 828-836.
- Bucci JB, WJ Showers, JF Levine, and B Usry. 2008. Valve gape response to turbidity in two freshwater bivalves (*Corbicula fluminea* and *Lampsilis radiata*). *Journal of Freshwater Ecology* 23: 479-483.
- Bundy JG, DJ Spurgeon, C Svendsen, PK Hankard, JM Weeks, D Osborn, JC Lindon, and JK Nicholson. 2004. Environmental metabonomics: Applying combination biomarker analysis in earthworms at a metal contaminated site. *Ecotoxicology* 13: 797-806.

- Bundy JG, Davey MP, Viant MR. 2009 Environmental metabolomics: a critical review and future perspectives. *Metabolomics* 5: 3-21.
- Burkhard B, Müller F, and A. Lill. 2008. Ecosystem Health Indicators. In SE Jorgensen and B Fath (Eds.) *Encyclopedia of Ecology*, pp1132-1138. Elsevier. Amsterdam.
- Burkhard MJ, S Leavell, RB Weiss, K Kuehnl, H Valentine, GT Watters, and BA Wolfe. 2009. Analysis and cytologic characterization of hemocytes from freshwater mussels (*Quadrula* sp.) *Veterinary Clinical Pathology* 38: 426-436.
- Campbell J, and RD Evans. 1991. Cadmium concentrations in the freshwater mussel (*Elliptio complanata*) and their relationship to water chemistry. *Archives of Environmental Contamination and Toxicology* 20: 125-131.
- Chittick B, M Stoskopf, M Law, R Overstreet, J Levine. 2001. Evaluation of potential health risks to Eastern Elliptio (*Elliptio complanata*) (Mollusca: Bivalvia: Unionida: Unionidae) and implications for sympatric endangered freshwater mussel species. *Journal of Aquatic Ecosystem Stress and Recovery* 9: 35-42.
- Cummings KE, and AE Bogan. 2006. Unionoida: Freshwater Mussels. In CF Sturm, TA Pearce and A Valdés (Eds.), *The Mollusks: A Guide to Their Study, Collection, and Preservation*. American Malacological Society. pp. 314-325. Universal Publishers. Boca Raton.
- Cummings KS, and DL Graf. 2010. Mollusca: Bivalvia. In JH Thorp and AP Covich (Eds.) *Ecology and Classification of North American Freshwater Invertebrates*. 3rd ed, p309-384. Elsevier. Boston.
- Cummings K, and J Cordiero. 2011. *Elliptio complanata* In: IUCN 2012, IUCN Red List of Threatened Species (ver. 2012.2) <www.iucnredlist.org>. (Accessed 07 March 2012)
- Day KE, JL Metcalfe, and SP Batchelor. 1990. Changes in intracellular free amino acids in tissues of the caged mussel, *Elliptio complanata*, exposed to contaminated environments. *Archives of Environmental Contamination and Toxicology* 19:816-827.
- Dove ADM, J Leisen, M Zhou, JJ Byrne, K Lim-Hing, HD Webb, L Gelbaum, MR Viant, J Kubanek, and F Fernández. 2012. Biomarkers of whale shark health: A metabolomic approach. *PLoS ONE* 7: e49379.

- Dunn WB, and DI Ellis. 2005. Metabolomics: Current analytical platforms and methodologies. *Trends in Analytical Chemistry* 24: 285-294.
- Eriksson L, H Antti, J Gottfries, E Holmes, E Johansson, F Lindgren, I Long, T Lundstedt, J Trygg, and S Wold. 2004. Using chemometrics for navigating in the large data sets of genomics, proteomics, and metabolomics (gpm). *Anal Bioanal Chem* 380: 419-429.
- Fan TWM, RM Higashi, AN Lane, and O Jardetzky. 1986. Combined use of ¹H-NMR and GC-MS for metabolite monitoring and in vivo ¹H-NMR assignments. *Biochimica et Biophysica Acta* 882: 154-167.
- Fan TWM (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog Nucl Mag Res Spec* 28: 161-219.
- Fan TWM, and AN Lane. 2008. Structure-based profiling of metabolites and isotopomers by NMR. *Prog Nucl Mag Res Spec* 52: 69-117.
- Gagne F, C Blaise, I Aoyama, R Luo, C Gagnon, Y Couillard, P Campbell, and M Salazar. 2002. Biomarker study of municipal effluent dispersion plume in two species of freshwater mussels. *Environmental Toxicology* 17: 149-159.
- Gewurtz SB, KG Drouillard, R Lazar, and GD Haffner. 2002. Quantitative biomonitoring of PAHs using the Barnes mussel. *Archives of Environmental Contamination and Toxicology* 43: 497-504.
- Gewurtz SB, R Lazar, and GD Haffner. 2003. Biomonitoring of bioavailable PAH and PCB water concentrations in the Detroit River using the freshwater mussel, *Elliptio complanata*. *Journal of Great Lakes Research* 29: 242-255.
- Gibb JOT, E Holmes, JK Nicholson, and JM Weeks. 1997. Proton NMR spectroscopic studies on tissue extracts of invertebrate species with pollution indicator potential. *Comp Biochem Physio.* 118B: 587-598.
- Grabarkiewicz JD, and WS Davis. 2008 Freshwater Mussels as Biological Indicators: Including Accounts of Interior Basin, Cumberlandian, and Atlantic Slope Species. U.S. Environmental Protection Agency, Office of Environmental Information, Washington DC. 122pp.

- Griffin JL. 2003. Metabonomics: NMR spectroscopy and pattern recognition analysis of body fluids and tissues for characterization of xenobiotic toxicity and disease diagnosis. *Current Opinion in Chemical Biology* 7: 648-654.
- Grivet JP, and AM Delort. 2009 NMR for microbiology: *In vivo* and *in situ* applications. *Progress in Nuclear Magnetic Resonance Spectroscopy* 54: 1-53.
- Gustafson LL, MK Stoskopf, AE Bogan, W Showers, TJ Kwak, S Hanlon, and JF Levine. 2005a. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Diseases of Aquatic Organisms* 65: 159-165.
- Gustafson LL, MK Stoskopf, W Showers, WG Cope, C Eads, R Linnehan, TJ Kwak, B Andersen, and JF Levine. 2005b. Reference ranges for hemolymph chemistries from *Elliptio complanata* of North Carolina. *Diseases of Aquatic Organisms* 65: 167-176.
- Holliman FM, D Davis, AE Bogan, TJ Kwak, WG Cope, and JF Levine. 2008. Magnetic resonance imaging of live freshwater mussels (Unionidae). *Invertebrate Biology* 127: 396-402.
- Hines A, GS Oladiran, JP Bignell, GD Stentiford, and MR Viant. 2007a. Direct Sampling Organisms from the Field and knowledge of their phenotype: Key recommendations for environmental metabolomics. *Environ. Sci. Technol* 41: 3375-3381.
- Hines A, WH Yeung, J Craft, M Brown, J Kennedy, J Bignell, GD Stentiford, and MR Viant. 2007b. Comparison of histological, genetic, metabolomics, and lipid-based methods for sex determination in marine mussels. *Analytical Biochemistry* 369: 175-186.
- Holmes E, and H Antti. 2002. Chemometric contributions to the evolution of metabonomics: mathematical solutions to characterizing and interpreting complex biological NMR spectra. *Analyst* 127: 1549-1557.
- Lankadurai BP, EG Nagato, and MJ Simpson. 2013. Environmental metabolomics: and emerging approach to study organism responses to environmental stressors. *Environ. Rev.* 21: 180-205.

- Leonard JA, WG Cope, MC Barnhart, RB Bringolf. 2014. Metabolomic, behavioral, and reproductive effects of the synthetic estrogen 17 α -ethinylestradiol on the unionid mussel *Lampsilis fasciola*. *Aquatic Toxicology* 150: 103-116.
- Lin CY, MR Viant, and RS Tjeerdema. 2006. Metabolomics: Methodologies and applications in the environmental sciences. *J. Pestic. Sci.* 31: 245-251.
- Malmendal A, J Overgaard, JG Bundy, JG Sorensen, NC Nielsen, V Loeschcke, and M Holmstrup. 2006. Metabolic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* 291: R205-R212.
- Martel P, T Kovacs, R Voss, and S Megraw. 2003. Evaluation of caged freshwater mussels as an alternative method for environmental effects monitoring (EEM) studies. *Environmental Pollution* 124:471-483.
- Master LL, BA Stein, S Kutner, GA Hammerson. 2000. Vanishing assets: Conservation status of U.S. species in the Powell River, Virginia. M.S. Thesis, Virginia Polytechnic Institute and State University, Virginia. 143pp.
- Metcalf-Smith JL. 1994. Influence of species and sex on metal residues in freshwater mussels (family Unionidae) from the St. Lawrence River, with implications for biomonitoring programs. *Environmental Toxicology and Chemistry* 13:1433-1443.
- Mierzykowski SE, and KC Carr. 2001. Total mercury and methyl mercury in freshwater mussels (*Elliptio complanata*) from the Sudbury River watershed, Massachusetts. USFWS. Special Project Report FY98-MEFO-2-EC. Old Town, ME.
- Miller MG. 2007. Environmental metabolomics: A SWOT analysis (strengths weaknesses opportunities and threats). *Journal of Proteome Research* 6: 540-545.
- Molinski TF. 2010. NMR of natural products at the ‘nanomole-scale’. *Nat. Prod. Rep.* 27: 321-329.
- Morrison N., D Bearden, JG Bundy, T Collette, F Currie, MP Davey, NS Haigh, D Hancock, OAH Jones, S Rochfort, SA Sansone, D Štys, Q Teng, D Field, and MR Viant. 2007. Standard reporting requirements for biological samples in metabolomics experiments: environmental context. *Metabolomics* 6: 203-210.

- Nedeau EJ. 2008. Freshwater Mussels and the Connecticut River Watershed. Connecticut Watershed Council, Greenfield Massachusetts. xvii+132pp.
- Nicholson JK, JC Lindon, and E Holmes. 1999. 'Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29: 1181-1189.
- O'Rourke S, KG Drouillard, and GD Haffner. 2004. Determination of laboratory and field elimination rates of polychlorinated biphenyls (PCBs) in the freshwater mussel, *Elliptio complanata*. *Archives of Environmental Contamination and Toxicology* 47: 74-83.
- Pelczar I. 2005. High-resolution NMR for metabonomics. *Current Opinion in Drug Discovery & Development* 8: 127-133.
- Rapport DJ, R Costanza, and AJ McMichael. 1998. Assessing Ecosystem Health. *Tree* 13: 397-402.
- Renaud CB, KLE Kaiser, ME Comba, and JL Metcalfe-Smith. 1995. Comparison between lamprey ammocoetes and bivalve mollusks as biomonitors of organochlorine contaminants. *Canadian Journal of Fisheries and Aquatic Sciences* 52: 276-282.
- Rosenblum ES, RS Tjeerdema, and MR Viant. 2006. Effects of temperature on host-pathogen-drug interactions in red abalone *Haliotis rufescens* determined by ¹H NMR metabolomics. *Environmental Science and Technology* 40: 707-7084.
- Schock TB, S Newton, K Brenkert, J Leffler, and DW Bearden. 2012. An NMR-based metabolomic assessment of cultured cobia health in response to dietary manipulation. *Food Chemistry* 133: 90-101.
- Schock TB, J Duke, A Goodson, D Weldon, J Brunson, JW Leffler, and DW Bearden. 2013. Evaluation of Pacific White Shrimp (*Litopenaeus vannamei*) health during a superintensive aquaculture growout using NMR-based metabolomics. *PLOS One*: e59521.
- Tessier L, G Vaillancourt, and L Pazdernik. 1994. Temperature effects on cadmium and mercury kinetics in freshwater molluscs under laboratory conditions. *Archives of Environmental Contamination and Toxicology* 26: 179-184.

- Thorsen WA, WG Cope, and D Shea. 2004. Bioavailability of PAHs: effects of soot carbon and PAH source. *Environmental Science and Technology* 38: 2029-2037.
- Tikunov AP, CB Johnson, H Lee, MK Stoskopf, and JM Macdonald. 2010. Metabolomic Investigations of American Oysters Using ¹H-NMR Spectroscopy. *Marine Drugs* 8: 2578-2596.
- Vaughn CC, and CC Hakenkamp. 2001. The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology* 46: 1431-1446.
- Vaughn CC, KB Gido, and DE Spooner. 2004. Ecosystem processes performed by unionid mussels in stream mesocosms: species roles and effects of abundance. *Hydrobiologia* 527: 35-47.
- Vaughn CC, SJ Nichols, and DE Spooner. 2008. Community and foodweb ecology of freshwater mussels. *Journal North American Benthological Society* 27: 409-423.
- Viant MR, ES Rosenblum, and RS Tjeerdema. 2003. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology* 37: 4982-4989.
- Viant MR. 2007. Metabolomics of aquatic organisms: The new 'omics' on the block. *Marine Ecology Progress Series* 332: 301-306.
- Villas-Bôas SG, JF Moxley, M Åkesson, G Stephanopoulos, and J Nielsen. 2005. High-throughput metabolic state analysis: The missing link in integrated functional genomics of yeasts. *Biochem. J.* 388: 669-677.
- Williams JD, ML Warren, KS Cummings, JL Harris, RJ Neves. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18: 6-22.
- Zhang L, X Liu, L You, D Zhou, Q Wang, F Li, M Cong, L Li, D Zhao, J Yu, and H Wu. 2011. Benzo(a)pyrene-induced metabolic responses in Manila clam *Ruditapes philippinarum* by proton nuclear magnetic resonance (¹H NMR) based metabolomics. *Environmental Toxicology and Pharmacology* 32: 218-225.

CHAPTER 2 - Tissue extraction methods for metabolic profiling of a freshwater bivalve, *Elliptio complanata* (Lightfoot, 1786)

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Abstract

Much is still unknown about why freshwater mussels (Unionidae) are particularly sensitive to environmental change. A better understanding of freshwater mussel metabolism is needed, and the field of environmental metabonomics holds the promise to inform these questions. A number of protocols exist for the extraction of metabolites for identification from animal tissues. As a first step in the application of environmental metabonomics to the study of freshwater mussels, we compared extraction protocols using an inorganic oxidizing acid (perchloric acid), an organic nitrile (acetonitrile), and a salt/water solution (Ringer's solution) to establish an uncomplicated, robust, repeatable and inexpensive tissue extraction protocol for freshwater mussel tissue. Perchloric acid resulted in notable extraction of energy-related nucleotides (AMP/ADP/ATP), yet had the lowest peak count of the three extraction methods and showed poor repeatability. Acetonitrile and Ringer's solution yielded

metabolite extraction results similar to each other with Ringer's solution having the greatest number of peaks particularly in the 3.0-4.5ppm sugar/amino acid range. Ringer's solution is simple to use, safe and consistent and bears consideration when selecting an extraction protocol for ^1H NMR experiments.

Introduction

The imperiled status of freshwater mussel populations worldwide is due to a suite of physical and chemical environmental changes including pollution, habitat change and loss of food resources (Bogan, 1993). One of the challenges in understanding the effects of these impacts is determining which single or combination of factors are affecting a given population or individual. A growing body of knowledge has aided in the diagnostic capability of investigators, yet there is still much we do not understand about freshwater mussel metabolism, the effects of noxious stimuli, and how to detect signs of illness, especially in the early stages.

Environmental metabonomics, defined as the measurement of metabolic response to external stimuli, characterizes the interaction between the organism and its environment by measuring physiological changes at a molecular level (Bundy et al., 2009; Morrison et al., 2007; Lankadurai et al., 2013). Environmental metabonomics techniques have been used to evaluate a wide range of taxa (Lankadurai et al., 2013). Among other contributions, metabonomics has been used to: 1) detect withering disease in abalone (*Haliotis rufescens* (Swainson, 1822)) (Viant et al., 2003), 2) characterize heat stress and recovery in fruit flies (*Drosophila melanogaster* (Meigen, 1830)) (Malmendal et al., 2006), 3) distinguish between earthworms (*Lumbricus rubellus* (Hoffmeister, 1843), *Lumbricus terrestris* (Linnaeus, 1758) and *Eisenia andrei* (Bouché, 1972)) from a metal contaminated and non-contaminated site (Bundy et al. 2004) and 4) suggest potential biomarkers for disease in whale sharks (*Rhincodon typus* (Smith, 1829)) (Dove et al., 2012). Data from environmental metabolomic studies can inform a wide range of ecological and environmental studies (Bundy et al. 2009; Miller 2007), and elucidate important life history questions (Dove et al., 2012; Tikunov et al.

2010). Metabonomic techniques evaluate tens to thousands of endogenous, low molecular weight (<1000 Da) organic metabolites as they occur within a cell, tissue, whole organism or biofluid, providing a snapshot of physiologic status for an organism (Bundy et al., 2009; Nicholson et al., 1999; Viant 2007). These measurements can then be mechanistically related to the organism's phenotype by considering established metabolic pathways to generate a robust multi-dimensional image of health status, in contrast to the limited information obtained when measuring a single biomarker. The value of the data yielded in this process is directly dependent on the use of reliable and repeatable sampling and processing techniques.

A metabolome is comprised of fatty and amino acids, vitamins, lipids, carbohydrates and other organic intermediate and end products of metabolic processes; each with distinct chemical characteristics which affect their stability and behavior in solution (Dunn and Ellis, 2005). A number of protocols exist to extract these metabolites from animal tissues (Table 2.1); however, to date there is no universally accepted technique (Liebke and Bundy, 2011; Lin et al., 2007; Viant, 2007; Lankadurai et al., 2013). For example, some extraction solvents selectively solubilize polar metabolites, while others are more efficient at extracting non-polar compounds (Lin et al., 2007). Still others precipitate large macromolecules that may interfere with identification of smaller metabolites resulting in improved spectral resolution (Lin et al., 2007). The ideal protocol for environmental metabolomic studies would be one that reproducibly and efficiently extracts a broad range of metabolites classes, does not destroy or modify the metabolites during processing, and is safe for the user and the environment (Barton et al., 2008; Liebke and Bundy; 2011, Lin et al., 2007; Maharjan and Ferenci, 2003).

As a preliminary step in the evaluation of metabolomics as a tool to evaluate freshwater mussel health and further our knowledge about freshwater mussel metabolism, we present a comparison of tissue extraction protocols for obtaining the ¹H -Nuclear Magnetic Resonance (NMR) metabolic profile of *Elliptio complanata*, a freshwater mussel common in the Eastern United States. To establish an uncomplicated, robust, repeatable and inexpensive

tissue extraction protocol for freshwater mussel tissue, we evaluated two solvents previously used to extract metabolites from other bivalve species, perchloric acid (Tikunov et al., 2010) and acetonitrile (Tuffnail et al., 2009), and a less toxic extraction protocol using Ringer's solution (Bundy et al., 2002; Gibb, 1997a,b). We compared the extraction protocols by: 1) analyzing the supernatants immediately after homogenization and after an additional 30 minute incubation time; 2) comparing the sum of the results of multiple serial extractions with the first extraction fraction of each sample and; 3) measuring the variability in the metabolic profiles observed between and within individual animals.

Table 2.1: Comparative studies of extraction methods. Asterisk indicates method(s) recommended by article authors.

Species	Extraction Method	Reference
Mammal cells	Acetonitrile*	Dietmair et al., 2012
	Methanol (freeze)*	
	Cold 50% methanol*	
	Methanol/chloroform*	
	Hot 80% methanol	
	Cold 100% methanol	
	Hot ethanol	
	Hot ethanol with HEPES	
	Cold ethanol	
	Hot water	
	Potassium hydroxide	
	Perchloric acid	
	Fish muscle and liver	
Acetonitrile/water (1:1)*		
Acetonitrile/water (2:1)*		
Methanol/water (1:1)		
Methanol/water (2:1)*		
Methanol/chloroform/water*		
Fish liver	Methanol/chloroform/water with KCl	Wu et al., 2008
	Step-wise methanol/chloroform/water	
	Two-step methanol/chloroform/water*	
Earthworms	All-in-one methanol/chloroform/water	Liebke and Bundy, 2011
	Chloroform/methanol	
	Aqueous acetonitrile/methanol*	
	Aqueous isopropanol/methanol	
Earthworms	Hot aqueous ethanol	Brown et al., 2008
	D2O buffer**	
	Acetonitrile-d3	
	Benzene-d6	
	Chloroform-d	
	Methanol-d4*	
DMSO-d6*		

Methodology

All chemicals were purchased through Fisher Scientific (Waltham, Massachusetts, USA).

Three adult Eastern Elliptio (*Elliptio complanata*) were collected from the Eno River, near Hillsborough, NC and transported in river water to the Aquatic Epidemiology and Conservation Laboratory at North Carolina State University College of Veterinary Medicine, Raleigh, NC. An oyster shucking knife was used to open the valves of the mussel and excise the mantle and adductor muscles from one valve. The anterior and posterior adductor muscles were sharply excised and placed into a 15ml polyethylene tube and snap frozen in liquid nitrogen within 10 seconds of opening the shell.

The muscle tissues from each animal were divided into eight portions and weighed to the nearest milligram. Each sample was placed individually into a 1.5ml polyethylene microcentrifuge tube and assigned a treatment by extraction solvent and time protocol (Table 2.2) and 500uL of the assigned extraction solvent (Ringer's solution, Acetonitrile 40%, Perchloric Acid 70%) was added. All solvents were maintained at room temperature, and samples were allowed to thaw during homogenization. Homogenization beads (200uL 0.9mm-1.6mm stainless steel beads, Next Advance) were added and the tubes were placed in a Bullet Blender homogenizer (Next Advance, Averill Park, New York, USA) and homogenized for 7 minutes. Additional beads (200uL 2mm zirconium oxide, Next Advance) were added to any samples that contained identifiable tissue pieces and an additional 3 minutes of homogenization was performed. Samples were considered completely homogenized once the tissue sample was reduced to a liquid consistency.

Table 2.2: Designation of treatment of each mussel by Solvent, Time Protocol, and Replicate. Includes tissue sample weight. * indicates a sample not included in final analysis due to poor spectral quality from contaminant or other signal interference.

Sample	Mussel W		Mussel X		Mussel Y	
	Solvent, Time Protocol, (Replicate)	Tissue weight (mg)	Solvent, Time Protocol, (Replicate)	Tissue weight (mg)	Solvent, Time Protocol, (Replicate)	Tissue weight (mg)
1	Ringer's, A (a)	119	Ringer's, A	47	Ringer's, A	84
2	Ringer's, A (b)	92	Ringer's, B	56	Ringer's, B	89
3	Ringer's, A (c)	108	Acetonitrile, A (a)	49	Acetonitrile, A	83
4	Ringer's, B	79	Acetonitrile, A (b)	65	Acetonitrile, B	155
5	Acetonitrile, A	85	Acetonitrile, A (c)	60	Perchloric Acid, A (a)	101
6	Acetonitrile, B	104	Acetonitrile, B	55	Perchloric Acid, A (b)	81
7	Perchloric Acid, A	81	Perchloric Acid, A	44	Perchloric Acid, A (c)	90
8	Perchloric Acid, B	97	Perchloric Acid, B	36	Perchloric Acid, B	130*

Samples designated for Time Protocol A were centrifuged (AccuSpin Micro 17, Fisher Scientific, Waltham, Massachusetts, USA) immediately after homogenization at 13,500rpm for 20 minutes to separate the tissue from the supernatant. The supernatant was collected and an additional 500uL of extraction solvent was added. The samples were then vortexed for approximately 30 seconds until the pellet was resuspended and incubated in a 4C refrigerator for 30min. Centrifugation was repeated, the supernatant collected and a third volume of solvent added. The vortexed samples were incubated for a second thirty minutes, centrifuged and supernatant collected.

Samples designated for Time Protocol B were vortexed and placed in a 4C refrigerator to allow the tissue to incubate in the extraction solvent for 30 minutes. After incubation, the samples were centrifuged at 13,500rpm for 20 minutes. The supernatant was collected, a second volume of solvent was added and the sample was incubated a second 30 minutes. After a second centrifugation and collection, the process was repeated a third time.

All collected Ringer's and acetonitrile extractions were frozen at -80C. Perchloric acid samples were buffered with potassium hydroxide to achieve a final pH of 7-7.4 then centrifuged at 13,500rpm for 20 minutes to remove any precipitate. The resulting supernatant was removed and frozen as the other samples.

Frozen samples were lyophilized overnight (Lyoph-Lock 18 Freeze Dry System, Laboconco, Kansas City, Missouri, USA), reconstituted with 700ul 10% deuterium oxide (D₂O) solution containing 0.1mM deuterated trimethylsilyl propionate (TSP) as an internal standard, then placed in microcentrifuge tubes. Samples were centrifuged at 13,500rpm for 20 minutes to separate any remaining solid material. Supernatant solutions were pipetted into 5mm borosilicate NMR tubes (Wilmad Labglass, Vineland, New Jersey, USA).

The pulsed field NMR experiments were performed on a Bruker AVANCE 500 MHz Spectrometer (1996) with Oxford Narrow Bore Magnet(1989), HP XW 4200 Host Workstation, and Topspin 1.3 Software version. The instrument is equipped with three Frequency Channels with Wave Form Memory and Amplitude Shaping Unit, three Channel Gradient Control Unit (GRASP III), variable Temperature Unit, Pre-Cooling and Temperature Stabilization Unit. A 5 mm ID 1H/BB (¹⁰⁹Ag-³¹P) Triple-Axis Gradient Probe (ID500-5EB, Nalorac Cryogenic Corp.) was used for all 1D ¹H presaturation experiments. The NMR probe was tuned to the ¹H frequency of 500.128 MHz in the 500 MHz spectrometer. All spectra were acquired at temperature 294 K. The instrument parameters for acquisition of the one-dimensional proton data and are listed in Table 2.3.

Table 2.3: ¹H data collection parameters

Parameter	¹ H value
Spectrometer frequency (MHz)	500.128
Spectral width (Hz) and (ppm)	13.2 ppm
Number of data points	32 K
Relaxation Delay (s)	1
Acquisition time (s)	2.47
Pulse width (□s) and tip angle	9.5 at 90°
Number of transients	128
Number of dummy scans	4
Presaturation delay	2sec
Presaturation power	58db

Data were analyzed using an ACD/Labs 12.0 1D NMR Processor (ACD/Labs, Toronto, Ontario, Canada). The ¹H spectra were zero-filled to 32,000 points, and line broadened using a 1.0 Hz exponential Gaussian function. The resulting spectra phase and baseline were corrected before integration using the ACD Intelligent Bucketing feature with a bin width of 0.04ppm excluding water and reference peaks (total 186 bins). Spectra were included for integration and additional analysis if a clear spectrum was obtained and the TSP peak was symmetrical with a half-peak width of 0.02ppm or less. The integral values for the first, second, and third extractions were added together for calculation of summed extraction analysis. Automatic peak counts were performed using a noise factor of 3.5 and minimum signal to noise ratio of 10.

The integral tables were normalized and Pareto scaled using Microsoft Excel 2010 (Microsoft, USA) to minimize inter-sample differences due to sample mass and to minimize effects of large amplitude differences respectively (Craig et al., 2006) The resulting transformed data was imported into SAS JMP v.10 (Cary, NC, USA) for principal components analysis (PCA). Through examination of a biplot PCA map, the contribution of metabolite peaks to the variation of the principal component line can be determined (Lin et al., 2007). Peak

identification was performed using Chenomx NMR Suite 7.6 (Chenomx, Edmonton, Alberta, Canada) and the Human Metabolome Database (www.hmdb.ca).

Results

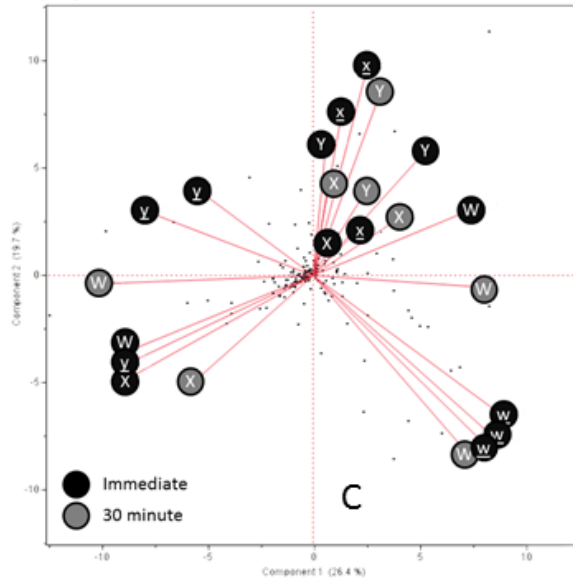
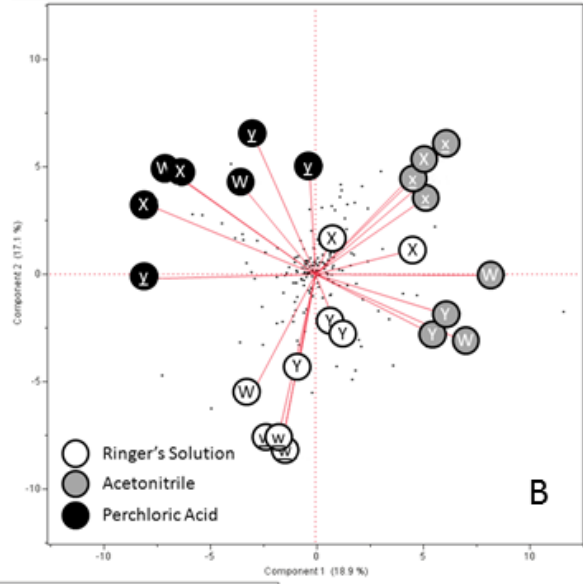
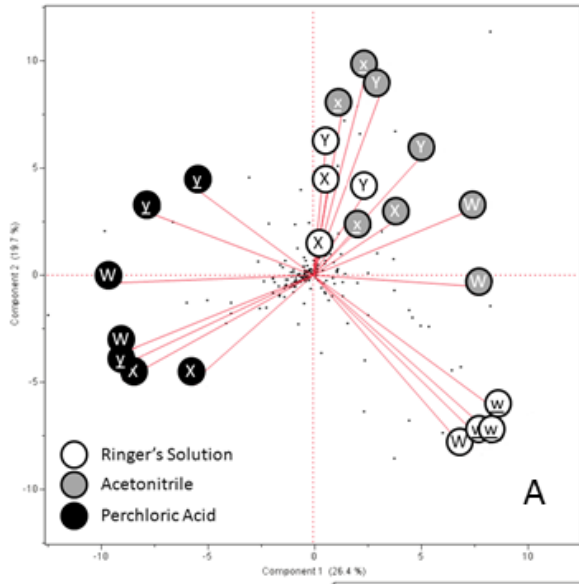
In this study, all three extraction solvents yielded metabolite profiles useable for metabolite identification and principal component analysis. One sample (Animal Y, 30 minute incubation) was not included in the analysis due to poor spectral quality characterized by poor detection of metabolites over background noise for all extraction fractions. For each solvent, the first component of a three fraction serial extraction yielded the greatest quantity and number of metabolites with the second and third fraction yielding few measurable metabolites. Solvent type accounted for the greatest variability between samples in both the first fraction and summed extractions (Figure 2.1 (A),(B)). Ringer's solution yielded the most peaks and the least variable profiles within the peaks obtained for an individual (Figure 2.1 (A),(B)). No obvious effect of time-to-first-extraction was evident (Figure 2.1 (C)).

Figure 2.1: Principal components biplots of ¹H-NMR spectra integrals showing clustering of samples with percentage variance. Letters within circles (W X Y) indicate the animal sampled lower case underlined letters indicate replicates of the same sample.

(A) Principal components map of solvent type calculated using first extraction of each sample. Perchloric acid samples (black) separate from other samples along PC 1. Ringer's solution (white) and acetonitrile (grey) group similarly however samples from Animal W group separately due to a strong glycogen peak.

(B) Principal components map of solvent type calculated using summed integrals from all three serial extractions of each sample with a particular solvent. With summation of the extraction fractions there is greater separation of the groups due to differences in the metabolite profile extracted by each solvent.

(C) Principal components map of time to first extraction calculated using the first extraction of each sample. There appears to be no correlation between metabolite profile and whether the extraction fluid is collected immediately after centrifugation (black) or incubated for 30 minutes prior to collection (grey).



Discussion

Comparison of solvents

Several studies looking at differences in extraction protocols for NMR analysis of animal tissues have displayed the diversity in techniques available and contrasting recommendations (Table 2.1). Prior NMR studies using molluscan tissue reflect this diversity with use of perchloric acid (Graham and Ellington, 1985, Rosenblum et al., 2006, Viant, 2003) and Ringer's solution (Gibb, 1997a) reported for gastropod tissue and perchloric acid (Tikunov et al., 2010), acetonitrile (Tuffnail et al., 2009), and methanol/chloroform with or without water (Hines et al., 2007; Jones et al., 2008; Kwon et al., 2012; Liu et al., 2011a,b,c; Zhang et al., 2011a,b) reported for marine bivalves. Animal tissues contain a large variety of metabolites with greatly differing physical and chemical characteristics, and no single technique currently known is capable of reliably yielding full extraction of all metabolites (Dietmair et al., 2012; Dunn and Ellis, 2005; Fan and Lane, 2008; Lin et al., 2007; Viant, 2003).

Often, mixtures of solvents are used to meet multiple protocol requirements (e.g., disrupt cell membranes, fractionate metabolite classes, solubilize metabolites, precipitate macromolecules) (Dietmair et al., 2012; Fan and Lane, 2008; Liebke and Bundy, 2011); however, this can increase the number of protocol steps and introduce variability. With this study we evaluated two commonly used extraction solvents (perchloric acid and acetonitrile) with a reported but less established physiologic solution (Ringer's solution) (Bundy et al., 2002; Gibb, 1997a,b). Our goal was to identify and characterize an easy to use, inexpensive, non-toxic protocol that gives high metabolite yield for global metabolite evaluation, and has good reproducibility.

Perchloric Acid

Perchloric acid (HClO_4) is a commonly used extraction solvent for NMR spectroscopy of biological samples, particularly for the extraction of nucleotides and other water-soluble metabolites including amines (Lin et al., 2006; Lin et al., 2007; Lowry et al., 1971; Shyrock et al., 1986). It is useful for metabolic profiling as it denatures and precipitates proteins and other macromolecules by itself or via precipitation with potassium hydroxide (KOH), removing the influence of these molecules on resonance spectra (Fan and Lane, 2008). However, other metabolites can be lost due to adsorption to perchlorate precipitates (Chen et al., 1977; Shyrock et al., 1986). As a highly volatile, strong acid, and a strong oxidizer at concentrations $>70\%$, perchloric acid requires special storage and handling and must be used in special ventilation hoods. Buffering steps are often included with perchloric acid protocols to minimize the effects of the low pH on the shift qualities of hydrogen metabolites in $^1\text{H-NMR}$ and to minimize destruction of acid-labile metabolites (Fan and Lane, 2008; Maharjan et al., 2003; Shyrock et al., 1986). This titration is time-consuming and can be technically difficult with small sample sizes, potentially resulting in wide alkaline and acid pH swings that can destroy metabolites of interest. Even if neutralized appropriately, peak shifting of metabolites due to acid effects is a major source of variation in perchloric acid protocols (Brown et al., 2008; Defernez and Colquhoun, 2003; Dietmair et al., 2012; Lin et al., 2007).

Our findings were consistent with these characterizations of perchloric acid extractions. Using principal components analysis to compare the methods, perchloric acid resulted in the most efficient extraction of energy-related nucleotides (AMP/ADP/ATP) along principal component 2. Perchloric acid samples had the lowest peak counts of the three extraction methods, suggesting lower numbers of metabolites extracted. We also found poor consistency between the replicate samples that did not improve with summation of the three extraction fractions (Figure 2.1).

Processing was more laborious for the perchloric acid samples than for the other methods. The perchloric acid samples were more likely to require additional homogenization time to achieve a liquefied sample, increasing the time needed for processing. Perchloric acid processing time was also increased 25-50 minutes per sample due to inclusion of the buffering step and subsequent centrifugation not needed for the acetonitrile or Ringer's solution protocols.

Acetonitrile

Acetonitrile (CH_3CN) is an aqueous, neutral, organic solvent with medium polarity that is usually better for extracting acid-labile metabolites than perchloric acid (Fan and Lane, 2008). Reproducibility is also improved for acetonitrile extractions as compared to perchloric acid (Lin et al., 2007). Acetonitrile is primarily useful for extraction of hydrophilic metabolites (Coen et al., 2003; Lin et al., 2007; Stentiford et al., 2005); however, it can also recover lipids and other macromolecules that result in superimposition of broad peaks on resonance spectra. These broad peaks complicate metabolite identification and quantification (Fan and Lane, 2008; Lin et al., 2007). Acetonitrile should be handled carefully, inhalation, ingestion and possibly skin absorption of acetonitrile can result in toxicity from its metabolite, hydrogen cyanide (Greenberg, 1999).

In our study, acetonitrile showed good reproducibility only after the three extraction fractions were summed (Figure 2.1). The 30-minute incubation showed greater peak numbers than the immediate collection after homogenization. This could be related to improved solubilization of metabolites over time. However, enzymatic activity resulting in the breakdown of macromolecules into smaller compounds (i.e., proteins to amino acids) cannot be ruled out. Principal components analysis showed similarities between acetonitrile and ringer's with overlap primarily due to putrescine and fructose metabolites.

Ringer's Solution

In the early 1880s, Ringer's solution was developed through investigation of the blood constituents needed to maintain contractility in frog cardiac muscle (Ringer, 1882; Ringer, 1883). As a salt solution isotonic to animal body fluids, this solution has become ubiquitous in physiologic studies and is used in modified form in human and veterinary medicine as an intravenous electrolyte solution. Ringer's solution is primarily a sodium chloride (NaCl), calcium chloride (CaCl₂) and potassium chloride (KCl) mixture with or without additives (Ringer's Solution, 2007). The composition of mineral salts is adjusted to accommodate individual species and meet their differing physiologic requirements for cell metabolism and osmolarity. This results in a slightly alkaline solution (pH 7.3-7.4), ideal for many NMR experiments. Mammalian and amphibian Ringer's solutions are inexpensive and readily obtainable through scientific supply companies, but can also be easily made in the laboratory (Ringer's Solution, 2007). The specific composition of a freshwater mussel Ringer's solution has not yet been determined, therefore an amphibian Ringer's solution composed of only NaCl, KCl, and CaCl₂ in water (Fisher Scientific, Waltham, Massachusetts, USA) was used in this study. The low concentration of paramagnetic elements (Ca²⁺⁺, Na⁺) in the Ringer's solution should have minimal effect on the NMR signal. Especially as any residual salt is further diluted through reconstitution of the sample with water (D₂O) during sample processing.

As with the use of phosphate buffer solutions (Brown et al., 2008; Bundy et al., 2009), detrimental effects on metabolites should be minimal and Ringer's solution should allow solubilization of metabolites at physiologic ratios. As an electrolyte solution that does not contain organic solvents or strong acids, this solution can be handled safely and disposed of without any special protocols. A suspected disadvantage is that Ringers has no quenching effect on enzymes and therefore may not maintain a temporally stable sample for analysis as long as extraction methods that denature proteins. It is possible that in a non-polar solution

such as Ringer's solution, hydrophobic components may settle with centrifugation (Brown et al., 2008).

In this study, Ringer's solution yielded the largest number of peaks, particularly in the 3.0-4.5ppm sugar/amino acid range. These metabolites were responsible for the separation of the majority of Ringer's samples from the samples processed with the two other extraction methods by principal component 1 (PC1). The replicate Ringer's samples had good consistency as shown by tight grouping on the principal components map when compared to all extraction methods and when only Ringer's samples were evaluated (Figure 2.1). This was seen in both the first extraction fraction and when the three extraction fractions were summed (Figure 2.1). Despite good reproducibility within an individual, PCA did show notable separation of mussel W from the other two individuals. This separation was not as apparent for the other extraction methods. Examination of the PCA biplot suggests that this grouping is due to variation in glycogen, indicating Ringer's may be a particularly good solution for extraction of this metabolite.

Other solvents

Methanol-based extraction protocols were not evaluated even though methanol/chloroform is well established for use in extractions of tissues of marine bivalves (Hines et al., 2007; Jones et al., 2008; Kwon et al., 2012; Liu et al., 2011a,b,c; Zhang et al., 2011a,b) and other species (Dietmair et al., 2012; Liebke and Bundy, 2011; Lin et al., 2007, Wu et al., 2008). The Ringer's method required fewer steps than needed for extraction with methanol/chloroform and a comparison of Ringer's solution with methanol/chloroform may be useful. In addition, Ringer's solution is less toxic and engineering controls needed to prevent exposure to methanol or chloroform are not needed when processing tissues with Ringer's solution.

Comparison of time protocol

Optimal time protocols varied with the extraction protocol. For the perchloric acid extractions, an immediate collection of supernatant following homogenization appeared to result in a greater number of spectral peaks than collection after 30 minutes incubation whereas acetonitrile yielded a greater peak number after incubation. For the Ringer's extraction, both time collections resulted in roughly the same number of peaks. Evaluation for time effect using principal components analysis, however, showed no apparent time effect suggesting that variation between individuals is greater than variation due to incubation time (Figure 2.1 (C)).

Comparison of serial extraction fractions

One extraction with 500uL of solvent per extraction was not adequate for retrieval of all metabolites, although many published extraction protocols extract tissues only once, with lower volume to tissue weight ratios and/or for shorter extraction times (Dietmair et al., 2012; Gibb et al., 1997a,b; Tikunov et al., 2010; Tuffnail et al., 2009). Although the peak numbers and intensities were notably lower on the second and third extractions than the first extraction, the addition of the three extractions was necessary to improve yield of metabolites for each extraction solvent tested. Extraction replicates had diminished variability when extractions were summed. These findings suggest that a minimum of three extractions are needed to adequately extract metabolites. However, increasing the ratio of solvent to tissue may achieve the same result if the solubilization of metabolites is not related to time of incubation, but rather saturation of the extraction solution.

Variation across individual mussels

An important quality of an extraction protocol for NMR is reproducibility (Brown et al., 2008; Dunn and Ellis, 2005; Lin et al., 2007). The introduction of variability through processing, such as pipetting errors, pH swings during titration, or temperature changes, can mask biological variation or create an artificially increased variation compared to actual

biological variation (Brown et al., 2008; Dunn and Ellis, 2005; Lin et al., 2007). In our study, Ringer's solution had the least intra-mussel variation based on principal components analysis. For Ringer's solution, the replicate variability was characterized by tight grouping of the replicate samples on both the first extraction and with all three extraction fractions summed. There was notable variability within the individual for the first extraction of acetonitrile, but reproducibility improved with the addition of the subsequent two extractions. Notable intra-mussel variability was seen with perchloric acid extraction that did not improve with the additional extractions. Variability related to the solvent utilized was greater than the individual variability between mussels, especially once extraction fractions were summed. (Figure 2.1 (A),(B)). As previously noted, with Ringer's solution extraction there was a difference in the amount of glycogen extracted from the tissues of mussel W.

Conclusions

Proton nuclear magnetic resonance spectroscopy using each of the three tested extraction solvents yields spectra that can be used for identifying metabolites in freshwater mussel adductor tissue. Determining effective protocols is the first step in the application of this technology to greater questions of freshwater mussel health. The strengths and weaknesses of each extraction procedure should be considered when selecting an extraction protocol for a metabolomics experiment. When specific metabolites are desired, selection of a specific protocol will result in optimal extraction of the markers of interest (Fan and Lane, 2008). In the early stages of many environmental metabolomic studies, a non-specific extraction is required as it may not be initially clear which are the metabolites of importance (Viant et al., 2003). The Ringer's solution protocol examined in this study is simple to use, safe and consistent, yielding a robust metabolite profile. Collection of supernatant immediately after homogenization is as efficient at collecting metabolites as allowing the homogenate to incubate thirty minutes prior to collection for this extraction solution. Three or more extractions are necessary to achieve complete extraction of metabolites when using 500uL of Ringer's per extraction. Ringer's solution bears consideration when selecting an extraction protocol for ^1H NMR experiments conducted to assess the health of freshwater mussels.

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References

- Barton, RH, JK Nicholson, P Elliott, and E Holmes. 2008. High-throughput ¹H NMR based metabolic analysis of human serum and urine for large-scale epidemiological studies: validation study. *International Journal of Epidemiology* 37: i31-i40.
- Bogan, AE. 1993. Freshwater Bivalve Extinctions (Mollusca: Unionoida): A search for causes. *American Zoologist* 33: 599-609.
- Brown SAE, AJ Simpson, and MJ Simpson. 2008. Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with *Eisenia fetida*. *Environmental Toxicology and Chemistry* 27: 828-836.
- Bundy, JG, EM Lenz, NJ Bailey, CL Gavaghan, C Svendsen, D Spurgeon, PK Hankard, D Osborn, JM Weeks, SA Trauger, P Speir, I Sanders, JC Lindon, JK Nicholson, and H Tang. 2002. Metabonomic assessment of toxicity of 4-fluoroaniline 35-difluoroaniline and 2-fluoro-4-methylaniline to the earthworm *Eisenia veneta* (Rosa): Identification of new endogenous biomarkers. *Environmental Toxicology and Chemistry* 21: 1966-1972.
- Bundy JG, DJ Spurgeon, C Svendsen, PK Hankard, JM Weeks, D Osborn, JC Lindon, and JK Nicholson. 2004. Environmental metabonomics: Applying combination biomarker analysis in earthworms at a metal contaminated site. *Ecotoxicology* 13: 797-806.
- Chen, SC, PR Brown, and D M Rosie. 1977. Extraction procedures for use prior to HPLC nucleotide analysis using microparticle chemically bonded packings. *Journal of Chromatographic Science* 15: 218-221.

- Coen, M, EM Lenz, JK Nicholson, ID Wilson, F Pognan, and JC Lindon. 2003. An integrated metabolomic investigation of acetaminophen toxicity in the mouse using NMR spectroscopy. *Chemical Research in Toxicology* 16: 295-303.
- Craig, A, O Cloarec, E Holmes, JK Nicholson, and JC Lindon. 2006. Scaling and normalization effects in NMR spectroscopic metabolomic data sets. *Analytical Chemistry* 78: 2262-2267.
- Defernez, M, and IJ Colquhoun. 2003. Factors affecting the robustness of metabolite fingerprinting using ^1H NMR spectra. *Phytochemistry* 62: 1009-1017.
- Dove ADM, J Leisen, M Zhou, JJ Byrne, K Lim-Hing, HD Webb, L Gelbaum, MR Viant, J Kubanek, and F Fernández. 2012. Biomarkers of whale shark health: A metabolomic approach. *PLoS ONE* 7: e49379.
- Dietmair, S, NE Timmins, PP Gray, L. K. Nielsen, and JO Krömer. 2012. Towards quantitative metabolomics of mammalian cells: Development of a metabolite extraction protocol. *Analytical Biochemistry* 404: 155-164.
- Dunn WB, and DI Ellis. 2005. Metabolomics: Current analytical platforms and methodologies. *Trends in Analytical Chemistry* 24: 285-294.
- Fan TWM, and AN Lane. 2008. Structure-based profiling of metabolites and isotopomers by NMR. *Prog Nucl Mag Res Spec* 52: 69-117.
- Gibb JOT, E Holmes, JK Nicholson, and JM Weeks. 1997. Proton NMR spectroscopic studies on tissue extracts of invertebrate species with pollution indicator potential. *Comp Biochem Physio.* 118B: 587-598.
- Gibb, JOT, C Svendsen, JM Weeks, and JK Nicholson. 1997b. ^1H NMR spectroscopic investigations of tissue metabolites biomarker response to Cu(II) exposor in terrestrial invertebrates: identification of free histidine as a novel biomarker of exposure to copper in earthworms. *Biomarkers* 2: 295-302.
- Graham, RA, and WR Ellington. 1985. Anaerobic aspartate metabolism and the formation of alanine in molluscan cardiac muscle: A ^{13}C NMR study. *Journal of Experimental Zoology* 236: 365-370.
- Greenberg, M. 1999. *Toxicological Review of Acetonitrile*. USEPA 35pg.

- Hines A, GS Oladiran, JP Bignell, GD Stentiford, and MR Viant. 2007. Direct Sampling Organisms from the Field and knowledge of their phenotype: Key recommendations for environmental metabolomics. *Environ. Sci. Technol* 41: 3375-3381.
- Jones, OAH, F Dondero, A Viarengo, and JL Griffin. 2008. Metabolic profiling of *Mytilus galloprovincialis* and its potential applications for pollution assessment. *Marine Ecology Progress Series* 369: 169-179.
- Kwon, YK, YS Jung, JC Park, J Seo, M Choi, and GS Hwang. 2012. Characterizing the effect of heavy metal contamination on marine mussels using metabolomics. *Marine Pollution Bulletin* 64: 1874-1879.
- Lankadurai BP, EG Nagato, and MJ Simpson. 2013. Environmental metabolomics: and emerging approach to study organism responses to environmental stressors. *Environ. Rev.* 21: 180-205.
- Liebke, M, and JG Bundy. 2011. Tissue disruption and extraction methods for metabolic profiling of an invertebrate sentinel species. *Metabolomics* 8: 819-830.
- Lin CY, MR Viant, and RS Tjeerdema. 2006. Metabolomics: Methodologies and applications in the environmental sciences. *J. Pestic. Sci.* 31: 245-251.
- Lin, CY, H Wu, RS Tjeerdema, and MR Viant. 2007. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics* 3: 55-67.
- Liu, X, L Zhang, L You, J Yu, J Zhao, Li, Q Wang, F Li, C Li, D Liu, and H Wu. 2011a. Differential toxicological effects induced by mercury in gills from three pedigrees of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. *Ecotoxicology* 20: 177-186.
- Liu, X, L Zhang, L You, M Cong, J Zhao, H Wu, C Li, D Liu, and J Yu. 2011b. Toxicological responses to acute mercury exposure for three species of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. *Environmental Toxicology and Pharmacology* 31: 323-332.
- Liu, X, L Zhang, L You, J Yu, M Cong, Q Wang, F Li, L Li, J Zhao, C Li, and H Wu. 2011c. Assessment of clam *Ruditapes philippinarum* as heavy metal bioindicators using NMR-based metabolomics. *Clean Soil Air Water* 39: 759-766.

- Lowry, OH, J Carter, JB Ward, and L Glaser. 1971. The effects of carbon and nitrogen sources on the level of metabolic intermediates in *Escherichia coli*. *Journal of Biological Chemistry* 246: 6511-6521.
- Maharjan, RM, and T Ferenci. 2003. Global metabolite analysis: the influence of extraction methodology on metabolome profiles of *Escherichia coli*. *Analytical Biochemistry* 313: 145-154.
- Malmendal, A, J Overgaard, JG Bundy, JG Sorensen, NC Nielsen, V Loeschcke, and M Holmstrup. 2006. Metabolic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* 291: R205-R212.
- Miller MG. 2007. Environmental metabolomics: A SWOT analysis (strengths weaknesses opportunities and threats). *Journal of Proteome Research* 6: 540-545.
- Morrison N, D Bearden, JG Bundy, T Collette, F Currie, MP Davey, NS Haigh, D Hancock, OAH Jones, S Rochfort, SA Sansone, D Štys, Q Teng, D Field, and MR Viant. 2007. Standard reporting requirements for biological samples in metabolomics experiments: environmental context. *Metabolomics* 6: 203-2010.
- Nicholson JK, JC Lindon, and E Holmes. 1999. ‘Metabonomics’: Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29: 1181-1189.
- Ringer, S. 1882. Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. *Journal of Physiology - London* 3: 380-383.
- Ringer, S. 1883. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *Journal of Physiology- London* 4: 29-42.
- Ringer’s Solution. 2007 *Cold Spring Harbor Protocols* doi:10.1101/pbd.rec110701
- Rosenblum ES, RS Tjeerdema, and MR Viant. 2006. Effects of temperature on host-pathogen-drug interactions in red abalone *Haliotis rufescens* determined by ¹H NMR metabolomics. *Environmental Science and Technology* 40: 707-7084.
- Shyrook, JC, R. Rubio, and RM Berne. 1986. Extraction of adenine nucleotides from cultured endothelial cells. *Analytical Biochemistry* 159: 73-81.

- Stentiford, GD, MR Viant, DG Ward, PJ Johnson, A Martin, W Wenbin, HJ Cooper, BP Lyons, and SW Feist. 2005. Liver tumors in wild flatfish: a histopathological proteomic and metabolomic study. *Omic*s 9: 281- 299.
- Tikunov AP, CB Johnson, H Lee, MK Stoskopf, and JM Macdonald. 2010. Metabolomic Investigations of American Oysters Using ¹H-NMR Spectroscopy. *Marine Drugs* 8: 2578-2596.
- Tuffnail, W, GA Mills, and P Cary, and R Greenwood. 2009. An environmental ¹H NMR metabolomics study of the exposure of the marine mussel *Mytilus edulis* to atrazine lindane hypoxia and starvation. *Metabolomics* 5: 33-43.
- Viant MR, ES Rosenblum, and RS Tjeerdema. 2003. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology* 37: 4982-4989.
- Viant, MR. 2003. Improved methods for the acquisition and interpretation of NMR metabolomic data. *Biochemical and Biophysical Research Communications* 310: 943-948.
- Viant MR. 2007. Metabolomics of aquatic organisms: The new ‘omics’ on the block. *Marine Ecology Progress Series* 332: 301-306.
- Wu, H, AD Southman, A Hines, and MR Viant. 2008. High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Analytical Biochemistry* 372: 204-212.
- Zhang L, X Liu, L You, D Zhou, Q Wang, F Li, M Cong, L Li, D Zhao, J Yu, and H Wu. 2011a. Benzo(a)pyrene-induced metabolic responses in Manila clam *Ruditapes philippinarum* by proton nuclear magnetic resonance (¹H NMR) based metabolomics. *Environmental Toxicology and Pharmacology* 32: 218-225.
- Zhang, L, X Liu, L You, D Zhou, H Wu, L Li, J Zhao, J Feng, and L Yu. 2011b. Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics. *Marine Environmental Research* 72: 33-39.

CHAPTER 3 - Key metabolites in tissue extracts of *Elliptio complanata* identified using ¹H Nuclear Magnetic Resonance (NMR) spectroscopy

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Abstract

We used ¹H-nuclear magnetic resonance (NMR) spectroscopy to describe the metabolome of the freshwater mussel, *Elliptio complanata*. Variability across tissue types was evaluated in mussels collected from the Eno River, Hillsborough, NC, and New Hope Creek, Durham, North Carolina, USA using principal components analysis. Muscle, digestive gland, mantle and gill tissues yielded identifiable but overlapping metabolic profiles. Digestive gland metabolic profiles varied between the two rivers characterized by differences in mono- and disaccharides. Mantle tissue metabolomes varied by gender. Nuclear magnetic resonance spectroscopy is a sensitive means to detect metabolites in the tissues of *Elliptio complanata* and holds promise as a tool for the investigation of freshwater mussel health and physiology.

Introduction

The ecological niche filled by suspension feeding bivalve mollusks makes them well suited to serve as biologic monitors of aquatic ecosystems. As filter feeders, they are exposed to suspended solids and dissolved chemicals as they process large quantities of water and aqueous solutes for food. The use of marine bivalves as biomonitors for contamination of the oceans is well established (Goldberg 1986, Páez-Osuna et al., 1993a, Páez-Osuna et al., 1993b, Liu et al., 2011, Zhang et al., 2011) and efforts have been made to use freshwater bivalves to monitor the health of surface waters (Won et al., 2005, Doyotte, et al., 1997, Grabarkiewicz and Davis, 2008). Current diagnostic methods to assess freshwater mussel health, such as tissue biopsies (Berg et al., 1995, Naimo et al. 1998), magnetic resonance imaging (Holliman et al., 2008) or hemolymph analysis (Gustafson et al., 2005b), need supportive research to tie the diagnostic results to an underlying state of health. Alternative targeted diagnostic methods, characteristic physiology and tissue responses, and the factors contributing to the response of metabolic indicators must be validated before freshwater bivalves can be effectively deployed as sentinels of ecosystem health.

Metabolomics, the study of an organism's profile of metabolites, has been used to study the physiologic responses of both terrestrial and aquatic organisms to changes in their environment. Metabolomic profiles have been successfully used to assess the response of bivalves (*Ruditapes philippinarum*) (Liu et al., 2011, Zhang et al., 2011) and earthworms (*Lumbricus rubellus*) (Bundy et al., 2007) to heavy metals, of abalone (*Haliotis rufescens*) (Rosenblum et al., 2006, Viant et al., 2003) to the causative agent of withering disease, and of fruit flies (*Drosophila melanogaster*) to heat stress (Malmendal et al., 2006). Environmental metabolomics shows promise as a tool to evaluate the physiologic responses of organisms to environmental degradation, changes in food web resources, and climate change (Bundy, et al., 2009, Peñuelas and Sardans, 2009, Ahuja et al., 2010).

Through spectrographic methods, a snapshot metabolic profile, can be obtained for a given tissue or biofluid at a specific time point. Potentially, patterns of metabolites could

suggest responses to particular stimuli; give information about exposure to toxicants or nutritional status, or identify the presence of an infectious disease. A broad range of metabolites can be detected with small samples collected through tissue biopsies or phlebotomy techniques. Prior to any studies of metabolic perturbation in response to a stressor, however, a basic knowledge of metabolic status and biochemical composition must be determined for the organism and tissue of interest, including the degree of variation expected in the unaffected population (Gibb et al., 1997). Core metabolites of individual tissues must be identified and confidence intervals for the range of responses to environmental variables that can be anticipated need to be documented before a particular tissue can be used to assess the health of an ecosystem.

We investigated the application of nuclear magnetic resonance spectroscopy (NMR) to examine the metabolome of Unionid freshwater mussels. *Elliptio complanata* from two river systems in the piedmont of North Carolina were sampled to evaluate metabolome differences based on tissue type. *Elliptio complanata* is a relatively common mussel species that inhabits many Atlantic slope rivers and holds promise both as a bioindicator for ecosystem health, and as a surrogate model for sympatric endangered mussel species (Chittick et al., 2001). As the first steps toward developing a baseline metabolic profile for *E. complanata* we evaluated metabolomics profiles obtained from *E. complanata* hemolymph, adductor muscle, foot muscle, gill, digestive gland and mantle tissue.

Methodology

Freshwater mussel collection and processing

In October of 2012, five adult *Elliptio complanata* (3 non-gravid females, 2 males) were taken from the Eno River, near Hillsborough, North Carolina, approximately 300m downstream from a highway bridge (Table 3.1). An additional five (5 males) were taken from the New Hope Creek, near Durham, North Carolina approximately 100m upstream of a road bridge (Table 3.1). Global positioning system coordinates were recorded for the study

sites (Figure 3.1). Mussels were processed streamside. Each individual was measured with calipers for height, length and width of shell. Tissue samples were collected and frozen as rapidly as possible, each mussel taking less than one minute to process completely. Hemolymph was aspirated from the anterior adductor muscle as described in Gustafson et al. 2005a and placed in a cryovial prior to placement on dry ice. The valves were gently pried open and soft tissues excised from the dorsal valve. Anterior and posterior adductor muscles (combined), foot muscle, mantle, right and left gill leaflets (combined) and digestive gland were excised, each tissue block then placed in weighed polyethylene tubes and placed in dry ice for transport and then held at approximately -80°C until analysis. A transverse section through the body cavity was taken and placed in formalin for histopathologic determination of gender and gravidity.

Table 3.1: Shell measurements (length, height, width) and median measurements for sampled *Elliptio complanata* in millimeters.

NH = New Hope Creek, E = Eno River, * = non-gravid female

	Length	Height	Width		Length	Height	Width
NH1	59	32	19	E1*	65	42	25
NH2	61	36	19	E2	64	39	23
NH3	70	42	21	E3*	72	41	23
NH4	58	33	22	E4	58	33	22
NH5	69	40	24	E5*	69	40	24
Median	61	36	21	Median	65	40	23

Water analysis

Monitoring equipment was placed within the stream channel for 7 days prior to the collection of mussels. A YSI 6920 (YSI, Inc., Yellow Springs, Ohio, USA) was placed mid-stream in each river system encased in an expanded metal cage for *in situ* measurement of temperature (C), conductivity (mS/cm), pH, turbidity (NTU), dissolved oxygen (% and mg/L). Measurements were recorded every 15 minutes for one week. An Isco water sampler (Teldyne Isco, Lincoln, Nebraska, USA) collection tube was placed next to the cage to

collect water samples every 8 hours for one week. Water samples were obtained at the time of mussel collection, transported to the laboratory and analyzed using a Hach analyzer (Hach Company, Loveland, Colorado, USA) for phosphate (ppm), ammonia (mg/L NH₃), nitrate (mg/L), nitrite (mg/L), alkalinity (mg/L CaCO₃), hardness (mg/L).

Tissue processing

Frozen tissues were pulverized using a Bullet Blender homogenizer (Next Advance, Averill Park, New York, USA) with 2:1 (v:w) Amphibian Ringer's solution (Fisher Scientific, Waltham, Massachusetts, USA) was added to the tissues 2ml solution to 1g tissue, vortexed, and incubated at 4 ° C overnight.

After incubation, the samples were centrifuged at 3450g for 20 minutes (Hermle Z300, Labnet International, Inc., Edison, New Jersey, USA). The supernatant was transferred into new polyethylene tubes, and frozen at -80C. All frozen samples, including the hemolymph samples were then lyophilized overnight (Lyoph-Lock 18 Freeze Dry System, Laboconco, Kansas City, Missouri, USA). Seven hundred microliters of 0.1mM deuterated trimethylsilyl propionate (TSP) in 10% D₂O was added to the dried samples, transferred to microcentrifuge tubes and centrifuged for 30 minutes at 5000g (AccuSpin Micro 17, Fisher Scientific, Waltham, Massachusetts, USA) to remove any remaining particulate matter. The supernatant was removed and pipetted into 5mm borosilicate Wilmad Labglass economy brand thin walled 5mm OD x 7 in length 100 MHz Rating NMR tube (Fisher Scientific, Waltham, Massachusetts, USA) for NMR analysis.

The pulsed field NMR experiments were performed on a Bruker (Billerica, Massachusetts, USA) AVANCE 500 MHz Spectrometer (1996) with Oxford (Abingdon, Oxfordshire, UK) Narrow Bore Magnet (1989), HP XW 4200 Host Workstation, and Topspin 1.3 Software version and processed into resonance spectra as described in Hurley-Sanders *et al.*, 2014. For NMR resonance spectra, the area under the peak was calculated as integrals, which correlates with metabolite concentration (Fan 1996). Integral tables were

calculated for the spectra using the Intelligent Bucketing (ACD Labs 12.0 1D NMR Processor, ACD Labs, Toronto, Ontario, Canada) feature with a bin width of 0.04ppm excluding water and TSP reference peaks (229 bins).

The integral tables were normalized and Pareto scaled using Microsoft Excel 2010 and then imported into SAS JMP v.10 for principal components analysis. Peak identification was performed using Chenomx NMR Suite 7.6 (Chenomx, Edmonton, Alberta, Canada), the Human Metabolome Database (www.hmdb.ca), and the Biological Magnetic Resonance Bank (www.bmrwisc.edu).

Differences in measured water quality parameters were examined using a two-tailed, two-sample unequal variance t-test (Windows Excel 2010) with a P-value of <0.05 considered significant.

Results

Table 3.2 presents the small molecular weight metabolites in multiple tissues of the freshwater mussel, *Elliptio complanata* we identified using ¹H nuclear magnetic resonance spectroscopy. Application of NMR techniques using an amphibian Ringer's solution extraction (Hurley-Sanders Chapter 2) successfully yielded spectra for metabolite identification in adductor muscle, foot muscle, gill, mantle, and digestive gland (Figure 3.2). Hemolymph samples yielded poor spectra with rare peaks above background noise and were not included in analyses. One Eno River adductor sample and one New Hope Creek mantle sample were lost during processing.

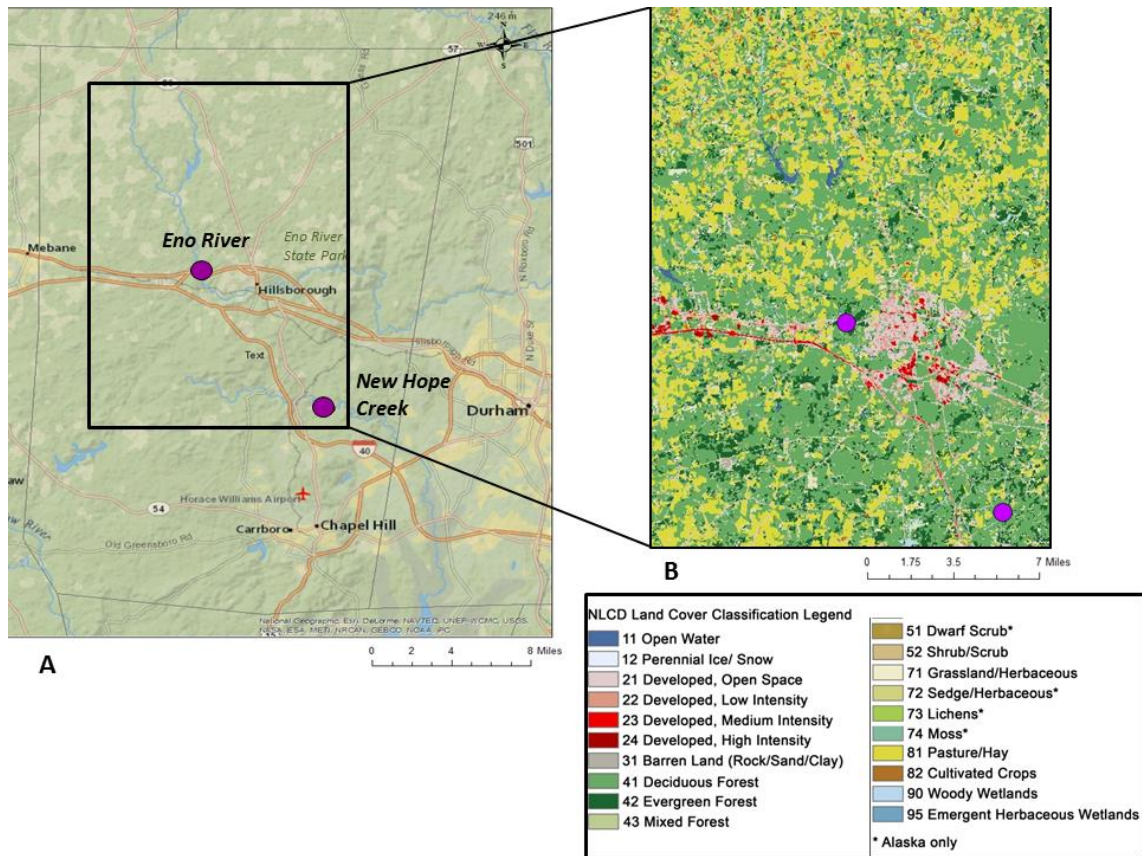


Figure 3.1: A) Orange County, North Carolina, United States of America with purple dots indicating sample sites. B) NLCD 2006 land cover map of enlarged area indicated by black outline showing primary land types upstream of sampling sites. Upstream regions are primarily Pasture/Hay, Deciduous Forest, Evergreen Forest for both locations.

Table 3.2: Suggested major metabolites and peak assignments. (s: singlet, br.s: broad singlet, d: doublet, dd: doublet of doublets, t: triplet, m: multiplet)

	Metabolites	Chemical shift and peak shape, ppm*	Reference
Amino acids	Alanine	1.46(d), 3.76(m)	[1] [2]
	Arginine	1.68(m), 1.90(m), 3.23(m), 3.74(m)	[1] [3]
	Glutamate	2.08(m), 2.34(m), 3.74(t)	[1] [3] [6]
	Glycine	3.54(s)	[1] [3]
	Isoleucine	0.92(t), 1.00(d), 1.26(m), 1.44(m), 1.96(m), 3.66(d)	[1] [3] [4]
	Leucine	0.94(d), 0.96(d), 1.66(m), 3.71(t)	[1] [3] [4]
	Serine	3.84(m), 3.96(m)	[1] [4]
	Taurine	3.25(t), 3.42(t)	[1] [5]
	Valine	0.98(d), 1.03(d), 2.25(m), 3.59(d)	[1] [3] [4]
Energy Related	Glucose	3.23(dd), 3.40(m), 3.46(m), 3.52(dd), 3.73(m), 3.82(m), 3.88(dd), 4.63(d), 5.22(d)	[1] [4]
	Glycogen	3.40(m), 3.60(m), 3.80(m), 3.96(br.s.), 5.40(br.s.)	[1] [2]
	Maltose	3.27(dd), 3.41(t), 3.58(m), 3.62(m), 3.66(m), 3.70(m), 3.76(m), 3.84(m), 3.90(dd), 3.93(d), 3.96(m), 5.22(d), 5.40(d)	[0]
	ADP	4.15(m), 4.16(m), 4.57(m), 5.94(m), 8.29(s), 8.54(s)	[1] [4] [6]
	ATP	4.21(m), 4.28(m), 4.39(m), 4.51(m), 4.62(t), 6.13(d), 8.24(s), 8.53(s)	[1] [4] [6]
	Acetate	1.91(s)	[2]
	2-oxoglutarate	2.43(t), 3.00(t)	[3] [6]
	Pyruvate	2.46(s)	[2]
	Lactate	1.32(d), 4.10(q)	[2]
Osmolytes/Organic acids			
	Betaine	3.25(s), 3.89(s)	[1] [4]
	β-alanine	2.55(t), 3.18(t)	[1]
	GABA	1.89(m), 2.28(t), 3.00(t)	[9]
Krebs Cycle Intermediates			
	Succinate	2.41(s)	[1] [7]
	Fumarate	6.51(s)	[7]
Fatty acid metabolism			
	Malonate	3.11(s)	[7]
	Carnitine	2.43(dd), 3.21(s), 3.42(m), 4.56(2)	[1]
Polyamine metabolism			
	Cadaverine	1.45(m), 1.71(m), 3.01(t)	[8]
	Ornithine	1.73(m), 1.83(m), 1.93(m), 3.05(t), 3.77(t)	[4]
	Putrescine	1.75(m), 3.04(t)	[4]
Unknown	Unknown 1	0.92(0.8 to 0.93 - multiple overlapping peaks)	Unknown peak 2 2.05(br.s)
	Unknown peak 3	2.39(t)	Unknown peak 6 3.19(s)
	Unknown peak 4	2.64(t)	Unknown peak 7 3.24(m)
	Unknown peak 5	2.84(m)	Unknown peak 8 3.63(br.s)

[1] Tikunov et al., 2010 [2] Gade and Wilps, 1975 [3] Hanson and Dietz, 1976 [4] Spann et al., 2011 [5] Bedford, 1973 [6] Hochachka and Mustafa, 1972 [7] Long et al., 1984 [8] Gasparini and Audit, 2000 [9] Gagne et al., 2007, [0] No definitive reference found for maltose in bivalves outside the digestive tract. *Chemical shifts identified using the HMDB, BMRB, and Chenomx NMR Suite 7.6

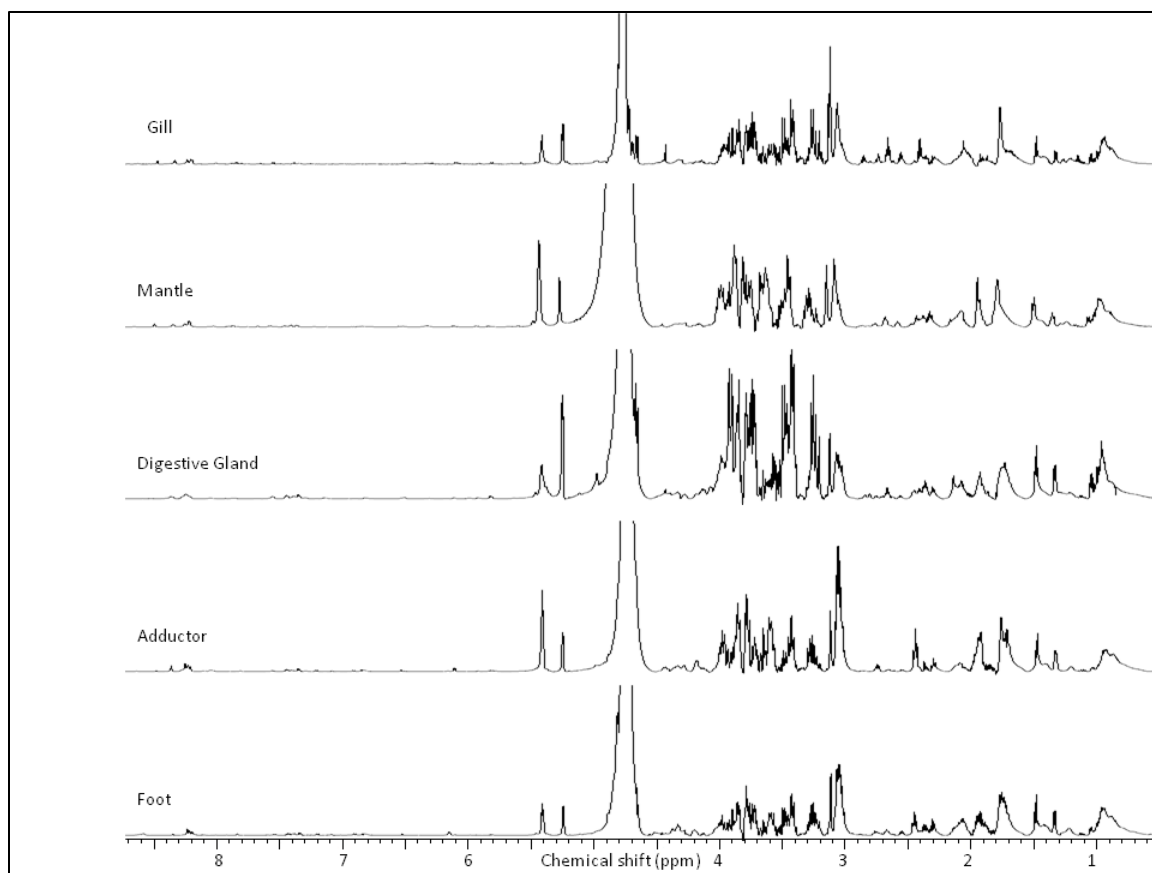


Figure 3.2: Representative ^1H -NMR spectra of each tissue from one individual.

Principal components analysis showed differentiation between tissue types (Figure 3.3). Variability along principal component 1 (PC1) partially separated the muscle tissues associated with amines and intermediates of amino acid metabolism from mantle, and digestive gland tissue associated with mono- and disaccharides and glycogen. Variability in principal component 2 (PC2) partially separated gill tissue associated with glutamate and several unidentified peaks from muscle tissues associated with amines and amino acid intermediates (Figure 3.4).

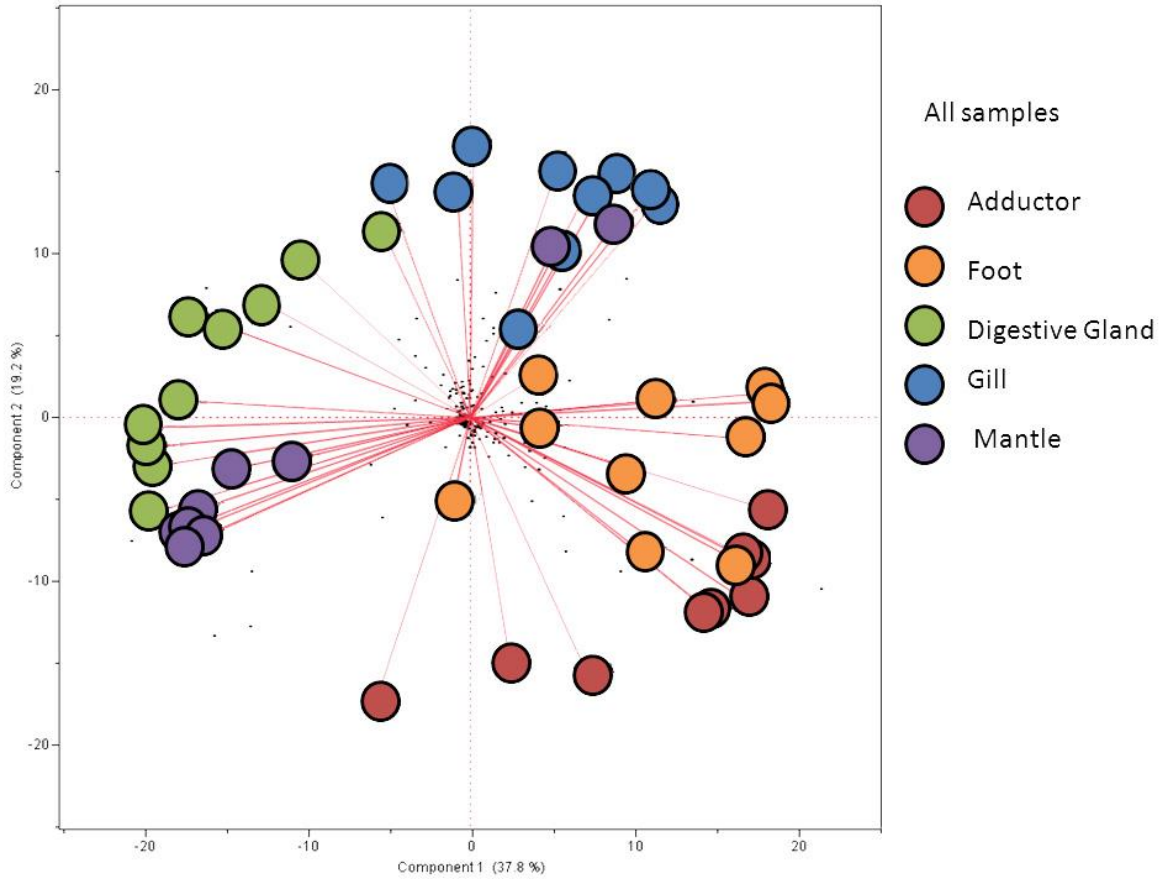


Figure 3.3: Two component principal components map of all samples from both rivers. Each tissue generally separates from the other indicating a characteristic metabolic profile for each tissue. The adductor and foot muscle profiles overlap indicating a relationship between the two muscle tissues. The two mantle samples that group with the gill samples are both female, this grouping may be due to seasonal reproductive changes to the mantle tissue.

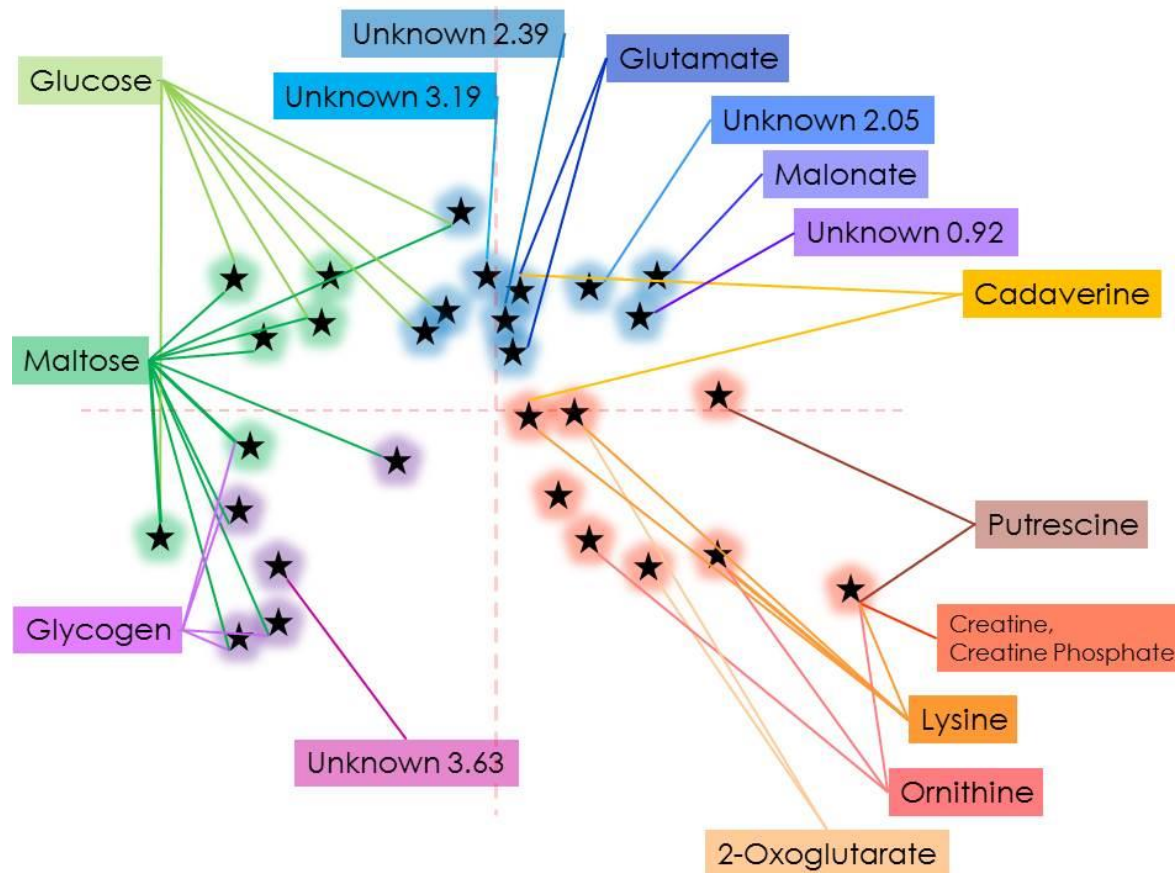


Figure 3.4: Loading plot of all tissue samples from both rivers. Blue stars correspond with scores within the gill grouping, green – digestive gland, purple – mantle, red – combined adductor and foot muscle.

Tissue associated variability was responsible for greater variation between samples than geographic location or sex when all samples were analyzed simultaneously. However, within tissue type, two variations were noted. A site effect for digestive gland samples was suggested by the noted variability along PC1 of glucose, maltose and glycogen, which separated the New Hope Creek digestive gland from the Eno River samples (Figure 3.5). Mantle tissue displayed an apparent sex effect. The three female samples grouped apart from the male samples regardless of site. This variability was noted along PC1 primarily in putrescine and two unknown resonances in the 0.85-0.93ppm ($-CH_3$) range and at 2.05ppm (likely $CH_2C=O$ or $CH_2C=C$ in structure) (Figure 3.6). The two mantle samples that grouped

with the gill tissue samples in the overall PCA map (Figure 3.3) were both female. Little to no site- or sex-associated difference was observed in other tissues.

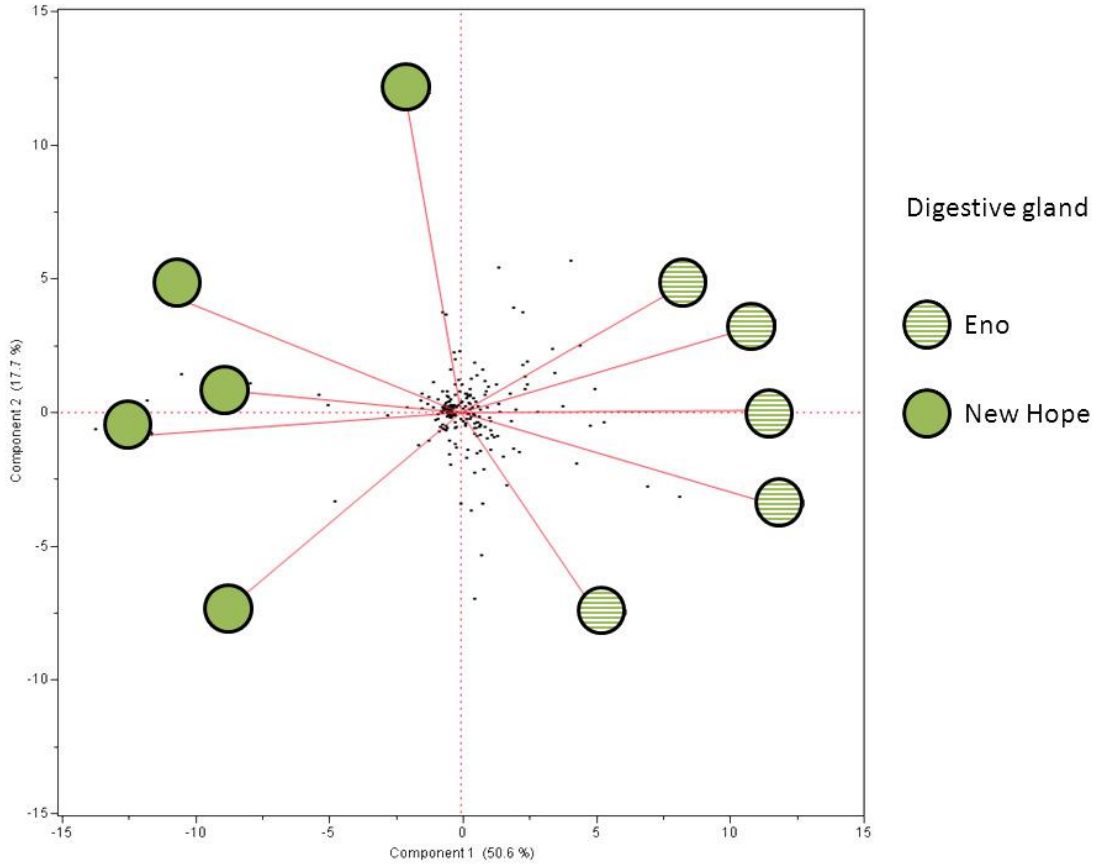


Figure 3.5: Tissue specific two-component principal components map of digestive gland samples. Eno River samples group separately from New Hope Creek samples suggesting location-specific effects on digestive gland metabolome.

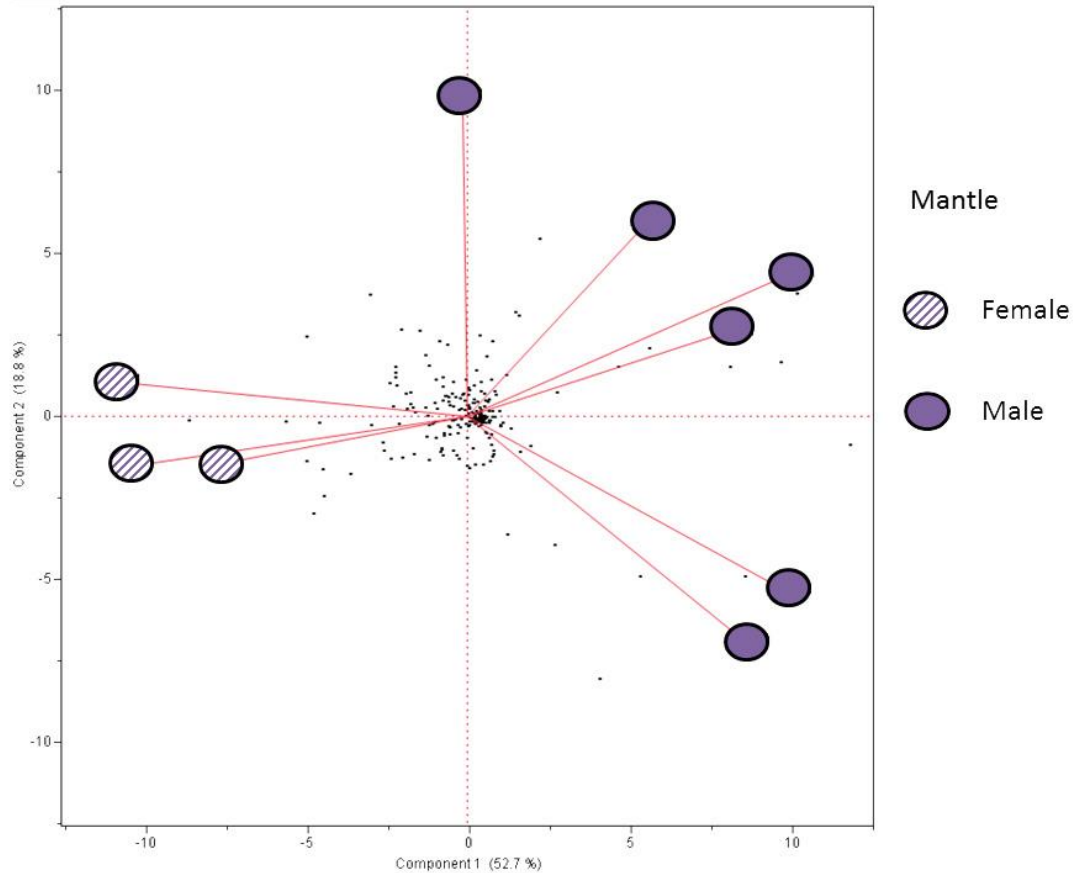


Figure 3.6: Tissue specific two-component principal components map of mantle samples. Samples from males group separately from female samples suggesting gender-specific effects on mantle metabolome.

Average water temperature, conductivity, pH, turbidity, oxygen and oxygen saturation measurements over the week prior to collection of mussels showed statistical differences between sites at the level of $p < 0.001$ ($n = 572$ per parameter, Table 3.3, Table 3.4). Statistical analysis to compare the water quality parameters collected by the Isco samples obtained at the study sites was not possible because only two samples were collected at the New Hope Creek site. The collection tube for that apparatus was discovered to have been obstructed after aspiration of the first sample. A second sample was collected at the time of mussel collection that provided beginning and end samples for phosphate, ammonia, nitrate, nitrite, alkalinity, and hardness ($n = 2$, Table 3.3). The measured values of these parameters

were similar to the samples collected at the same time points as the Eno River and occurred within the range of the Eno River samples (n=18, n=9 for phosphate, Table 3.3). No measurements from either the Eno River or the New Hope Creek were above EPA criteria for drinking water (www.epa.gov).

Table 3.3: Descriptive statistics for water quality parameters collected from the Eno River and New Hope Creek. Median, mean and standard deviation not calculated for New Hope samples collected by ISCO due to small sample number, one sample was collected at time of equipment deployment and at time of equipment collection a week later. (Temp=temperature, Cond=conductivity, pH, Turbid=turbidity, ODOsat=optical dissolved oxygen saturation, ODO=optical dissolved oxygen, NO₃=nitrate, NO₂=nitrite, Alk=alkalinity (as CaCO₃), Hard=hardness, NH₃=ammonia, PO₄=phosphate).

Eno River												
	YSI 6920						ISCO					
	Temp	Cond	pH	Turbid	ODO sat	ODO	NO3	NO2	Alk	Hard	NH3	PO4
	C	mS/cm		NTU	%	mg/L	mg/L	mg/L	mg/mL	mg/L	mg/L	ppm
mean	18.25	0.078	7.04	-0.144	84.83	8.01	0.22	0.02	35	40.9	-0	0
max	24.7	0.189	7.22	9.5	101.1	8.86	1.6	0.03	40	51.3	0.2	0.1
median	18.3	0.077	7.03	-0.1	84.4	8.02	0.15	0.02	35	34.2	-0	0
min	14.2	0.009	6.52	-5.4	81.1	7.04	-0.9	0.01	30	34.2	-0.1	0.1
stdev	2.95	0.007	0.07	4.32	2.4	0.49	0.65	0.01	2.42	2.02	0.1	0
N	572	572	572	572	572	572	18	18	18	18	18	9
New Hope Creek												
	YSI 6920						ISCO					
	Temp	Cond	pH	Turbid	ODO sat	ODO	NO3	NO2	Alk	Hard	NH3	PO4
	C	mS/cm		NTU	%	mg/L	mg/L	mg/L	mg/mL	mg/L	mg/L	ppm
mean	16.76	0.085	7.31	4.554	96.64	9.44						
max	21.2	0.103	7.62	142.1	114.2	11.9	0.8	0.02	40	51.3	-0	0.1
median	16.2	0.085	7.31	0.5	99.35	9.68						
min	13.3	0.002	7.16	-10.1	83	7.69	-0.2	0.02	40	51.3	-0	0.1
stdev	2.56	0.011	0.04	17.08	6.87	1.1						
N	572	572	572	572	572	572	2	2	2	2	2	2

Table 3.4: Parameters and averages measured by YSI 6920 every 15 minutes over the span of one week between rivers. All parameters were significantly different between the two rivers at a $p < 0.001$.

Welch Two Sample t-test					
	N	Eno mean	NH mean	p-value	95% CI
Temperature (C)	572	18.25	16.76	<0.001	(1.17, 1.82)
Conductivity (mS/cm)	572	0.0779	0.0848	<0.001	(-0.0079, -0.0058)
pH	572	7.04	7.31	<0.001	(-0.27, -0.26)
Turbidity (NTU)	572	0.1442	4.554	<0.001	(-6.145, -3.252)
ODOsat (%)	572	84.83	96.63	<0.001	(-12.41, -11.21)
ODO (mg/L)	572	8.02	9.44	<0.001	(-1.53, -1.33)

Discussion

This study serves as a preliminary investigation of the use of metabolomic techniques for the study of freshwater mussels. We sampled *Elliptio complanata*, a freshwater mussel common in North Carolina from two watersheds in Orange County, NC (Figure 3.1) to identify the predominant endogenous metabolites in several tissues.

Hemolymph samples extracted from the anterior adductor muscle of mussels did not yield useful spectra in our study. Few peaks were visible above baseline noise potentially because of low concentrations of metabolites in this biofluid. Hemolymph extraction and analysis has been effectively shown to identify physiologic parameters in freshwater mussels (Gustafson et al., 2005b) and additional efforts at using hemolymph for metabolic analysis are warranted. It is possible that aspiration of a larger volume of hemolymph would allow for concentration techniques to be employed to yield a more useful metabolomic profile.

All identified metabolites were found in all tissues other than hemolymph, with each tissue having a characteristic pattern of metabolites (Figures 3.2, 3.3). Many of the metabolites found in *E. complanata* have been identified in American oyster (*Crassostrea virginica*) tissues using proton NMR, however, there were two notable differences in the

bivalve metabolomes. First, the high concentrations of osmolytes seen in the oyster (Tikunov et al., 2010) were not seen in *E. complanata*. This absence of osmolytes may reflect evolutionary adaptations of the mussel to the low osmolarity of their freshwater environment (Dietz and Branton, 1975). Second, *Elliptio complanata* tissues contained predominant peaks of the polyamines putrescine and cadaverine not identified in oyster tissue (Tikunov et al., 2010). The physiologic significance of these polyamines in freshwater mussels is unknown.

In this study, despite significant differences ($p < 0.001$) in temperature, conductivity, pH, turbidity, and dissolved oxygen, principal components analysis of all spectra simultaneously suggested that tissue type influenced sample grouping more than river site (Table 3.4, Figure 3.3). This may suggest that the water quality and food resources of both rivers fell within a range that allows normal metabolic functioning for *E. complanata*. It may also indicate that the study sites are functionally very similar despite statistical differences in water parameters noted. Both sites were located in wooded areas, upstream from a road bridge and the land-use of the upstream watershed is similar (Figure 3.1). Little is reported regarding a “normal” range of abiotic habitat requirements for *E. complanata*, however, our water quality measurements for both rivers were within the ranges of what has been measured in other studies using *E. complanata* or other unionids (Matteson, 1948, Griffiths and Cyr, 2006, Kessler et al., 2007, Cummings and Graf, 2010, Aldridge et al., 1987). In this preliminary analysis, our study sampled animals from only two sites, one in each of two similar rivers. To identify geographic effects on the metabolism of *E. complanata* more study sites would need to be studied.

The principal components groupings by tissue likely reflect differences in physiologic function. Analysis of the principal components loadings plot highlights the metabolites that characterize each tissue grouping (Figure 3.4).

Digestive gland, characterized along PC1 by the carbohydrate metabolites glycogen, maltose and glucose, primarily functions as an organ of digestion but also serves as a site for carbohydrate storage (Thompson et al., 1974, Tikunov et al., 2010). This tissue was the only

tissue where a metabolomic difference by location was noted based on single tissue principal component analysis. A clear division by location was seen, primarily along PC1, separating Eno from New Hope samples by relative concentrations of glucose, maltose and glycogen (Figure 3.5) with New Hope samples having a greater relative concentration of maltose. This difference could be due to site-associated variation in nutritional status as measured by carbohydrate storage or reflect the gastrointestinal contents. Bacterioplankton and bacterial sediment populations vary greatly in different streams (Crump and Hobbie, 2005, Bucci et al., 2014) and the measured metabolites may not reflect a primary difference in the metabolome of the mussels themselves. The influence of microbial metabolomes or metabolism, either in the gut or on epidermal surfaces, on the metabolome of the mussel is to the best of our knowledge, unknown.

Muscle tissue makes primary use of carbohydrates to meet energy needs during aerobic metabolism, however, bivalves may switch to protein stores under periods of stress resulting in increased pools of free amino acid intermediates and end products (De Zwann and Wijsman, 1976, Zurburg and De Zwann, 1981). In this study *Elliptio complanata* muscle tissues were differentiated from other tissue samples by the relative concentrations of the amines lysine, ornithine, putrescine, and cadaverine and the Krebs cycle intermediate, 2-oxoglutarate. Polyamines such as ornithine-derived putrescine and lysine-derived cadaverine have been found in a wide variety of animal and plant tissues and have been shown to participate in a large number of metabolic reactions, however their role as intermediate metabolites remains unclear (Jänne et al., 2004, Tabor and Tabor, 1975). They appear to have multiple functions in cellular metabolism including both promotion of cell proliferation and induction of apoptosis (Jänne et al., 2004). The prominence of putrescine deserves further investigation to better understand the role of polyamines in freshwater mussel physiology. Levels of putrescine have been shown to transiently decrease in response to paraquat exposure in the gastropod mollusk *Biomphalaria glabrata* (Cochón et al., 2007) suggesting that putrescine concentrations in tissues may be a useful biomarker for toxicant-associated oxidative damage.

Gill tissue is used by freshwater bivalves for respiration, food prehension and sorting, and in reproduction. The diversity of these functions makes it reasonable to postulate the need for gill tissue to produce a wide variety of metabolites. The prominent unknown peaks in the 0-6ppm range consistent with methyl groups (-CH₃) and alkenes (C_nH_{2n}) found in our study suggest that gill tissue may have relatively increased levels of lipoproteins or steroid hormones (Fan and Lane, 2008). These metabolites are too large for adequate detection and identification using the ¹H-NMR techniques used in our study, but it would not be unexpected for portions of these molecules to overlap the low-molecular weight spectrum (Brown et al., 2008).

Like gill tissue, unionid mantle tissue is seasonally involved in reproduction, particularly in females. When ready to release their larvae, many gravid female freshwater mussel species transform the margins of their mantle into elaborate displays to attract fish that act as a host for the transformation of the larvae into juvenile mussels (Grabarkiewicz and Davis, 2008). Our mantle samples were characterized primarily by carbohydrate metabolites, which reflects the role of the mantle as a primary site of carbohydrate storage for bivalves (Patterson et al., 1999, Ojea et al., 2004, Tikunov et al., 2010), however, two of the mantle samples from the Eno River grouped with the gill samples based on principal components analysis. This may be related to the influence of macromolecular lipids or steroid hormones related to reproductive status, as both of these samples were female (De Zwann and Wijsman, 1976, Cavaletto and Gardner, 1999). When only mantle tissue is evaluated, all three female samples separate from the male samples, regardless of location (Figure 3.6). The three female mussels separated from the male mussels along PC1 with glycogen, glucose and maltose peaks associated with the male mussels. Relative differences in putrescine, cadaverine, and lysine peaks along with Unknown 1, which is suspected to be lipoprotein overlap, characterize female mantle samples. Earlier studies also noted a gender-related division by principal components analysis in the mantle metabolome of *Mytilus galloprovincialis* using (Hines et al., 2007), although the metabolites associated with gender varied from those found in our study with the exception of lysine. Longitudinal studies to

assess seasonal changes in the metabolome are needed to better characterize the impact of reproductive status on the metabolome of freshwater mussels.

Conclusion

With the use of proton NMR, we observed relative concentration differences in metabolites in different tissues. More extensive study that can establish necessary confidence intervals for baseline values is needed to identify the metabolomic responses of different tissues to environmental changes. It will be important to determine which tissue sample would best reflect metabolic changes in response to a given stimulus and to identify the most appropriate tissues for assessing particular environmental impacts. Our studies represent an initial step toward understanding the prominent metabolites that characterize freshwater mussel tissue and understanding how metabolomic studies can inform efforts to understand freshwater mussel health and disease.

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References

- Aldridge DW, BS Payne, and AC Miller. 1987. The Effects of Intermittent Exposure to Suspended solids and turbulence on three species of freshwater mussels. *Environ Pollut* 45: 17-28.
- Ahuja I, RCH de Vos, AM Bones, and RD Hall. 2010. Plant molecular stress responses face climate change. *Trends Plant Sci* 15: 664-680.
- Bayne B. 1973. Aspects of the metabolism of *Mytilus edulis* during starvation. *Neth J Sea Res* 7: 399-410.
- Beckonert O, HC Keun, TMD Ebbels, J Bundy, E Holmes, JC Lindon, and JK Nicholson. 2007. Metabolic profiling, metabolomics and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature Protocols* 2: 2692-2703.
- Bedford JJ. 1973. Osmotic relationships in a freshwater mussel, *Hyridella menziesi* Gray (Lamellibranchia: Unionidae). *Arch Int Physio Biochem* 81: 819-831
- Berg DJ, WR Haag, SI Guttman, and JB Sickel. 1995. Mantle biopsy: a technique for nondestructive tissue-sampling of freshwater mussels. *J N Am Benthol Soc* 14: 577-581.
- Bogan AE. 1993. Freshwater Bivalve Extinctions (Mollusca: Unionoida): A search for causes. *American Zoologist* 33: 599-609.
- Brown SAE, AJ Simpson, and MJ Simpson. 2008. Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with *Eisenia fetida*. *Environmental Toxicology and Chemistry* 27: 828-836.
- Bucci JP, AJ Szempruch, JM Caldwell, JC Ellis, and JF Levine. 2014. Seasonal changes in microbial community structure in freshwater stream sediment in a North Carolina river basin. *Diversity* 6: 18-32.
- Bundy JG, HC Keun, JK Sidhu, DK Spurgeon, C Svendsen, P Kille, and AJ Morgan. 2007. Metabolic profile biomarkers of metal contamination in a sentinel terrestrial species are applicable across multiple sites. *Environ Sci Technol* 41: 4458-4464.

- Bundy JG, Davey MP, Viant MR. 2009. Environmental metabolomics: a critical review and future perspectives. *Metabolomics* 5: 3-21.
- Cavaletto JF, and WS Gardner. 1999. Seasonal dynamics of lipids in freshwater benthic invertebrates. In MT Arts and BC Wainman (Eds.) *Lipids in Freshwater Ecosystems*, pp. 109-126. Springer. New York.
- Cloarec O, ME Dumas, A Craig, RH Barton, J Trygg, J Hudson, C Blancher, D Gauguier, JC Lindon, E Holmes, and JK Nicholson. 2005. Statistical Total Correlation Spectroscopy: An exploratory approach for latent biomarker identification from metabolic ^1H NMR data sets. *Anal Chem* 77: 1282-1289.
- Chittick B, M Stoskopf, M Law, R Overstreet, J Levine. 2001. Evaluation of potential health risks to Eastern Elliptio (*Elliptio complanata*) (Mollusca: Bivalvia: Unionida: Unionidae) and implications for sympatric endangered freshwater mussel species. *Journal of Aquatic Ecosystem Stress and Recovery* 9: 35-42.
- Cochón AC, AB Della Penna, G Kristoff, MN Piol, LC San Martín de Viale, and NR Verrengia Guerrero. 2007. Differential effects of paraquat on oxidative stress parameters and polyamine levels in two freshwater invertebrates. *Ecotox Environ Safe* 68: 286-292.
- Crump BC, and JE Hobbie. 2005. Synchrony and seasonality of bacterioplankton communities of two temperate rivers. *Limnol Oceanogr* 50: 1718-1729.
- Cummings KS, and DL Graf. 2010. Mollusca: Bivalvia. In JH Thorp and AP Covich (Eds.) *Ecology and Classification of North American Freshwater Invertebrates*. 3rd ed, p309-384. Elsevier. Boston.
- De Zwann A, and TCM Wijsman. 1976. Anaerobic metabolism in Bivalvia (Mollusca): Characteristics of anaerobic metabolism. *Comp Biochem Physiol* 54B: 313-324.
- Dietz TH. 1974. Body fluid composition and aerial oxygen consumption in the freshwater mussel, *Ligumia subrostrata* (Say): Effects of dehydration and anoxic stress. *Biol Bull* 147: 560-572.
- Dietz TH, and WD Branton. 1975. Ionic regulation in the freshwater mussel, *Ligumia subrostrata* (Say). *J Comp Physio* 104: 19-26.

- Doyette A, C Cossu, M Jacquin, M Babut, P Vasseur. 1997. Antioxidant enzymes, glutathione, and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve, *Unio tumidus*. *Aquat Toxicol* 39: 93-110.
- Fan TWM (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog Nucl Mag Res Spec* 28: 161-219.
- Fan TWM, and AN Lane. 2008. Structure-based profiling of metabolites and isotopomers by NMR. *Prog Nucl Mag Res Spec* 52: 69-117.
- Gäde G, and H Wilps. 1975. Glycogen degradation and end products of anaerobic metabolism in the fresh water bivalve *Anodonta cygnea*. *J Comp Physiol* 104: 79-85.
- Gagné F, P Cejka, C André, R Hausler, and C Blaise. 2007. Neurotoxicological effects of a primary and ozonated treated wastewater on freshwater mussels exposed to an experimental flow-through system. *Comp Biochem Phys C*: 460-470.
- Gasparini S, and C Audit. 2000. The free guanidine and polyamine pools of bivalve mollusks in relation to their ecology. *Biochem Sys Ecol* 28: 209-218.
- Gibb JOT, E Holmes, JK Nicholson, and JM Weeks. 1997. Proton NMR spectroscopic studies on tissue extracts of invertebrate species with pollution indicator potential. *Comp Biochem Physio.* 118B: 587-598.
- Goldberg ED. 1986. The Mussel Watch Concept. *Environ Monitor Assess* 7: 91-103.
- Grabarkiewicz JD, and WS Davis. 2008 Freshwater Mussels as Biological Indicators: Including Accounts of Interior Basin, Cumberlandian, and Atlantic Slope Species. U.S. Environmental Protection Agency, Office of Environmental Information, Washington DC. 122pp.
- Griffiths NA, and H Cyr. 2006. Are there hot spots for *Elliptio complanata* in the shallow littoral zone of a large Canadian Shield lake? *Can J Fish Aquat Sci* 63: 2137-2147.
- Gustafson LL, MK Stoskopf, AE Bogan, W Showers, TJ Kwak, S Hanlon, and JF Levine. 2005a. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Diseases of Aquatic Organisms* 65: 159-165.

- Gustafson LL, MK Stoskopf, W Showers, WG Cope, C Eads, R Linnehan, TJ Kwak, B Andersen, and JF Levine. 2005b. Reference ranges for hemolymph chemistries from *Elliptio complanata* of North Carolina. *Diseases of Aquatic Organisms* 65: 167-176.
- Hanson JA, and TH Dietz. 1976. The role of free amino acids in cellular osmoregulation in the freshwater bivalve *Ligumia subrostrata* (Say). *Can J Zool* 54: 1927- 1931.
- Hines A, GS Oladiran, JP Bignell, GD Stentiford, and MR Viant. 2007. Direct Sampling Organisms from the Field and knowledge of their phenotype: Key recommendations for environmental metabolomics. *Environ. Sci. Technol* 41: 3375-3381.
- Hochachka PW, and T Mustafa. 1972. Invertebrate facultative anaerobiosis. *Science* 178: 1056-1060.
- Holliman FM, D Davis, AE Bogan, TJ Kwak, WG Cope, and JF Levine. 2008. Magnetic resonance imaging of live freshwater mussels (Unionidae). *Invertebrate Biology* 127: 396-402.
- Jänne J, L Alhonen, M Pietilä, and TA Keinänen. 2004. Genetic approaches to the cellular functions of polyamines in mammals. *Eur J Biochem* 271: 877-894.
- Kessler DH, TJ Newton, and L Green. 2007. Long-term monitoring of growth in the Eastern Elliptio, *Elliptio complanata* (Bivalvia: Unionidae), in Rhode Island: a transplant experiment. *J N Am Benthol Soc* 26: 123-133.
- Liu, X, L Zhang, L You, J Yu, M Cong, Q Wang, F Li, L Li, J Zhao, C Li, and H Wu. 2011. Assessment of clam *Ruditapes philippinarum* as heavy metal bioindicators using NMR-based metabolomics. *Clean Soil Air Water* 39: 759-766.
- Long SD, GE Rodrick, and FE Friedl. 1984. Succinate dehydrogenase in various tissues of *Anodonta couperiana*, *Elliptio buckleyi* and *Mercenaria campechiensis* (Mollusca: Bivalvia). *Comp Biochem Physiol* 78B: 467-472.
- Matteson MR. 1948. Life History of *Elliptio complanatus* (Dillwyn, 1817). *Am Midl Nat* 40: 690-723.
- Naimo TJ, ED Damschen, RG Rada, and EM Monroe. 1998. Nonlethal evaluation of the physiological health of unionid mussels: methods for biopsy and glycogen analysis. *J N Am Benthol Soc* 17: 121-128.

- Ojea J, AJ Pazos, D Martínez, S Novoa, JL Sánchez, and M Abad. 2004. Seasonal variation in weight and biochemical composition of the tissues of *Ruditapes decussatus* in relation to the gametogenic cycle. *Aquaculture* 238: 451-468.
- Páez-Osuna F, JI Osuna-López, G Izaguirre-Fierro, and HM Zazueta-Padilla. 1993a. Heavy metals in oysters from a subtropical coastal lagoon associated with an agricultural drainage basin. *Bull Environ Contam Toxicol* 50: 696-702.
- Páez-Osuna F, G Osuna-López JI, G Izaguirre-Fierro, and HM Zazueta-Padilla. 1993b. Heavy metals in clams from a subtropical coastal lagoon associated with an agricultural drainage basin. *Bull Environ Contam Toxicol* 50: 915-921.
- Patterson MA, BC Parker, and RJ Neves. 1999. Glycogen concentration in the mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. *Am Malacol Bull* 15: 47-50.
- Peñuelas J, and J Sardans. 2009. Elementary Factors. *Nature* 460: 803-804.
- Spann N, DC Aldridge, JL Griffin, and OAH Jones. 2011. Size-dependent effects of low level cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*. *Aquat Toxicol* 105: 589-599.
- Rosenblum ES, RS Tjeerdema, and MR Viant. 2006. Effects of temperature on host-pathogen-drug interactions in red abalone *Haliotis rufescens* determined by ¹H NMR metabolomics. *Environmental Science and Technology* 40: 707-7084.
- Tabor CW, and H Tabor. 1976. 1,4-Diaminobutane (putrescine), spermidine, and spermine. *Annu Rev Biochem* 45: 285-306.
- Thompson RJ, NA Ratcliffe, and BL Bayne. 1974. Effects of starvation on structure and function in the digestive gland of the mussel (*Mytilus edulis* L.). *J Mar Biol Assoc UK* 54: 699-712.
- Tikunov AP, CB Johnson, H Lee, MK Stoskopf, and JM Macdonald. 2010. Metabolomic Investigations of American Oysters Using 1H-NMR Spectroscopy. *Marine Drugs* 8: 2578-2596.
- Vaughn CC, and CC Hakenkamp. 2001. The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology* 46: 1431-1446.

- Viant MR, ES Rosenblum, and RS Tjeerdema. 2003. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology* 37: 4982-4989.
- Williams JD, ML Warren, KS Cummings, JL Harris, RJ Neves. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18: 6-22.
- Won SJ, A Novillo, N Custodia, MT Rie, K Fitzgerald, M Osada, and I Callard. 2005. The Freshwater Mussel (*Elliptio complanata*) as a Sentinel Species: Vitellogenin and Steroid Receptors. *Integr Comp Biol* 45: 72-80.
- Zhang, L, X Liu, L You, D Zhou, H Wu, L Li, J Zhao, J Feng, and L Yu. 2011b. Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics. *Marine Environmental Research* 72: 33-39.
- Zurburg W, and A De Zwann. 1981. The role of amino acids in anaerobiosis and osmoregulation in bivalves. *J Exp Zool* 215: 315-325.

CHAPTER 4 – Variable Impact of Diet on Carbohydrate and Polyamine Metabolites in *Elliptio complanata* as Assessed Using Proton Nuclear Magnetic Resonance Spectroscopy

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Abstract

Nutrition is an important aspect of the captive management of freshwater mussels, yet many questions remain about how best to feed them while they are held in aquaria. Proton nuclear magnetic resonance imaging was used to evaluate the effect of seven diet treatments on the metabolome of the freshwater mussel *Elliptio complanata*. Diet treatments included 1) *Ankistrodesmus* spp., 2) *Ankistrodesmus* and *Bacillus subtilis*, 3) *Bacillus subtilis*, 4) Nutrient Broth, 5) a commercial shellfish diet, 6) water with no dietary supplement, and 7) filtered water with no dietary supplement. Over a 20 day period, individual variation was found to have more impact on the overall metabolome than any individual diet treatment.

Carbohydrate energy sources glycogen and glucose, and the polyamines putrescine and cadaverine varied by diet, with significant differences between the *Ankistrodesmus*+*Bacillus subtilis* treatment and the commercial shellfish diet.

Introduction

Approximately 70% of freshwater mussel species world-wide are endangered, threatened or at risk (Williams et al., 1993; Bogan, 1993). In response, programs have been developed to support declining populations through captive propagation and population augmentation (Jones et al., 2006). One challenge to captive maintenance of mussels is our limited understanding of freshwater mussel nutrition (Vaughn et al., 2008; Hernández-Zárate and Olmos-Soto, 2006). The use of bacteria and algae by freshwater mussels is generally accepted, however, differences in the species used across mussel taxa are likely (Gatenby et al., 1996; Nichols and Garling, 2000; Vaughn and Hakenkamp, 2001). Other food sources such as fine particulate organic matter or dissolved carbon are also likely, but their dietary contribution is not clear (Stephens and Manahan, 1984; Gatenby et al., 1996; Nichols and Garling, 2000). Determination of resource limitations that may affect mussel habitat selection and contribute to population declines, as well as insights into the potential influence of invasive species on mussel populations all depend on a better understanding of the diet of freshwater mussels. Understanding the metabolic effects of food resource availability, food resource depletion and their effects on freshwater mussel health are essential to efforts to conserve remaining populations (Tuffnail et al., 2009; Viant et al., 2003).

In the laboratory diet formulation and the impact of food deprivation on freshwater mussel physiology is particularly important to our understanding of experimental outcomes. Although reductions in glycogen stored in mantle tissues are well documented in as little as seven days when mussels are not fed (Patterson et al., 1997; Patterson et al., 1999), freshwater mussel toxicology research is often performed under fasted conditions for up to three weeks when the toxicity of chronic exposures is examined (ASTM, 2006). In the field, food deprivation can affect susceptibility to other disease processes such as parasitism (Jokela et al., 2005).

Nutritional metabonomics, the study of metabolism as it is affected by diet, (Gibney et

al., 2005; Rezzi et al., 2007; Wishart, 2008) can be used to determine metabolic effects of a particular diet or diet component (Schock et al., 2012; Fardet et al., 2008) through the application of spectrographic techniques, including nuclear magnetic resonance spectroscopy (NMR) to identify and quantify small molecular weight metabolites in biofluids or tissue extracts.. These techniques have been used to identify markers of fed versus fasted status in rats (Gürdeniz et al., 2012) and to characterize starvation in marine mussels (*Mytilus edulis*) and abalone (*Haliotis rufescens*) secondary to toxins exposure or infectious disease (Tuffnail et al., 2009; Viant et al., 2003).

The objective of this study was to evaluate the contribution of food sources to the metabolic status of the freshwater mussel *Elliptio complanata*, through ¹H-NMR techniques. We evaluated two food items currently fed in laboratories rearing freshwater mussels in aquaria, one a commercial diet formulated for oyster mariculture and the other an in-house cultured algae, *Ankistrodesmus*, obtained from local water sources. *Ankistrodesmus* is a common, easily cultured algae species, described as a potential food source for other freshwater mussel species, *Quadrula pustulosa* and *Amblema plicata* (Patterson et al., 1999). To explore the potential role of bacteria in freshwater mussel diets we evaluated *Bacillus subtilis*, a ubiquitous aerobic, gram-positive, rod-shaped bacterium found in soil and water (Stein, 2005) and of potential interest as a food for freshwater mussels managed in captivity. Unspeciated *Bacillus* have been cultured from freshwater bivalves, including *E. complanata* (Chittick et al., 2001; Toews et al., 1993; Hatha et al., 2005) and the clearance and assimilation of *Bacillus subtilis* has been reported for zebra mussels (*Dreissena polymorpha*) (Frischer et al., 2000; Silverman et al., 1995). *Bacillus subtilis* has been used as probiotic in human and animal diets based on potential immunostimulatory effects on the gastrointestinal system (Salinas 2005) and has been reported to improve water quality and disease resistance in farmed shrimp (Vaseeharan and Ramasamy 2003).

Methodology

Study animals

Forty adult *Elliptio complanata* were collected from the Eno River near Hillsborough, North Carolina, USA and transported to the Aquatic Epidemiology and Conservation Laboratory, North Carolina State University College of Veterinary Medicine, Raleigh, North Carolina, USA (AECL). The mussels were then rinsed with dechlorinated city water and gross debris scrubbed from the outer shells to minimize epibiota load. The mussels were then placed as a group in a holding tank and acclimated for 5 days. City water dechlorinated with sodium thiosulfate (ProLine Chlorine Neutralizer, Aquatic Eco-Systems, Inc., Apopka, Florida, USA), a minimum of 24 hours before use, was used in all study aquaria.

After acclimation, 36 mussels were cleaned a second time. Shell dimensions and wet weight (Molina et al., 2005) were recorded and the animals were then divided into Treatments 1-6 (Table 4.1). A seventh treatment was added the next day due to evidence of native bacteria in the water-only group, which raised concerns about the native bacteria acting as a food source. The duration of the experiment was 20 days. A 12 hour light:dark cycle was maintained throughout the study period. Each mussel was housed individually in approximately 6 liters of water. These tanks had been scrubbed with dish soap (Natural Dish Liquid, Seventh Generation, Burlington, Vermont, USA) and rinsed thoroughly with water prior to initial use. Supplemental air was provided through airstones and the containers were covered with aluminum foil to minimize evaporation and contamination with airborne particulates. Animals were fed twice daily (Table 4.1). The volume and frequency of feeding were based on current laboratory (AECL) feeding practices and information from reports of other studies using unionids (Gatenby et al., 1996; Patterson et al., 1999). Total water changes were performed three times weekly to limit bacterial and/or algae growth within the treatment tanks with each water change occurring immediately prior to a feeding. The interior walls of the holding tanks were wiped down with a sponge under running water before refilling to remove possible biofilm from the tank walls.

Diet Treatments

Treatments 1 and 2 were fed a laboratory grown *Ankistrodesmus* spp. (Table 4.1). The algae was cultured continuously in 5 gallon plastic bottles under fluorescent light. Dechlorinated city water was used as a base with Kent F/2 Algal Formula (Aquatic Eco-Systems, Inc., Apopka, Florida, USA) added as per product instructions. Daily cell counts were performed using a hemocytometer.

Table 4.1: Treatment description including final sample counts with sex ratios. One mortality occurred in the bacteria treatment early in the experiment. Two samples were lost during processing, and four samples were eliminated from final analysis due to poor spectral quality characterized by large, un-resolvable water peaks resulting in peak obliteration and baseline deformation.

Treatment	Amount fed per feeding (All counts are approximate)	Adductor		Mantle	
		n	Male:Female	n	Male:Female
1 Algae	5×10^7 cells/L <i>Ankistrodesmus</i>	5	4:1	6	4:2
2 Algae/Bacteria	2.5×10^7 cells/L <i>Ankistrodesmus</i> + 1.7×10^9 CFU <i>Bacillus subtilis</i>	6	4:2	6	4:2
3 Bacteria	1.7×10^9 CFU <i>Bacillus subtilis</i> 1ml Difco Nutrient Broth	5	3:2	4	2:2
4 Nutrient Broth	233000	5	2:3	4	0:4
5 Shellfish diet	1×10^8 cells/L Shellfish Diet 1800	5	3:2	6	4:2
6 Water-only	No food.	6	3:3	6	3:3
7 Filtered water	No food.	4	3:1	4	3:1

Treatments 2 and 3 were fed laboratory cultured *Bacillus subtilis* grown in Nutrient Broth (Difco Nutrient Broth 23000, Becton, Dickenson and Company, New Jersey, USA). Nutrient broth was made according to product instructions then autoclaved. One liter of Nutrient Broth was inoculated with 2ml of stock culture (1×10^8 CFU/ml) and incubated overnight at 37°C. This culture was maintained in approximate stasis at 4°C. Culture counts were performed 1-2 times weekly using Petrifilm (3M, St. Paul, Minnesota, USA). Bacteria were fed suspended in 1ml Nutrient Broth. The Nutrient Broth treatment (Treatment 4) was fed autoclaved broth with no bacterial inoculation. Two of the females in this group released

glochidia within 48 hours of initiation of the experiment.

Treatment 5 was fed Shellfish Diet 1800 (Reed Mariculture, California, USA) composed of four marine microalgae: 40% *Isochrysis* sp., 15% *Pavlova* sp., 25% *Tetraselmis* sp., 20% *Thalassiosira weissflogii*. These algae are not live and manufacturer reported total cell count per milliliter was verified prior to starting the experiment using a hemocytometer.

Initially, a treatment group in dechlorinated city water (Treatment 6) was to act as the negative control, or nutrition depleted group, however, culture of the water grew unidentified bacteria within 24 hours of initiation of the experiment. A filtered water group (Treatment 7) was added the second day of the study to serve as another control and to evaluate the effect of these native bacteria. For this group, city water was filtered through a 0.45um membrane filter (Fisherbrand, Fisher Scientific, Waltham, Massachusetts, USA) immediately prior to a water change. The unfiltered dechlorinated city water was used for all the other treatments, making it possible that a bacterial source of nutrition was available to all treatments except the filtered group. Contamination by epibiota is possible for all treatments as sterilization of the mussels was not feasible.

We hypothesized that the combined feeding of live algae and bacteria would provide a tissue metabolic profile significantly different from a treatment including no supplemental feeding. We expected the commercial algae diet tested would provide a tissue profile more similar to the *Ankistrodesmus* algae-alone treatment than to other non-algae treatments. In addition, we also expected the bacteria-only group to differentiate metabolically from the algae-alone diets and for the single item diets to result in a metabolic response with characteristics of starvation.

Tissue collection and processing

At the end of the experiment, the animals were remeasured and reweighed. An oyster shucking knife was used to open the valves and excise the mantle and adductor muscles from the dorsal valve. Within 10 seconds, the anterior adductor muscle was excised and placed in a pre-weighed cryovial then snap frozen using liquid nitrogen. Immediately thereafter, the dorsal mantle was excised and preserved in the same manner. A transverse section of the body cavity was placed in 10% neutral buffered formalin for histopathological determination of sex.

The frozen tissues were weighed. For metabolite extraction, amphibian Ringer's solution (Fisher Scientific, Waltham, Massachusetts, USA) (Hurley-Sanders et al., Chapter 2) and 0.05mm zirconium oxide beads (Next Advance, New York, USA) were added to the tissue at a ratio of 2ml:1g tissue and 100uL:1g tissue respectively. The samples were then placed in a Bullet Blender (Next Advance, New York, USA) and homogenized for 5 minutes at speed 10 until all tissue was liquefied. This solution was incubated at 4°C for 30 minutes and then centrifuged at 13,500g for 10 minutes (AccuSpin Micro 17, Fisher Scientific, Waltham, Massachusetts, USA). The supernatant was then collected and retained. Additional 2:1 (v:w) Ringer's solution was added and the pellet resuspended. A second incubation, centrifugation and collection was performed. The process was repeated a third time to yield a total of three extractions of each tissue sample. The three extractions were then combined.

The extraction solution was frozen at -20C and then lyophilized overnight (Lyoph-Lock 18 Freeze Dry System, Laboconco, Kansas City, Missouri, USA). The resulting powder was dissolved in 700uL 10% D₂O containing 0.1mM deuterated trimethylsilyl propionate (TSP) (Cambridge Isotope Laboratories, Inc., Tewksbury, Massachusetts, USA) and centrifuged to remove any remaining particulate. The supernatant was then transferred to 7" 5mm 100 MHz rating borosilicate NMR tubes (Wilmad Labglass, Vineland, New Jersey, USA).

Proton nuclear magnetic resonance spectroscopy (NMR) was performed on each sample using a Bruker (Billerica, Massachusetts, USA) AVANCE 500MHz electromagnet (1996) with Oxford (Abingdon, Oxfordshire, UK) Narrow Bore Magnet (1989), HP XW 4200 Host Workstation, and Topspin 1.3 Software version at the NCSU BioNMR Facility, Raleigh, North Carolina, USA. A one-dimensional presaturation protocol was used with 128 transients as described in Chapter 2. The recorded resonance data was processed into spectra and integral tables calculated with ACD Labs 1D NMR Processor Academic Edition 12.0 (Toronto, Ontario, Canada) software. Peak integrals, calculated from the area under the spectral peaks, correlate with metabolite concentration (Fan, 1996). Integral tables were calculated for the spectra using the Intelligent Bucketing feature with a bin width of 0.04ppm excluding water and TSP reference peaks (223 bins).

The resulting peak integrals were exported to Microsoft Excel 2010 and each spectrum was normalized to unity to eliminate dilution differences due to different tissue sample sizes. Principal components analysis and hierarchical clustering analysis were performed using SAS JMP Pro 9 (SAS, Cary, NC) to assess the similarity of the seven treatment groups. Wilcoxon signed rank comparison for each pair was applied to compare peak integrals by paired treatments to determine statistically significant differences between treatment groups. This non-parametric method was used as many of the sample responses were not normally distributed. To identify pertinent trends in our data that would prompt additional research, a statistical significance level of $p < 0.1$, was used to improve detection of key relationships over a traditional p-value of 0.05.

To visually compare the overall effect of treatments, median-centered graphs were created. The median value was determined for each peak across treatments for each tissue. These median values were then used to median-center the associated peak integrals. For demonstrative purposes, the median values per treatment of these median-centered peaks were graphed to give a relative comparison of diet effect per peak. This technique is similar to the significant difference spectra described in Schock et al., 2013.

Peak identification was performed using Chenomx 7.6, the Human Metabolome Database (www.hmdb.ca), the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu) and the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/).

Results

At the end of the experiment, all animals appeared to be in good body condition characterized by consistent coloration of tissues, firm tissue texture and plump appearance of the body. No animal showed evidence of tissue wasting. Most animals were slightly heavier at the end of the experiment with the greatest median weight gain in the Nutrient Broth group (median = 0.75g, equivalent to an approximately 4% median weight gain). The shellfish diet and the water-only group showed the lowest weight gain with the median gain for both groups equaling 0.2g (equivalent to approximately 0.7% median weight gain). No change was evident in pre- and post-experiment shell measurements.

Metabolomic profiles with identifiable peaks for analysis were generated for animals in each of the treatment groups consistent with previous reports of *Elliptio complanata* (Hurley-Sanders et al Chapter 2 and 3) (See Figure 4.1 for an example). In general, single item diets showed median peak values similar to the nutritionally deplete treatments of water-only or filtered water treatments.

Whole spectrum comparison via principal component or hierarchical clustering analysis failed to result in statistical differences between treatment groups for either tissue, however, multiple individual peak values for mantle and adductor tissue were found to be statistically significant, particularly between the algae/bacteria and shellfish diet treatments (Figure 4.2). Statistically significant differences ($p < 0.1$) were found in adductor tissue at 5.40 ppm (glycogen) between the algae/bacteria treatment and both the shellfish diet and algae treatments with the algae/bacteria treatment having the greater median concentration of glycogen (Figure 4.3(A)). At 5.22 ppm (glucose), statistically significant differences ($p < 0.1$)

were found between the shellfish diet treatment and the water-only and algae/bacteria diets (Figure 4.4(A)) with a lower median concentration of glucose in shellfish diet fed animals. For mantle tissue, statistically significant differences ($p < 0.1$) were found at 5.40 ppm (glycogen) between the Nutrient Broth treatment and the algae and shellfish diet treatments (Figure 4.3(B)) with greater median concentrations in the Nutrient Broth fed animals. No significant differences were found for mantle tissue at 5.22 ppm (glucose) (Figure 4.4(B)). Statistically significant differences ($p < 0.1$) were also seen between the shellfish diet and algae/bacteria diet in putrescine (1.75 ppm resonance) (Figure 4.5) and cadaverine (1.70 and 3.02 ppm resonance) (Figure 4.6) for both mantle and adductor tissues with the shellfish diet having the greater concentration of both polyamines.

Due to the complex nature of tissue extracts, significant differences were not found at all resonance peaks of these metabolites. Where a hydrogen resonance appears on the spectrum is related to the chemical environment surrounding the hydrogen, and these resonances can overlap with hydrogen resonances of other, similarly structured metabolites (Figure 4.7). This overlap can obscure differences between treatments for just that metabolite peak (Fan, 1996).

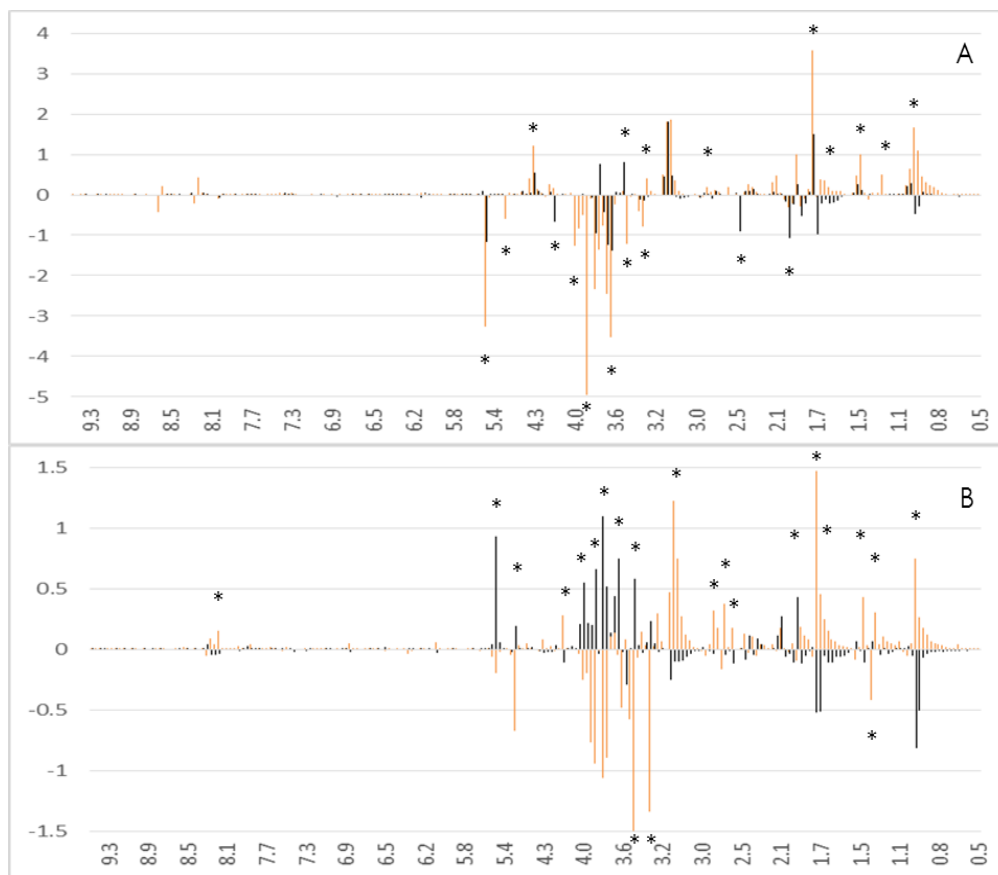


Figure 4.1: A) Adductor, B) Mantle. Median-centered graphs of tissue resonance peaks (ppm) in response to algae/bacteria (black) and shellfish (orange) diets showing overall pattern of metabolome response to diet. Y-axis is peak height as measured by peak integral (unitless). Asterisk (*) indicates area of statistical difference at a significance of $p < 0.1$.

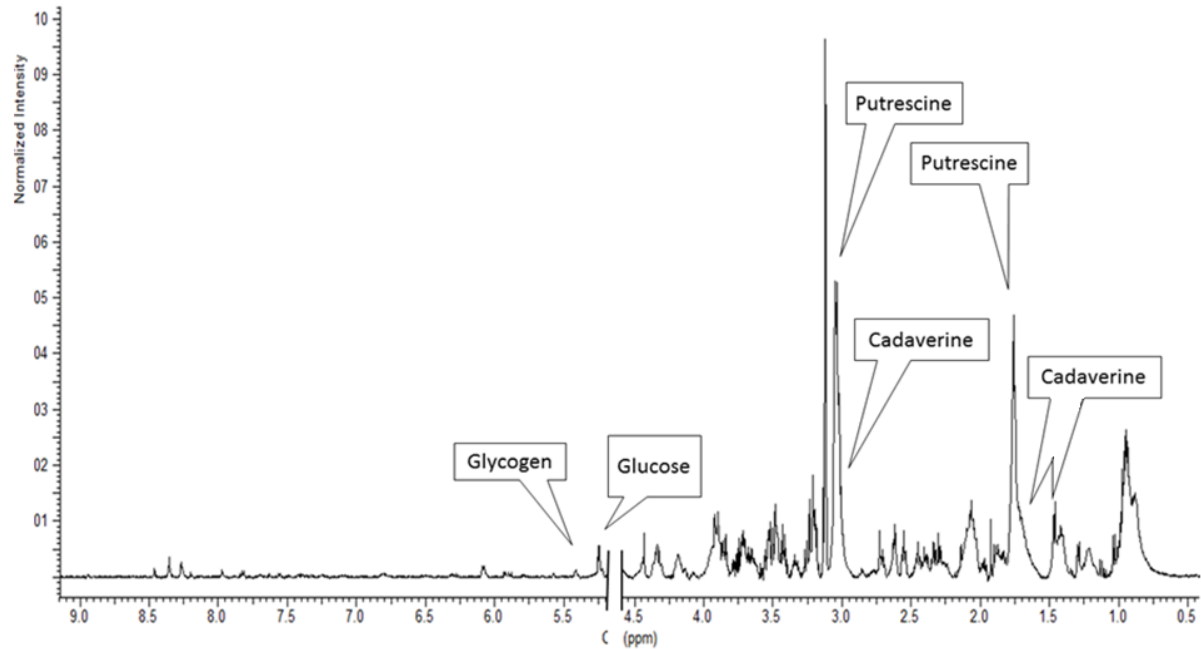


Figure 4.2: Sample mantle tissue proton spectrum from the shellfish diet treatment with peak resonances of interest marked. Water peak centering on 4.77 ppm has been removed.

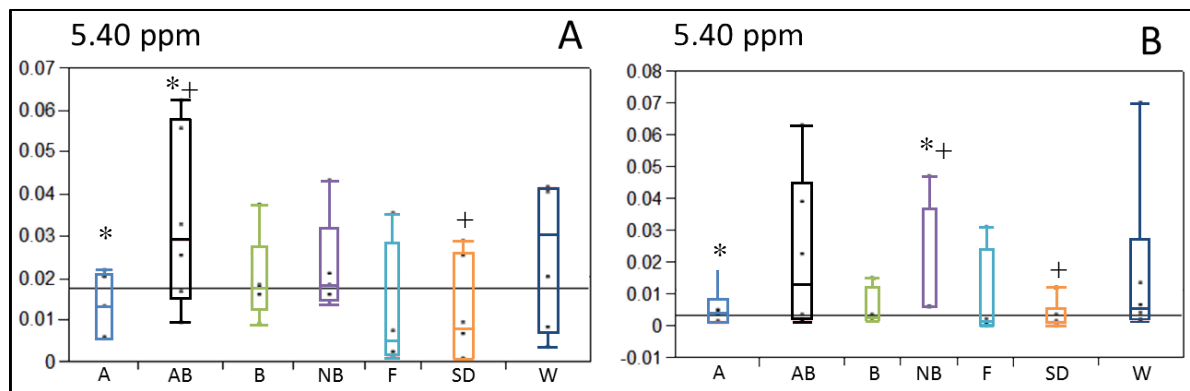


Figure 4.3: A) Adductor, B) Mantle. Distribution of responses by diet at glycogen (5.40 ppm) peak with minimum, maximum, median, 25th, and 75th quantiles. Treatments are abbreviated A = Algae, AB = Algae/Bacteria, B = bacteria, NB = Nutrient Broth, F = Filtered water, SD = Shellfish Diet, W = Water-only. Y-axis shows peak integral value (unitless). Across-treatment median is marked by horizontal black line. *,+ indicate statistically different treatment groups ($p < 0.1$).

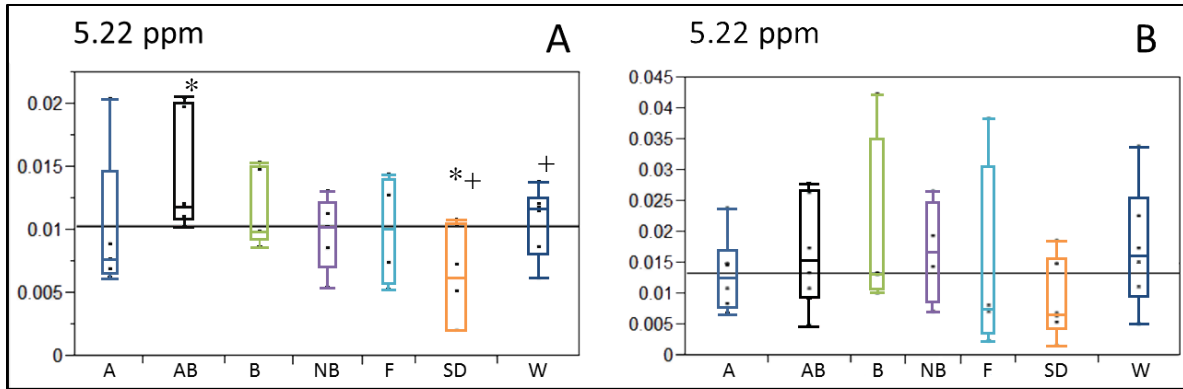


Figure 4.4: A) Adductor, B) Mantle. Distribution of responses by diet at glucose (5.22) peak with minimum, maximum, median, 25th, and 75th quantiles. Treatments are abbreviated A = Algae, AB = Algae/Bacteria, B = bacteria, NB = Nutrient Broth, F = Filtered water, SD = Shellfish Diet, W = Water-only. Y-axis shows peak integral value (unitless). Across-treatment median is marked by horizontal black line. *,+ indicate statistically different treatment groups ($p < 0.1$).

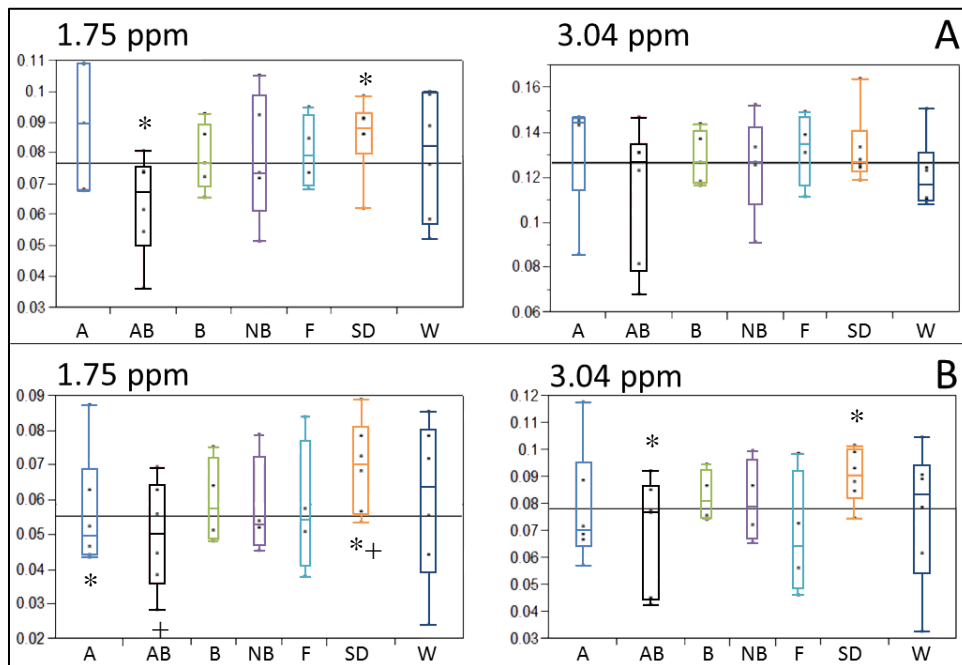


Figure 4.5: A) Adductor, B) Mantle. Distribution of responses by diet for putrescine (1.75 ppm and 3.04 ppm) with minimum, maximum, median, 25th, and 75th quantiles. Treatments are abbreviated A = Algae, AB = Algae/Bacteria, B = bacteria, NB = Nutrient Broth, F = Filtered water, SD = Shellfish Diet, W = Water-only. Y-axis shows peak integral value (unitless). Across-treatment median is marked by horizontal black line. *,+,# indicate statistically different treatment groups ($p < 0.1$).

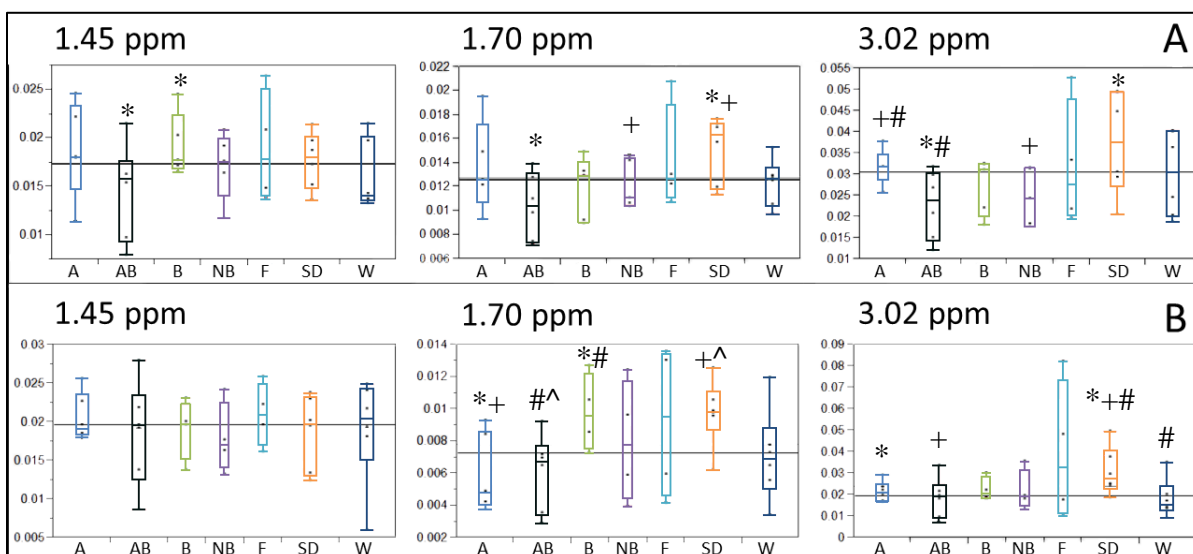


Figure 4.6: A) Adductor, B) Mantle. Distribution of responses by diet for cadaverine (1.45 ppm, 1.70 ppm and 3.02 ppm) with minimum, maximum, median, 25th, and 75th quantiles. Treatments are abbreviated A = Algae, AB = Algae/Bacteria, B = bacteria, NB = Nutrient Broth, F = Filtered water, SD = Shellfish Diet, W = Water-only. Y-axis shows peak integral value (unitless). Across-treatment median is marked by horizontal black line. *, +, #, ^ indicate statistically different treatment groups ($p < 0.1$).

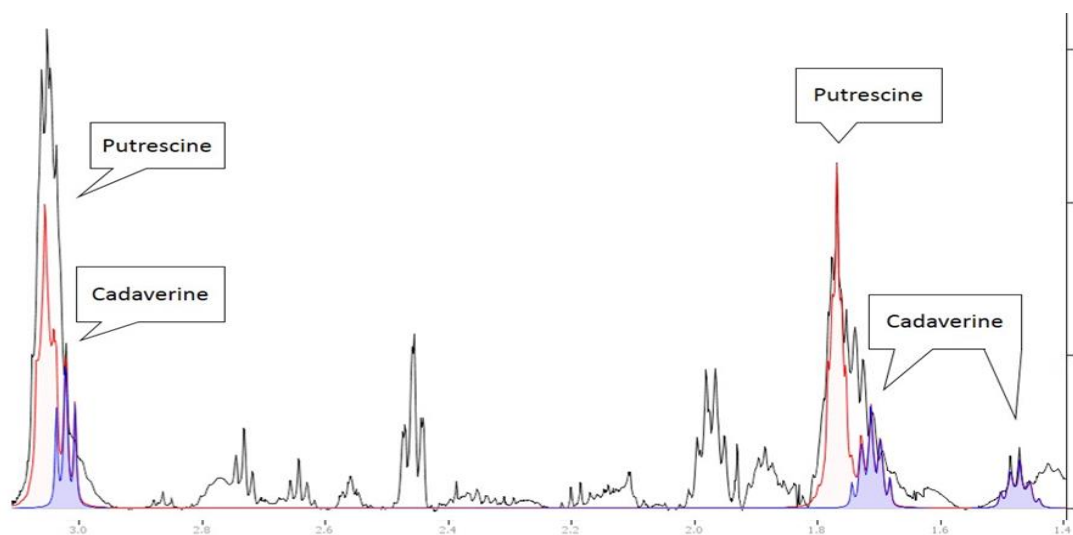


Figure 4.7: Representative proton spectrum showing peak-fitting of putrescine and cadaverine peaks using Chenomx Profiler 7.6. Putrescine 3.04 ppm peak shows overlap from other metabolites whereas the 1.75 ppm peak is less obscured by other metabolites.

Discussion

This report presents the first investigation of freshwater mussel nutrition using nuclear magnetic resonance spectroscopy. Metabolite profiles were successfully obtained for both adductor and mantle tissue yielding basic metabolic information for *Elliptio complanata* in each of seven diet treatments. This expands our knowledge of the metabolic effect of diet beyond traditional methods of monitoring growth and weight gain (Gatenby et al., 1996; Castel and Trider, 1974). Peak-by-peak comparisons of diet effect support previous work using glycogen as a marker of nutritional status (Patterson 1996; Patterson 1999; Baker and Hornbach, 2004) and suggests additional metabolites for further study. Expected clear differences between fed and unfed treatment groups were not seen using principal components or hierarchical clustering analysis, as substantial individual animal metabolic variability minimized the potential to detect an overall treatment effect. Principal components analysis is primarily a pattern recognition technique, and is susceptible to instrumental and spectral artifacts, metabolite overlap, and operates under the assumption of normal distribution (Hotelling, 1933, Weljie et al., 2006). Gender (Hurley-Sanders Chapter 3; and pre-experiment nutritional status (Patterson et al., 1997; Liang, 1993) may have contributed to metabolic profile and individual response to diet more than anticipated. The influence of natural biofilm is unknown, however, one study suggests freshwater mussels do not use biofilm as a food sources (Christian et al., 2004)

Glycogen stores in mantle tissue have been used as an indicator of nutritional condition in bivalves (Patterson et al., 1997; Patterson et al., 1999; Naimo et al., 1998; Vaughn and Hakenkamp, 2001). The concentration of glycogen varies seasonally relative to gametogenesis (Bayne, 1973; Albentosa et al., 2007; Ojea et al., 2004) and food availability (Vaughn and Hakenkamp, 2001; Patterson et al., 1999). In marine bivalves, total body analysis of carbohydrates, lipids and proteins in response to starvation suggest a primary reliance on carbohydrate stores as a main energy reserve in the initial stages of starvation (Bayne, 1973; Albentosa et al., 2007). We identified the 5.40 ppm peak of glycogen and the

5.22 ppm peak of glucose as potentially associated with diet. Both 5.40 ppm and 5.22 ppm were readily identifiable peaks with little confounding overlap from other metabolites identified in *E. complanata* or *Crassostrea virginica* (Hurley-Sanders Chapter 3, Tikunov et al., 2010).

Maintenance of glycogen mantle stores in the Nutrient Broth treatment suggests that soluble organic carbons in the broth may be biologically available to the mussel. Conversely, the lower relative glycogen concentrations in both adductor and mantle tissue may indicate that the commercial shellfish diet is inadequate to maintain glycogen status alone. The lower concentration of tissue glycogen and glucose in adductor suggests depletion of carbohydrate resources in response to this diet. The shellfish diet is composed of killed marine algae that may not be readily sorted as food by *E. complanata*, may not be digestible, or may be nutritionally incomplete (Ren et al., 2014). The live algae diet provided in this experiment, as noted above, also had statistically significantly lower glycogen concentrations than the algae/bacteria diet in adductor tissue, providing additional evidence that algae-only diets are inadequate for *E. complanata*. The improved maintenance of glycogen concentrations in the algae/bacteria diet treatment suggests that digestion, assimilation or nutritional profile of the live algae diet is improved by addition of *Bacillus subtilis*. Additional investigation of the shellfish diet supplemented with *B. subtilis* and potentially other bacteria that may have a probiotic effect on nutrition is warranted.

Although tissues from the shellfish diet treatment had comparatively low glycogen concentrations, this treatment group had the highest median concentrations at chemical shift 0.86-1.01ppm, which is a complex region where a variety of metabolites have resonances including lipids and lipoproteins (Fan and Lane, 2008; Brown et al., 2008). There were statistically significant differences between the shellfish diet and algae/bacteria treatments for adductor, and between the shellfish diet and the water-only treatment for mantle tissue in this region. Lipid content has been shown to vary based on algae species (Gatenby et al., 2003) and tissue levels of lipid have been shown to increase with dietary lipid in mollusks (Mai et

al., 1995). It is possible that in response to the multi-algae combination of the shellfish diet, mussels are maintaining lipid stores yet depleting carbohydrate stores in their efforts to maintain energy balance.

Another category of markers worthy of additional investigation in freshwater mussels are the polyamines, particularly putrescine and cadaverine. Polyamines are organic cations that have been used as markers of normal and pathological cell growth in vertebrates. They are associated with nucleic acid and protein synthesis and apoptosis (Stuck et al., 1996; Jänne et al., 2004), however, their role as intermediate metabolites continues to remain unclear (Davis, 2004). Polyamines have been investigated as markers of diet in the European sea bass (*Dicentrarchus labrax*) (Orlandini et al., 1989), and of starvation in shrimp (*Penaeus vannamei*) (Stuck 1996) and rodents (Domschke and Söling, 1973; Brosnan et al., 1983; Sieler et al., 1981). In the sea bass, the concentrations of polyamines putrescine, spermine and spermidine vary by protein content of the diet, and are influenced by environmental temperature (Orlandini et al., 1989). In rat liver, putrescine and spermidine decrease with starvation and returned to normal after re-feeding (Domschke and Söling, 1973). In other studies, putrescine has been shown to increase during starvation in mouse livers (Seiler et al., 1981), and the mammary tissues of lactating rats (Brosnan et al., 1983).

In our study, putrescine and cadaverine were the only two polyamines identified, although many others have been reported in marine bivalves (Gasparini and Audit, 2000; Hamana et al., 1991). We saw statistically significant differences ($p < 0.1$) in putrescine (1.75 ppm resonance) and cadaverine (1.70 and 3.02 ppm resonance) concentrations between the shellfish diet and the algae/bacteria diet for both mantle and adductor tissue with the shellfish diet having the greater concentration of both polyamines. This may indicate a shift from carbohydrate to protein catabolism with depletion of glycogen in the shellfish diet treatments. However, the variable response of polyamine concentrations to starvation in vertebrate species (Domschke and Söling, 1973; Seiler et al., 1981, Brosnan et al., 1983) indicates a need for additional research clarifying the relationship between polyamines and catabolism in

freshwater mussels.

Conclusion

When evaluating the metabolic response of freshwater mussels to alternative diets, carbohydrate energy sources and polyamine cations should be considered as potential markers of nutritional status. Glycogen and glucose are suggested as positive markers of nutritional status, whereas putrescine and cadaverine concentrations appear to be negatively associated and may serve as viable metabolic markers of declining nutritional status and health. A laboratory cultured combination of *Ankistrodesmus* and *Bacillus subtilis* appeared to result in greater maintenance of glycogen stores and reduced levels of polyamines in both adductor and mantle tissue than either component alone or a commercial algae-based shellfish diet. General treatment effects of diets were masked by individual variation, highlighting the importance of considering and assessing pre-experiment nutritional condition when assembling research cohorts for nutritional or other experiments. Much additional work is needed to further define the daily nutritional maintenance requirements of freshwater mussels held in captivity and the role of dietary resource availability in freshwater mussel health in surface waters. Metabolomic profiling provides another potentially useful tool for assessing the health of remaining freshwater mussel populations.

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References

- Albentosa M, MJ Fernández-Reiriz, U Labarta and A Pérez-Camacho. 2007. Response of two species of clams, *Ruditapes decussatus* and *Venerupis pullastra*, to starvation: Physiological and biochemical parameters. *Comparative Biochemistry and Physiology, Part B* 146: 241-249.
- American Society for Testing and Materials (ASTM). 2006. Standard guide for conducting laboratory toxicity tests with freshwater mussels. E2455-06. In *Annual Book of ASTM Standards*, Vol. 11.06. Philadelphia, PA, pp. 1393–1444.
- Baker SM, and DJ Hornbach. 2001. Seasonal metabolism and biochemical composition of two unionid mussels, *Actinonaias ligamentina* and *Amblema plicata*. *J Moll Stud* 67: 407-416.
- Bayne B. 1973. Aspects of the metabolism of *Mytilus edulis* during starvation. *Neth J Sea Res* 7: 399-410.
- Bogan AE. 1993. Freshwater Bivalve Extinctions (Mollusca: Unionoida): A search for causes. *American Zoologist* 33: 599-609.
- Brosnan ME, R Farrell, H Wilansky, and DH Williamson. 1983. Effect of starvation and refeeding on polyamine concentrations and ornithine decarboxylase antizyme in mammary gland of lactating rats. *Biochem J* 212: 149-153.
- Castel JD, and DJ Trider. 1974. Preliminary feeding trials using artificial diets to study the nutritional requirements of oysters (*Crassostrea virginica*). *Journal Fisheries Research Board of Canada* 31: 95-99.
- Chittick B, M Stoskopf, M Law, R Overstreet, J Levine. 2001. Evaluation of potential health risks to Eastern Elliptio (*Elliptio complanata*) (Mollusca: Bivalvia: Unionida: Unionidae) and implications for sympatric endangered freshwater mussel species. *Journal of Aquatic Ecosystem Stress and Recovery* 9: 35-42.
- Christian AD, BN Smith, DJ Berg, JC Smoot, and RH Findlay. 2004. Trophic position and potential food sources of 2 species of unionid bivalves (Mollusca: Unionidae) in 2

- small Ohio streams. *Journal of the North American Benthological Society* 23: 101-113.
- Davis R. 2004. Management of polyamine pools and the regulation of ornithine decarboxylase. *Journal of Cellular Biochemistry* 44: 199-205.
- Domschke S, and HD Söling. 1973. Polyamine metabolism in rat liver: Effect of starvation and refeeding. *Horm Metab Res* 5: 97-101.
- Fan TWM (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog Nucl Mag Res Spec* 28: 161-219.
- Fan TWM, and AN Lane. 2008. Structure-based profiling of metabolites and isotopomers by NMR. *Prog Nucl Mag Res Spec* 52: 69-117.
- Fardet A, R Llorach, A Orsoni, JF Martin, E Pujos-Guillot, C Lapierre, and A Scalbert. 2008. Metabolomics provide new insight on the metabolism of dietary phytochemicals. *J Nutr* 138: 1282-1287.
- Frischer ME, SA Nierzwicki-Bauer, RH Parsons, K Vathanodorn, and KR Waitkus. 2000. Interactions between zebra mussels (*Dreissena polymorpha*) and microbial communities. *Can J Fish Aquat Sci* 57: 591-599.
- Gasparini S, and C Audit. 2000. The free guanidine and polyamine pools of bivalve mollusks in relation to their ecology. *Biochemical Systematics and Ecology* 28: 209-218.
- Gatenby CM, RJ Neves, and BC Parker. 1996. Influence of sediment and algal food on cultured juvenile freshwater mussels. *Journal of the North American Benthological Society* 15: 597-609.
- Gatenby CM, DM Orcutt, DA Kreeger, BC Parker, VA Jones, and RJ Neves. 2003. Biochemical composition of three algal species proposed as food for captive freshwater mussels. *Journal of Applied Phycology* 15: 1-11.
- Gibney MJ, M Walsh, L Brennan, HM Roche, B German, and B van Ommen. 2005. Metabolomics in human nutrition: opportunities and challenges. *Am J Clin Nutr* 82: 497-503.

- Gürdeniz G, M Kristensen, T Skov, and LO Dragsted. 2012. The effect of LC-MS data preprocessing methods on the selection of plasma biomarkers in fed vs. fasted rats. *Metabolites* 2: 77-99.
- Hamana K, M Nitsu, K Samejima, and S Matsuzaki. 1991. Novel tetraamines, pentaamines and hexaamines in sea urchin, sea cucumber, sea squirt and bivalves. *Comp Biol Physiol* 100B: 59-62.
- Hatha AAM, KS Christi, R Singh, and S Kumar. 2005. Bacteriology of the fresh water bivalve clam *Batissa violacea* (Kai) sold in the Suva market. *South Pacific Journal of Natural Science* 23: 48-50.
- Hernández-Zárate G, and J Olmos-Soto. 2006. Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent *in situ* hybridization and polymerase chain reaction. *Journal of Applied Microbiology* 100: 664-672.
- Hines A, GS Oladiran, JP Bignell, GD Stentiford, and MR Viant. 2007. Direct Sampling Organisms from the Field and knowledge of their phenotype: Key recommendations for environmental metabolomics. *Environ. Sci. Technol* 41: 3375-3381.
- Hotelling H. 1933. Analysis of a complex of statistical variables into principal components. *Journal of Educational Psychology*. 24: 417-441.
- Jänne J, L Alhonen, M Pietilä, and TA Keinänen. 2004. Genetic approaches to the cellular functions of polyamines in mammals. *Eur J Biochem* 271: 877-894.
- Jokela J, J Taskinin, P Mutikainen, and K Kopp. 2005. Virulence of parasites in hosts under environmental stress: Experiments with anoxia and starvation. *Oikos* 108: 156-164.
- Jones JW, EM Hallerman, and RJ Neves. 2006. Genetic management guidelines for captive propagation of freshwater mussels (Unionoidea). *Journal of Shellfish Research* 25: 527-535.
- Liang, I. 1993. The response of the Manila clam, *Tapes philippinarum*, juveniles to nutritive stress. *J Exp Mar Biol Ecol* 173: 111-121.
- Mai K, JP Mercer, and J Donlon. 1995. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. III. Response of abalone to various levels of dietary lipid. *Aquaculture* 134: 65-80.

- Naimo TJ, ED Damschen, RG Rada, and EM Monroe. 1998. Nonlethal evaluation of the physiological health of unionid mussels: methods for biopsy and glycogen analysis. *J N Am Benthol Soc* 17: 121-128.
- Nichols SJ, and D Garling. 2000. Food-web dynamics and trophic-level interactions in a multispecies community of freshwater unionids. *Can J Zool* 78: 871-882.
- Ojea J, AJ Pazos, D Martínez, S Novoa, JL Sánchez, and M Abad. 2004. Seasonal variation in weight and biochemical composition of the tissues of *Ruditapes decussatus* in relation to the gametogenic cycle. *Aquaculture* 238: 451-468.
- Orlandini G, N Reali, ME Soldi, F Bacciottini, R Vivani, and A Casti. 1989. Effect of temperature and diet on polyamine concentrations of the European Sea Bass (*Dicentrarchus labrax* L.) *Comp Biochem Physiol* 94B: 581-585.
- Patterson MA, BC Parker, and RJ Neves. 1997. Effects of quarantine times of glycogen levels of native freshwater mussels (Bivalvia: Unionidae) previously infested with zebra mussels. *Am Malacol Bull* 14: 75-79.
- Patterson MA, BC Parker, and RJ Neves. 1999. Glycogen concentration in the mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. *Am Malacol Bull* 15: 47-50.
- Ren Y, W Liu, CM Pearce, I Forster, and RS McKinley. 2014. Effects of selected mixed-algal diets on growth and survival of early postset juveniles of the Pacific geoduck clam, *Panopea generosa* (Gould, 1850). *Aquaculture nutrition* doi:10.1111/12145.
- Rezzi S, Z Ramadan, LB Fay, and S Kochhar. 2007. Nutritional metabonomics: Applications and perspectives. *Journal of proteome research* 6: 513-525.
- Schock TB, S Newton, K Brenkert, J Leffler, and DW Bearden. 2012. An NMR-based metabolomic assessment of cultured cobia health in response to dietary manipulation. *Food Chemistry* 133: 90-101.
- Schock TB, J Duke, A Goodson, D Weldon, J Brunson, JW Leffler and DW Bearden. 2013. Evaluation of Pacific white shrimp (*Litopenaeus vannamei*) health during a superintensive aquaculture growout using NMR-based metabolomics. *PLOS ONE* 8: e59521.

- Seiler N, FN Bolkenius, and S Sarhan. 1981. Formation of acetylpolyamines in the liver of fasting animals. *International Journal of Biochemistry* 13: 1205-1214.
- Silverman H, EC Achberger, JW Lynn, and TH Dietz. 1995. Filtration and utilization of laboratory-cultured bacteria by *Dreissena polymorpha*, *Corbicula fluminea*, and *Carunculina texasensis*. *Biol Bull* 189: 308-319.
- Stein T. 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*. 56: 845-857.
- Stephens GC, and DT Manahan. 1984. Technical advances in the study of nutrition of marine molluscs. *Aquaculture* 39: 155-164.
- Stuck KC, SA Watts, and SY Wang. 1996. Biochemical responses during starvation and subsequent recovery in postlarval Pacific white shrimp, *Penaeus vannamei*. *Marine Biology* 125: 33-45.
- Tikunov AP, CB Johnson, H Lee, MK Stoskopf, and JM Macdonald. 2010. Metabolomic Investigations of American Oysters Using ¹H-NMR Spectroscopy. *Marine Drugs* 8: 2578-2596.
- Toews S, M Beverly-Burton, and T Lawrimore. 1993. Helminth and protest parasites of zebra mussels, *Dreissena polymorpha* (Pallas, 1771), in the Great Lakes region of southwestern Ontario, with comments on associated bacteria. *Can J Zool* 71: 1763-1766.
- Tuffnail, W, GA Mills, and P Cary, and R Greenwood. 2009. An environmental ¹H NMR metabolomics study of the exposure of the marine mussel *Mytilus edulis* to atrazine lindane hypoxia and starvation. *Metabolomics* 5: 33-43.
- Vaseeharan B, and P Ramasamy. 2003. Control of pathogenic *Vibrio* spp. By *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Letters in Applied Microbiology* 36: 83-87.
- Vaughn CC, and CC Hakenkamp. 2001. The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology* 46: 1431-1446.
- Vaughn CC, SJ Nichols, and DE Spooner. 2008. Community and foodweb ecology of freshwater mussels. *Journal North American Benthological Society* 27: 409-423.

- Viant MR, ES Rosenblum, and RS Tjeerdema. 2003. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology* 37: 4982-4989.
- Weljie AM, J Newton, P Mercier, E Carlson, and CM Slupsky. 2006. Targeted profiling: Quantitative analysis of ^1H NMR metabolomics data. *Anal Chem* 78: 4430-4442.
- Williams JD, ML Warren, KS Cummings, JL Harris, RJ Neves. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18: 6-22.
- Wishart DS. 2008. Metabolomics: applications to food science and nutrition research. *Trends in Food Science and Technology* 19: 482-492.

CHAPTER 5 – Metabolomics in the study of freshwater health

This dissertation serves as a preliminary step in the application of nuclear magnetic resonance-based metabolomics and metabonomics to the study of freshwater mussel health. Multiple extraction protocols have been used to obtain useable proton spectra of freshwater mussel adductor tissue, with Ringer's solution proving to be a safe, inexpensive option (Chapter 2). Differences in tissue metabolomes for several different organs have been established (Chapter 3), and potentially important metabolites in the understanding of diet and nutritional status have been identified, validated and quantified (Chapter 4).

This dissertation developed into a metabolomics project when it became apparent that not enough was known about basic mussel physiology to adequately investigate why freshwater mussels are particularly sensitive to ammonia. Nuclear magnetic resonance-based metabolomics was initially intended to be one of several diagnostic techniques to be used to better understand the mussel's physiologic responses to ammonia because the unbiased nature of NMR allows assessment of suspected and unexpected metabolic responses simultaneously. In the initial applications of this technology to freshwater mussel physiology studies, however, many methodological questions arose that needed to be addressed.

Proton NMR is the primary modality of NMR-based metabolomic investigations because tens to hundreds of hydrogen-based metabolites can be detected simultaneously, allowing a broad snapshot of metabolic status in a single sample. There are a number of protocols to prepare animal tissues for this type of spectrographic analysis. Major differences in the protocols are primarily related to the solutions used to extract metabolites from the tissue. My project started shortly after completion of related work in oysters in the Marine Metabolomics Laboratory at North Carolina State University's Center for Marine Sciences and Technology (CMAST), and my initial protocols mirrored their work using perchloric acid (Tikunov et al., 2010) and acetonitrile (unpublished). It quickly became clear that these and other published tissue extraction protocols have limitations due to handling, storage, and

safety concerns, as well as some labor intensive steps including pH titration. My goal with Chapter 2 was to compare the techniques learned from our colleagues to another published protocol (Bundy et al., 2002) that eliminated these labor intensive steps and safety concerns. As discussed in Chapter 2, Ringer's solution yielded spectra of an acceptable quality when compared to perchloric acid and acetonitrile extractions and this became the extraction protocol I chose for subsequent experiments. With more experience using the Ringer's extraction protocol, however, some limitations of this protocol have become apparent. Though the salts in the Ringer's solution are likely not at great enough concentrations in the final sample to create resonance interference within the NMR magnet, further work needs to be done to determine if this is true, and to determine if there is any benefit of using Ringer's solution over using only water or deuterated water alone. Preliminary work in our lab and published reports (Brown et al., 2008; Fan and Lane, 2008) suggests a loss of $-OH$ and $-NH$ metabolite signals when a sample is reconstituted with 100% deuterated water suggesting this should be of concern with deuterated water extractions (Appendix A). Ringer's extracted samples also contain what are likely macromolecule (lipid, protein, long-chain carbohydrate) resonances that overlap the low-molecular weight spectrum potentially obscuring metabolite peaks of interest in our analyses. Further investigation is also needed to refine the solution-to-tissue ratio and number of extraction cycles needed to optimally solubilize tissue metabolites for analysis.

A preliminary trial with the use of 1:1 v:v methanol:water as an extraction solution (Appendix B) has shown a simplification of spectral peaks in the 0.85-1.00ppm and 3.00-4.50ppm regions of the proton spectrum (Figure 5.1) as compared to Ringer's extractions, supporting the concern that spectra obtained with Ringer's solution contains lipid resonances. The methanol extraction appears to result in the loss of the glycogen peak at 5.40ppm, which has been noted in other methanol extractions (Tikunov et al., 2013). Future extraction with methanol to remove lipid interference should use a lower percentage methanol mixture.

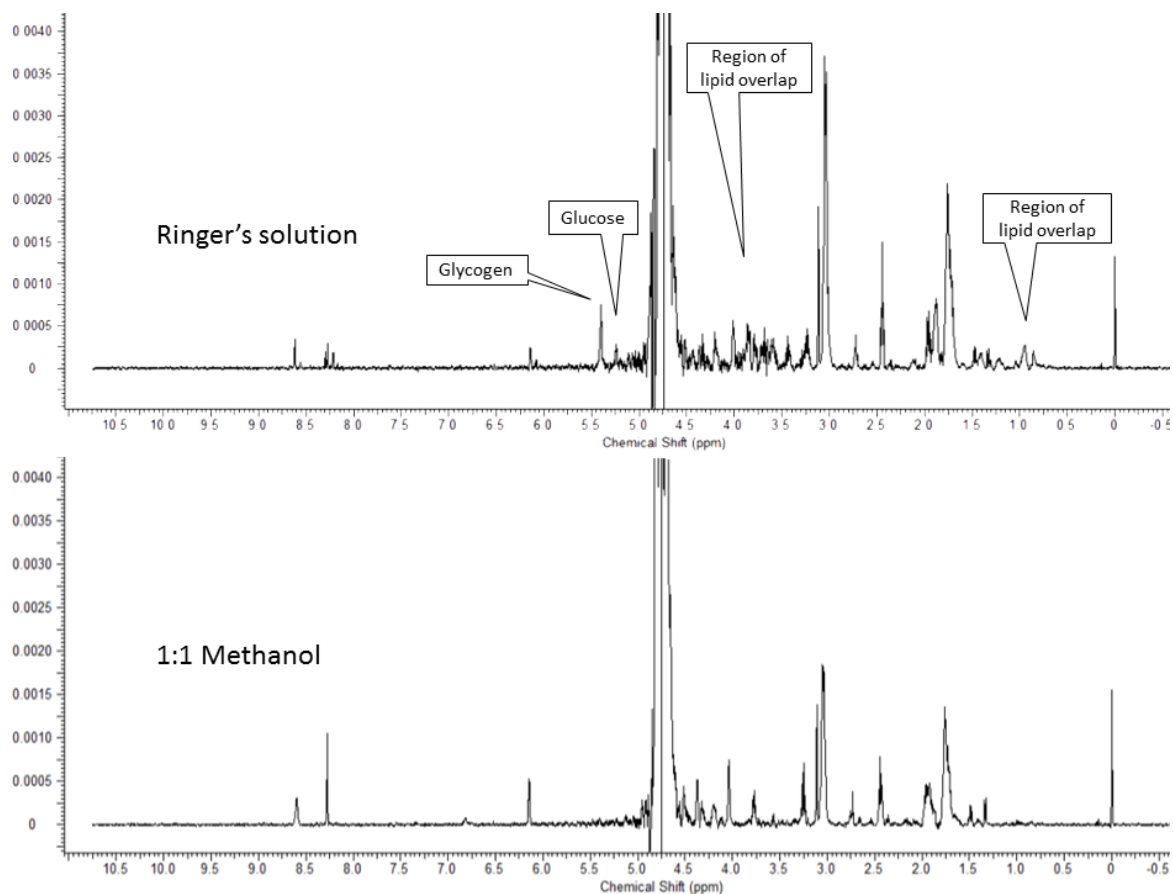


Figure 5.1: Comparative proton NMR spectra of *Elliptio complanata* adductor tissue. Adductor tissue from one individual was divided, half was processed with a Ringer's solution (top) and half was processed with a 1:1 (v:v) methanol solution. Similar peak profiles are noted, however, the glucose (5.22 ppm) and glycogen (5.40 ppm) peaks are absent in the methanol extraction. Simplification of the spectrum is evident at 0.80-1.00 ppm and 3.00-4.50 ppm in the methanol extraction due to removal of lipids with this protocol.

After a processing protocol was selected, a description of the metabolome of a freshwater mussel (Chapter 3) was needed as a baseline for any further studies that would include a physiologic stressor. By describing the metabolome of several *Elliptio complanata* tissues collected from two local sites, we aimed to highlight the physiologic character of the tissues, and hypothesized that hemolymph would be a viable sample for metabolomic testing. Preliminary testing with *Lampsilis radiata* yielded encouraging hemolymph spectra (Figure 5.2). Our analysis in Chapter 3 showed that *E. complanata* hemolymph contains metabolites

at such low concentrations that spectral analysis is not reliable and therefore, as sampled, hemolymph failed to be useful. Hemolymph continues to be a sample of particular interest due to its minimally invasive collection technique and further work focused on improving hemolymph protocols could be particularly rewarding. Use of larger samples to allow concentration of metabolites, the use of 3mm NMR (Wilmad Lab Glass, Vineland, New Jersey, USA), or Shigemi NMR microtubes (Shigemi Inc., Allison Park, Pennsylvania, USA) which facilitate use of very small amounts of sample, may yield better results from this tissue.

Additional work also needs to be done to develop reference ranges for normal concentrations of tissue metabolites for adductor, foot, mantle, and gill tissues in healthy *E. complanata*. By applying metabolomic techniques to a larger number of mussels, the population variation of individual metabolites could be determined as has been done for hemolymph chemistries (Gustafson et al., 2005). This would improve the capabilities of NMR-based metabolomics as a diagnostic tool; an individual sample could then be compared to an expected range of values for measured metabolites. Application of two-dimensional NMR techniques, such as total correlation spectroscopy (TOCSY) (Figure 5.3) and heteronuclear single quantum correlation spectroscopy (HSQC) will aid in identification of unknown metabolites through correlation of peak resonances. A greater geographical distribution of sampling and longitudinal sampling over time would be required to appreciate the variability introduced by both the mussel's environment and reproductive status. Digestive gland tissue was the only tissue in our investigation to show a potential site effect in the two sites we examined. We hypothesize that this could be related to a difference in local microbial fauna, but correlative work with microbial community analysis is needed to further understand this observation.

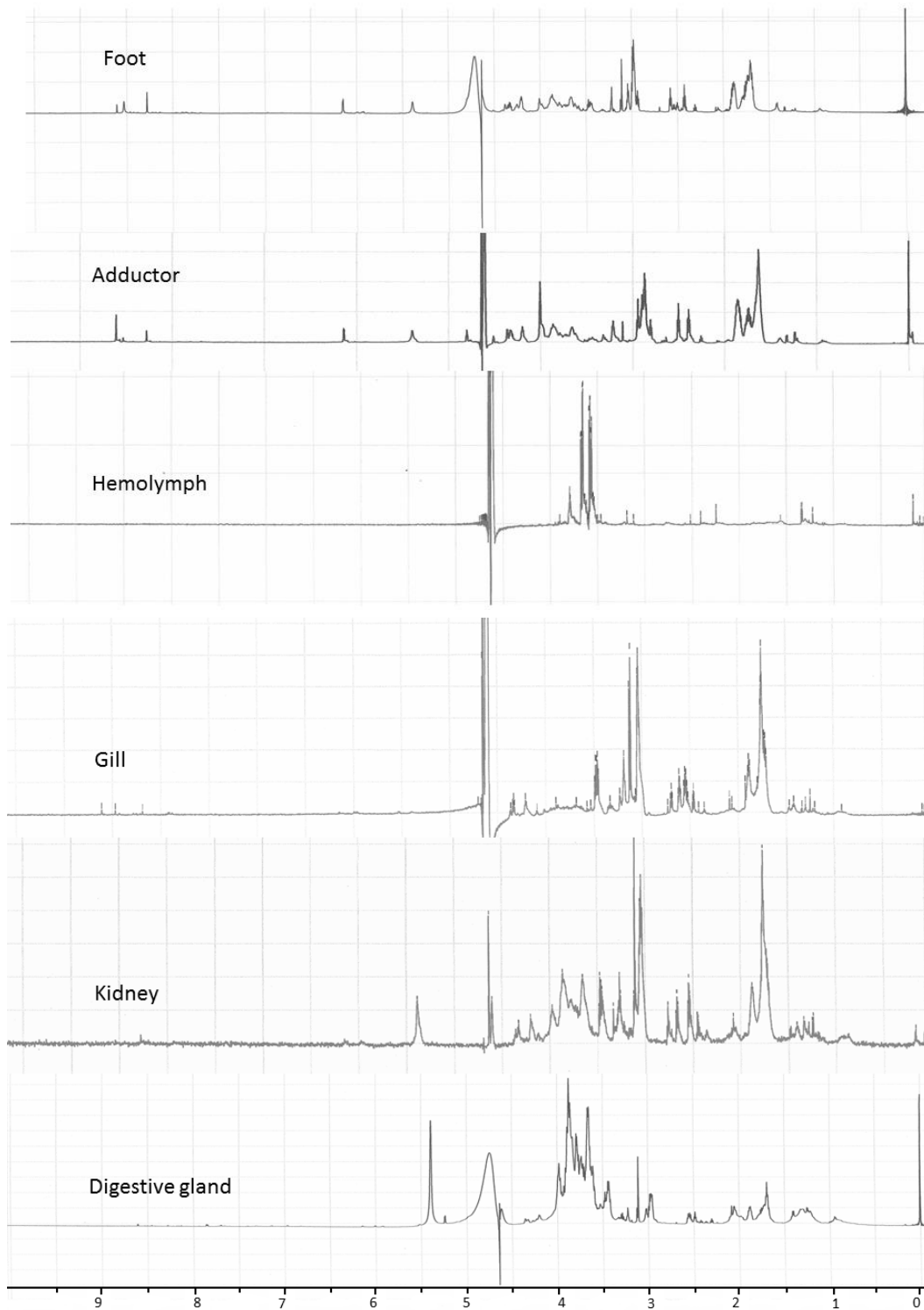


Figure 5.2: Preliminary tissue spectra from an adult *Lampsilis radiata*.

These investigations also need to be applied to other freshwater mussel species. Our preliminary work with freshwater mussel metabolomics included *Lampsilis radiata* which did not yield any immediately obvious differences from *E. complanata* (Figure 5.2). However, differences in both sample preparation and analytical protocols (Appendix C) precluded any direct comparison of spectra from these species.

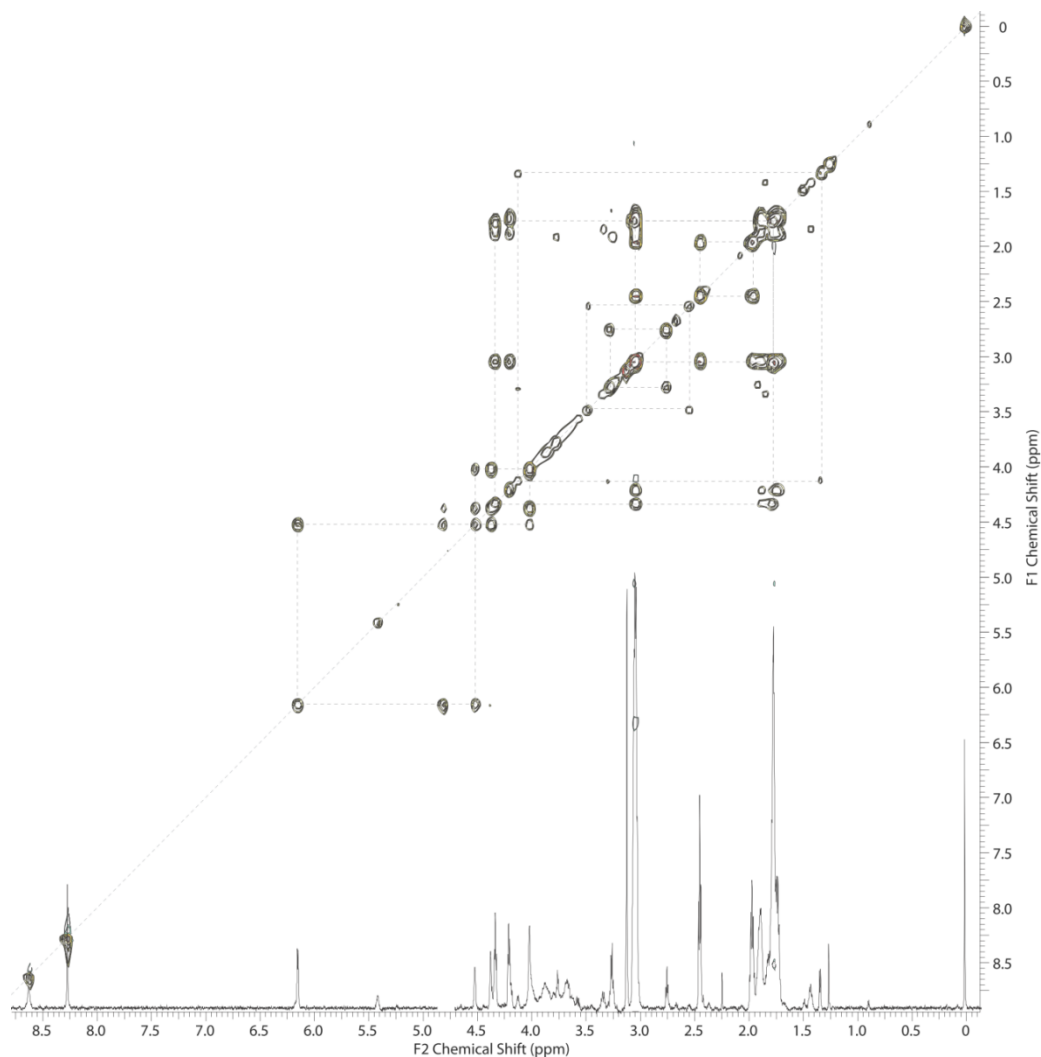


Figure 5.3: Example of total correlation spectroscopy (TOCSY) two-dimensional proton spectra on *Elliptio complanata* adductor tissue. (Image credit – Andrey Tikunov)

The next step in the application of metabolomic techniques is to detect how the metabolome changes in response to a stimuli. Concurrent work building a diluter and attempted preliminary ammonia exposures highlighted the challenges of using ammonia as an initial stimulus. An opportunistic trial which had provided early samples for the protocol experiments looked at fasted laboratory quarantined mussels and the resulting spectra suggested a direction of particular interest to our laboratory (Aquatic Epidemiologic and Conservation Laboratory (AECL)) with subjective spectral differences between wild-caught versus one-week fasted *E. complanata* (Appendix D). Nutrition of freshwater mussels has been a particular challenge in captive propagation facilities and this became an opportunity to look at the metabolic effects of captive diets and to investigate possible markers associated with food resource depletion. Initially we wanted to look at the difference between fasted animals and animals fed *Bacillus subtilis*. This bacterium has been under investigation in other studies in our laboratory (AECL) as a supplement to current feeding practices. To determine if *E. complanata* made use of *B. subtilis* we first grew the bacteria on U13-glucose (Cambridge Isotope Laboratories, Inc., Tewksbury, Massachusetts, USA) supplemented growth media and evaluated the uptake of marked carbons by *B. subtilis* using ^{13}C -NMR (Appendix E). These labeled bacteria were then fed to *E. complanata* and subjectively different ^{13}C spectra were obtained between mussels fed the marked bacteria, the individual exposed to marked broth alone, and the individual offered nothing (Figure 5.4) (Appendix F) with the broth-only sample appearing more similar to the fasted sample. Concurrent experiments with ^{13}C -labeling of a laboratory grown algae (Appendix E) did not yield evidence of uptake of the labeled sodium bicarbonate (Cambridge Isotope Laboratories, Inc., Tewksbury, Massachusetts, USA), further investigation was discontinued. Carbon labeling of food has been used successfully in *Crassostrea virginica* (Tikunov et al., 2010) and warrants further application in freshwater mussel research. Based upon the carbon labeling experiment, a feeding trial of bacteria in broth versus broth alone was initiated (Appendix G), yet principal components analysis of the proton spectra showed no clear effect attributable to supplementing with bacteria.

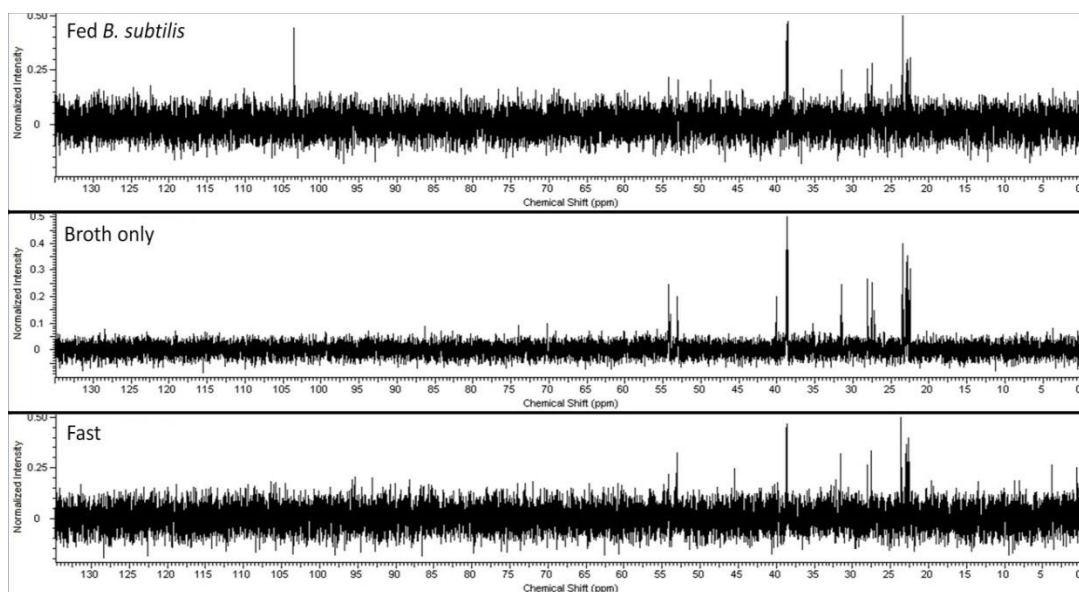


Figure 5.4: Carbon spectra from *Elliptio complanata* adductor tissue. Variation in spectral resonances seen in mussel fed *Bacillus subtilis* grown on U13-glucose supplemented nutrient broth for one week (top) as compared to mussel fed U-13 supplemented nutrient broth for one day and a mussel given no supplemental nutrition for one week (bottom).

Metabolic effects of diet have been successfully analyzed using proton NMR in non-human species (Schock et al., 2012, Shock et al., 2013) and the field of nutritional metabolomics is expanding in the world of human medicine (Gibney et al., 2005). The expansion of our preliminary feeding trials in Chapter 4 provides a groundwork for future application of nutritional metabolomics in freshwater mussels, and highlights design considerations that can be used for similar investigation in other species as well. The potential for future work is enormous and discussed in part in the fourth chapter. One additional consideration would be to include a treatment that includes a “wild diet,” either through a cohort that is maintained in-situ, or to transport river water for laboratory fed animals. Comparison to an in-situ group would be challenging due to differences in temperature, water quality, and other parameters that could have a confounding metabolic effect. However, a comparison to a “wild diet” could clarify if any of our diet treatments in

Chapter 4 were nutritionally complete or if all animals in the study were malnourished, improving our understanding of diet-related metabolic markers.

Further investigation in both wild and laboratory reared mussels is needed to determine role of putrescine in the metabolism of *E. complanata* and other freshwater mussels. Putrescine was one of the first metabolites we identified in mussel tissue due to its clear and prominent peaks on proton NMR spectra and had not been identified in the preceding experiments with *C. virginica* (Tikunov et al., 2010). As discussed in other chapters, the role of putrescine and other polyamines in intermediate metabolism is unclear for any species, and the relative concentration found in *E. complanata* tissues seems subjectively to be unusual. Through this dissertation, we have found that putrescine appears to vary based on tissue type, gender and nutritional status. Whether the polyamine acts as a secondary metabolite, deterring predation by giving mussel tissue a putrescent odor or flavor, or if it plays an integral role in core freshwater mussel metabolism is unknown.

In conclusion, the techniques explored and refined while conducting this dissertation have a breadth of potential applications for studying freshwater mussels, and most any other species of interest. This dissertation applied the techniques of NMR-based metabolomics to basic physiologic questions. It has also laid a foundation for additional explorations in environmental and nutritional metabolomics. Continued efforts focused on the metabolome of freshwater mussels will broaden our diagnostic tool box for studying their health, and support their conservation.

References

- Brown SAE, AJ Simpson, and MJ Simpson. 2008. Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with *Eisenia fetida*. *Environmental Toxicology and Chemistry* 27: 828-836.
- Bundy JG, EM Lenz, NJ Bailey, CL Gavaghan, C Svendsen, D Spurgeon, PK Hankard, D Osborne, JM Weeks, SA Trauger, P Speir, I Sanders, JC Lindon, JK Nicholson, and H Tank. 2002. Metabonomic assessment of toxicity of 4-fluoroaniline, 3,5-difluoroaniline and 2-fluoro-4methylaniline to the earthworm *Eisenia veneta* (Rosa): Identification of new endogenous biomarkers. *Environmental Toxicology and Chemistry* 21: 1966-1972.
- Fan TWM (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog Nucl Mag Res Spec* 28: 161-219.
- Fan TWM, and AN Lane. 2008. Structure-based profiling of metabolites and isotopomers by NMR. *Prog Nucl Mag Res Spec* 52: 69-117.
- Gibney MJ, M Walsh, L Brennan, HM Roche, B German, and B van Ommen. 2005. Metabolomics in human nutrition: opportunities and challenges. *Am J Clin Nutr* 82: 497-503.
- Gustafson LL, MK Stoskopf, W Showers, WG Cope, C Eads, R Linnehan, TJ Kwak, B Andersen, and JF Levine. 2005b. Reference ranges for hemolymph chemistries from *Elliptio complanata* of North Carolina. *Diseases of Aquatic Organisms* 65: 167-176.
- Schock TB, S Newton, K Brenkert, J Leffler, and DW Bearden. 2012. An NMR-based metabolomic assessment of cultured cobia health in response to dietary manipulation. *Food Chemistry* 133: 90-101.
- Schock TB, J Duke, A Goodson, D Weldon, J Brunson, JW Leffler and DW Bearden. 2013. Evaluation of Pacific white shrimp (*Litopenaeus vannamei*) health during a superintensive aquaculture growout using NMR-based metabolomics. *PLOS ONE* 8: e59521.

Tikunov AP, CB Johnson, H Lee, MK Stoskopf, and JM Macdonald. 2010. Metabolomic Investigations of American Oysters Using ¹H-NMR Spectroscopy. *Marine Drugs* 8: 2578-2596.

Tikunov AP, JH Winnike, K Tech, RE Jeffries, CT Semelka, J Martin, R McClelland, LM Graves, JM Macdonald. 2013. Fluxomics by NMR Spectroscopy from Cells to Organisms Focusing on Liver. *Current Metabolomics* 1: 128-159.

APPENDICES

Appendix A – 10% vs 100% D₂O

Reconstitution with 10% Deuterated water versus 100% Deuterated water

- 1) Laboratory housed, moribund but responsive, sub-adult *Lampsilis fasciola* was removed from its shell and snap frozen in liquid nitrogen
- 2) Whole frozen body was pulverized with mortar and pestle, kept frozen with intermittent addition of liquid nitrogen during pulverization
- 3) 1.03g powdered tissue transferred to a pre-weighed polyethylene tube and 2.1ml perchloric acid was added, vortexed, and incubated in 4°C refrigerator overnight.
- 4) Sample was centrifuged at 3.5g for 15 minutes and supernatant was transferred to a 50ml polyethylene tube.
- 5) Supernatant buffered with potassium chloride to a pH of 7.03, centrifuged for 20 minutes.
- 6) Supernatant was pipetted into a 5ml tube and placed in -80°C overnight.
- 7) The sample was then lyophilized.
- 8) Half the sample was reconstituted with 1ml 0.1mM TSP in 100% D₂O, and the other half was reconstituted with 0.1mM TSP in 10% D₂O. D₂O had been diluted with deionized water.
- 9) Proton NMR spectroscopy performed on 500Hz Bruker electromagnet at NCSU BioNMR Facility as described in Chapter 1.

Appendix B –Methanol Extraction

Methanol Extraction

- 1) Twelve adult *Elliptio complanata* were collected from the Eno River and transported to NCSU. The mussels were kept overnight in river water with an aerator.
- 2) Each animal was opened as described in Chapter 1. Anterior adductor muscles were excised, divided in approximately half, placed in a polyethylene tube and snap frozen in liquid nitrogen.
- 3) One half of each adductor muscle was pulverized in 1ml amphibian Ringer's solution, and the other half was pulverized in 1:1 v:v methanol:deionized water using a NextAdvance Bullet Blender for 5 minutes at speed 10. Methanol samples required a second 5 minute blend to achieve a homogenous solution.
- 4) These samples were then placed in a 4°C refrigerator to incubate for 30 minutes.
- 5) Samples were then centrifuged at 12g for 15 minutes, supernatant collected into microcentrifuge tubes and placed in a -80°C freezer.
- 6) A second 1ml of extraction solution was added to the pellet, vortexed and incubated. After thirty minutes, the samples were centrifuged, supernatant collected and frozen as above.
- 7) Frozen samples were transferred to the Hamner institute for lyophilization.
- 8) Lyophilized samples were reconstituted with 700uL 0.1mM TSP in 10% D2O, centrifuged for 10 minutes at 12g. Supernatant was transferred to NMR tubes.
- 9) Proton NMR spectroscopy was performed on 500Hz Bruker electromagnet at NCSU BioNMR Facility as described in Chapter 1.

Appendix C - Lampsilis radiata

Lampsilis radiata

- 1) A total of five laboratory housed adult *Lampsilis radiata* were used for initial trials over approximately one month.
- 2) Hemolymph was collected from the anterior adductor muscle as described in Gustafson 2005. Each animal was then opened as described in Chapter 1. On the first two animal tissues were collected in this order: anterior adductor muscle, foot muscle, gill, digestive gland. From the next animals, tissues were collected as listed: adductor, foot, gill, mantle, kidney, digestive gland. Tissues were placed in polyethylene tubes and snap frozen in liquid nitrogen. Tubes had been pre-weighed.
- 3) Frozen tissues were pulverized in a stainless steel mortar and pestle with intermittent addition of liquid nitrogen to keep the tissues frozen and returned to their polyethylene tubes.
- 4) Perchloric acid (PCA) was added at a ratio of 2ml PCA to 1g tissue, vortexed, and incubated in 4°C refrigerator overnight.
- 5) Samples were then centrifuged at 3500g for 15 minutes and supernatant was transferred to a 50ml polyethylene tube.
- 6) Supernatant was buffered with potassium chloride to a pH of 7.0-7.4, centrifuged for 20 minutes.
- 7) Supernatant was then collected and stored at -80°C overnight.
- 8) The sample was then lyophilized.
- 9) Initial samples were reconstituted with 700µL 1mM TSP in 100% D2O, but it was quickly determined that this concentration was more than was necessary for the muscle tissues. Following samples were reconstituted with 0.1mM TSP in 100% D2O.
- 10) Proton NMR spectroscopy performed on 700MHz Varian electromagnet through the UNC/NCSU Joint Department of Biomedical Engineering.

Appendix D – Fed/Fasted

Elliptio complanata Fed/Fasted

- 1) Six *Elliptio complanata* were collected from the Eno River and transported to NCSU. Three animals were sacrificed immediately upon arrival (Fed), and three were housed in 2 gallons deionized water for 1 week with aeration via airstone and no supplemental feeding (Fasted).
- 2) Each animal was then opened as described in Chapter 1. Tissues were excised in the following order: anterior adductor muscle, foot muscle, kidney, digestive gland, gill, mantle. Tissues were placed in polyethylene tubes and snap frozen in liquid nitrogen. Tubes had been pre-weighed.
- 3) Frozen tissues were pulverized in a stainless steel mortar and pestle with intermittent addition of liquid nitrogen to keep the tissues frozen and returned to their polyethylene tubes.
- 4) Perchloric acid (PCA) was added at a ratio of 2ml PCA to 1g tissue. The samples were then vortexed, and incubated in 4°C refrigerator overnight.
- 5) Samples were then centrifuged at 3500g for 10 minutes and supernatant was transferred to a 50ml polyethylene tube.
- 6) Supernatant was buffered with potassium chloride to a pH of 7.0-7.8, centrifuged for 20 minutes.
- 7) Supernatant was then collected and placed in -80°C overnight.
- 8) The sample was then lyophilized and placed in the -80°C freezer.
- 9) Fed samples were reconstituted 6 days after lyophilization with 800uL 0.1mM TSP in 100% D₂O. Fasted samples were reconstituted 3 days after lyophilization. The reconstituted samples were centrifuged at 3500g for 20 minutes, supernatant removed and pipetted into NMR tubes.
- 10) Samples were refrigerated at 4°C (24 days Fed, 12 days Fasted) in 5mm NMR tubes prior to spectroscopy.
- 11) Proton NMR spectroscopy performed on 700MHz Varian electromagnet at UNC.

Appendix E – Carbon Labeling Bacteria and Algae

Carbon Labeling

In-house culture of *Bacillus subtilis*:

Ongoing culture kept in 4°C refrigerator.

When new culture needed, 1ml of old culture (at approximately 10^7 CFU/ml) added to previously autoclaved and cooled 1L Difco 233000 Nutrient Broth. The inoculated broth was then incubated at 37°C overnight and returned to the refrigerator.

In-house culture of *Polymorpha* spp:

Ongoing culture kept under fluorescent lights.

When new culture needed, 1L of old culture (cell count varies) is added to approximately five gallons of dechlorinated city water with Kent F/2 Algal Formula (Aquatic Eco-Systems, Inc., Apopka, Florida, USA) added per product instructions.

1) Determining uptake of labeled carbon by *B. subtilis*:

- a. 200µL stock + 50ml DNB + 0.19g U13-glucose (=20mM glucose) (Cambridge Isotope Laboratories, Inc., Tewksbury, Massachusetts, USA) was placed in 37°incubator.
- b. Samples collected at 30, 60, 90, 120, 180, 240, and 300 minutes
 - i. 1ml placed on Petrifilm for colony forming unit counts. All samples were too numerous to count.
 - ii. 1ml filtered on 0.45µm membrane filter. Filtrate was rinsed with 15ml DI water to remove any residual Nutrient Broth containing U13-glucose. Filter was then placed in a polyethylene tube and snap frozen in liquid nitrogen. Samples were stored at -80°C.

- 2) Determining uptake of for labeled carbon by *Polymorpha* spp.:
- a. 2ml *Polymorpha* stock (3.76×10^6 cells/ml) was added to 13ml water containing Kent F/2 Algal Formula plus 0.003g deuterated sodium bicarbonate (Cambridge Isotope Laboratories, Inc., Tewksbury, Massachusetts, USA) to make a 2.5mM NaD(CO₃) solution.
 - b. 2ml stock +125ml treated water + 0.83g NaD(CO₃) (=80mM NaD(CO₃))
 - i. All solutions incubated under grow lights overnight.
 - ii. 1ml filtered on 0.45µm membrane filter. Filtrate was rinsed with 15ml DI water to remove any residual Nutrient Broth containing NaD(CO₃). Filter was then placed in a polyethylene tube and snap frozen in liquid nitrogen. Samples were stored at -80°C.
- 3) Sample preparation for NMR
- a. Frozen filters were pulverized and 2ml 0.7M perchloric acid was added to each sample. This solution incubated overnight at 4°C. (Note, filter had previously been evaluated via NMR for evidence of confounding resonances. No peaks were seen on filter-only samples.)
 - b. The next day, samples were centrifuged at 3500g for 30 minutes and the supernatant collected. Supernatant was pH balanced to pH 7-7.3 with potassium hydroxide then centrifuged at 3500g for 30 minutes.
 - c. Resulting supernatant was frozen at -80°C then lyophilized overnight.
 - d. Powder was resuspended in 700µL 0.1mM TSP in 10% D₂O, centrifuged at 3000g for 20 minutes and supernatant transferred to 5mm NMR tubes.
 - e. Carbon spectra were collected at NCSU BioNMR Facility using the 500MHz Bruker electromagnet described in Chapter 1, tuned to the ¹³C frequency of 125.77. Instrument parameters are listing in the table below.

Table E.1: ^{13}C data collection parameters

Parameter	Value
Acquisition Time (sec)	1.0813
Nucleus	^{13}C
Number of Transients	40960
Original Points Count	32768
Pulse sequence	zgdc
Receiver Gain	11585.2
SW(cyclical) (Hz)	30303.03
Spectrum offset (Hz)	1275.78
Sweep Width (Hz)	30302.11
Temperature (degree C)	20.6

- f. Recorded resonances were processed using ACD/Labs 12.0 1D NMR Processor (ACD/Labs, Toronto, Ontario, Canada).

Appendix F – Carbon labeling *Elliptio complanata*

Carbon labeling of *Elliptio complanata*

- 1) Four adult *Elliptio complanata* were collected from the Eno River and transported to NCSU-AECL.
- 2) Animals were housed together in 2 gallons Unionid River Water (URW) (Christopher Owens personal communication with request to withhold recipe until published) for four days to acclimate and fast them prior to the experiment. URW is deionized water supplemented with mineral based on the chemistry of two rivers in Kentucky to minimize osmotic stress on the freshwater mussels.
- 3) After four days, each was placed in 750ml URW with an airstone for aeration. Tops of the beakers were covered with aluminum foil to minimize evaporation and airborne contamination.
- 4) *Bacillus subtilis* was labeled with U13-glucose as described in Appendix E. Inoculated broth had been incubated overnight at 37°C.
- 5) Feeding trial lasted one week. One mussel received no supplementation for the duration of the trial (Fast). A second mussel received 2ml of labeled *B. subtilis* in U13-glucose nutrient broth spaced approximately 12 hours apart and was sacrificed 7 hours after the second feeding (Fed 1 day). The third mussel was fed a total of 6 times before sample collection (Fed 3 days) and the fourth was fed 14 times prior to the end of the week (Fed 7 days).
- 6) A month after these were collected and processed as described in step 7, an additional *E. complanata* was collected, fasted 4 days, fed 2ml U13-glucose in Nutrient Broth twice daily for one day, then sacrificed (Broth 1 day).
- 7) Each animal was opened and sampled immediately at the end of their trial time as described in Chapter 1. Tissues were excised in the following order: anterior adductor muscle, digestive gland, mantle, gill. Tissues were placed in polyethylene tubes and snap frozen in liquid nitrogen. Tubes had been pre-weighed.

- 8) Frozen tissues were pulverized in a stainless steel mortar and pestle with intermittent addition of liquid nitrogen to keep the tissues frozen and returned to their polyethylene tubes.
- 9) Perchloric acid (PCA) was added at a ratio of 2ml PCA to 1g tissue. The samples were then vortexed, and incubated in 4°C refrigerator overnight.
- 10) Samples were then centrifuged at 2500g for 20 minutes and supernatant was transferred to a 50ml polyethylene tube.
- 11) Supernatant was buffered with potassium chloride to a pH of 7.0-7.22, centrifuged for 20 minutes.
- 12) Supernatant was then collected and placed in -80°C until frozen.
- 13) The sample was then lyophilized overnight.
- 14) Fed samples were reconstituted after lyophilization with 700uL 0.1mM TSP in 10% D₂O. The reconstituted samples were centrifuged at 3500g for 20 minutes, supernatant removed and pipetted into NMR tubes.
- 15) Proton and ¹³C-NMR was performed on all samples at the NCSU BioNMR facility as described in Chapter 1 and Appendix E respectively.

Appendix G – *Bacillus* Feeding Trial

Elliptio complanata Feeding Trial: *Bacillus subtilis* vs Nutrient Broth

- 1) *Bacillus subtilis* culture prepared by adding 0.4ml stock culture to 100ml autoclaved and cooled Difco Nutrient Broth. The inoculated broth was incubated at 37°C overnight.
- 2) Ten adult *Elliptio complanata* were collected from the Eno River and transported in river water to NCSU-AECL. Upon arrival they were rinsed under deionized water and placed individually placed into 2L Unionid River Water. It was determined that one of the mussels was dead.
- 3) After two days, the mussels were rinsed and scrubbed and placed into 1L beakers containing URW. Four animals were fed *B. subtilis* in Nutrient Broth and 3 animals were fed 3ml Nutrient Broth alone twice daily for a week. The first day, animals were given 3ml of diet per feeding, the second day 4ml, and the remaining five days 2ml. The initial increase was to ensure the animals were fed adequately, but it was then decreased due to cloudiness of the water indicating the animals were overfed and bacterial overgrowth was occurring.
- 4) Water changes were done daily prior to the PM feeding with URW.
- 5) On day seven, hemolymph and adductor tissue were collected as described in Chapter 3 and snap frozen in liquid nitrogen. Frozen samples were stored at -80°C for 12 days prior to additional processing.
- 6) Hemolymph samples were lyophilized overnight, reconstituted with 700µl 0.1mM TSP in 10% D₂O, and centrifuged at 13,000g for 10 minutes. Supernatant was then collected and pipetted into 5mm NMR tubes for spectroscopy.
- 7) Adductor samples were transferred to microcentrifuge tubes, 1ml:1g Ringer's solution and 100µl 0.5mm zirconium oxide beads were added. Samples were then homogenized at speed 10 for a total of 10 minutes. Samples were then stored at -20°C overnight.
- 8) Adductor samples were allowed to partially thaw.

- 9) After thawing, the samples were centrifuged at 13,000g for 10 minutes, supernatant collected and stored at 4°C. The pellet was resuspended in Ringer's at the initial volume and incubated at room temperature (24°C) for 30 minutes.
- 10) Step 9 was repeated and centrifuged a final time to collect a third supernatant. The supernatant fractions were added together to create one extraction sample per tissue sample.
- 11) Extraction samples were then frozen at -80°C, then lyophilized, reconstituted, and placed in NMR tubes as in Step 6.
- 12) All samples were then analyzed using proton NMR at the NCSU BioNMR Facility as described in Chapter 1.