

Key metabolites in tissue extracts of *Elliptio complanata* identified using ¹H nuclear magnetic resonance spectroscopy

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We used ¹H nuclear magnetic resonance spectroscopy to describe key metabolites of the polar metabolome of the freshwater mussel, *Elliptio complanata*. Principal components analysis documented variability across tissue types and river of origin in mussels collected from two rivers in North Carolina (USA). Muscle, digestive gland, mantle and gill tissues yielded identifiable but overlapping metabolic profiles. Variation in digestive gland metabolic profiles between the two mussel collection sites was characterized by differences in mono- and disaccharides. Variation in mantle tissue metabolomes appeared to be associated with sex. Nuclear magnetic resonance spectroscopy is a sensitive means to detect metabolites in the tissues of *E. complanata* and holds promise as a tool for the investigation of freshwater mussel health and physiology.

Key words: Freshwater mussels, metabolites, nuclear magnetic resonance spectroscopy

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Introduction

The ecological niche filled by suspension-feeding bivalve molluscs makes them well suited to serve as biological 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, b; Lehmann *et al.*, 2007; Liu *et al.*, 2011; Zhang *et al.*, 2011), and efforts have been made to use freshwater bivalves

to monitor the health of surface waters (Doyotte *et al.*, 1997; Won *et al.*, 2005; Grabarkiewicz and Davis, 2008).

Metabolomics, the study of an organism's profile of metabolites, has been used to study the physiological responses of both terrestrial and aquatic organisms to changes in their environment. The techniques allow a broad range of metabolites to be quantified in small samples collected by tissue biopsy or phlebotomy. Metabolomic profiles have been successfully used to assess the response of bivalves to heavy metals and endocrine disruptors (Liu *et al.*, 2011; Zhang *et al.*, 2011;

Leonard *et al.*, 2013, 2014). The use of patterns of metabolites to characterize responses to particular environmental perturbations has the potential greatly to increase our understanding of the pathogenesis of environmental impacts on key bivalve species.

As a preliminary investigation, we conducted a non-targeted nuclear magnetic resonance spectroscopy (NMR) examination of the metabolome of a relatively common Unionid mussel species, *Elliptio complanata*. This mussel is found in many Atlantic slope rivers and has the potential to be useful as a bioindicator for ecosystem health and as a surrogate model for sympatric endangered mussel species (Chittick *et al.*, 2001). *Elliptio complanata* from two river systems in the piedmont of North Carolina were sampled to evaluate differences in metabolome across tissue types. We collected haemolymph, adductor muscle, foot muscle, gill, digestive gland and mantle tissue to examine the following hypotheses: (i) individual variation would not obscure metabolic profile variations across tissue types; and (ii) variations in metabolic profile associated with tissue type would be compatible with known physiological functions of the tissues.

Materials and methods

Freshwater mussel collection and processing

In October 2012, five adult *E. complanata* (three non-gravid females and two males) were taken from the Eno River, near Hillsborough (NC, USA), ~300 m downstream from a highway bridge (Table 1). An additional five (five males) were taken from the New Hope Creek, near Durham (NC, USA), ~100 m upstream of a road bridge (Table 1). Global positioning system coordinates were recorded for the study sites. 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 (<1 min to process from removal from the water to the last tissue being placed in dry ice). As much haemolymph as could be retrieved (0.5–1.5 ml) was aspirated from the anterior adductor muscle as described by Gustafson *et al.* (2005a) and placed in a cryovial 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 was then placed in preweighed polyethylene tubes and placed in dry ice for transport. Samples were held at approximately -80°C until analysis. A transverse section through the body cavity was taken and placed in formalin for histopathological determination of sex and gravidity. The fixed tissues were embedded in paraffin wax, sectioned into 5 mm slices and placed on glass microscope slides. Haematoxylin and eosin stains were applied, and the tissues were analysed using an Olympus CK2 light microscope (Olympus Corporation, Center Valley, PA, USA).

Water analysis

A YSI 6920 (YSI, Inc., Yellow Springs, OH, USA) encased in an expanded metal cage was placed mid-stream in each river system within the stream channel for 7 days prior to the collection of mussels. Measurements of temperature (in degrees Celsius), conductivity (in millisiemens per centimetre), pH, turbidity [nephelometric turbidity units (NTU)] and dissolved oxygen (expressed as a percentage and in milligrams per litre) were recorded every 15 min for 1 week.

Tissue processing

Frozen tissues were pulverized using a Bullet Blender homogenizer (Next Advance, Averill Park, NY, USA). A 2:1 (v/w; 2 ml solution to 1 g tissue) Amphibian Ringer solution (Fisher Scientific, Waltham, MA, USA) was added to the tissues, vortexed, and incubated at 4°C overnight. Amphibian Ringer solution was selected as an extraction medium because it is readily available commercially and free of lactate found in other available Ringer solutions. Incubation in Ringer solution results in solubilization of polar metabolites for NMR analysis.

After incubation, the samples were centrifuged at $3450g$ for 20 min (Hermle Z300; Labnet International, Inc., Edison, NJ, USA). The supernatant was transferred into new polyethylene tubes and frozen at -80°C . All frozen samples, including the haemolymph samples, were then lyophilized overnight (Lyoph-Lock 18 Freeze Dry System; Laboconco, Kansas City, MO, USA). Seven hundred microlitres of 0.1 mM deuterated trimethylsilyl

Table 1: Shell measurements (length, height and width) and median measurements for sampled *Elliptio complanata* (in millimetres)

	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

Abbreviations: E, Eno River; NH, New Hope Creek. *Non-gravid female; all other mussels were male.

propionate in 10% deuterium oxide was added to the dried samples, transferred to microcentrifuge tubes and centrifuged for 30 min at 5000g (AccuSpin Micro 17; Fisher Scientific) to remove any remaining particulate matter. The supernatant was removed and pipetted into 5 mm borosilicate Wilmad Labglass economy brand thin-walled (5 mm outer diameter × 7 mm in length) 100 MHz Rating NMR tube (Fisher Scientific) for NMR analysis. One Eno River adductor sample and one New Hope Creek mantle sample were lost during processing.

Pulsed field NMR experiments were performed on a Bruker (Billerica, MA, 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 by Hurley-Sanders *et al.* (2015). 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 feature (ACD Labs 12.0 1D NMR Processor; ACD Labs, Toronto, Ontario, Canada) with a bin

width of 0.04 ppm excluding water and deuterated trimethylsilyl propionate 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.bmrb.wisc.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

Application of NMR techniques using an amphibian Ringer solution extraction (Hurley-Sanders *et al.*, 2015) successfully yielded spectra for metabolite identification in adductor muscle, foot muscle, gill, mantle and digestive gland (Fig. 1).

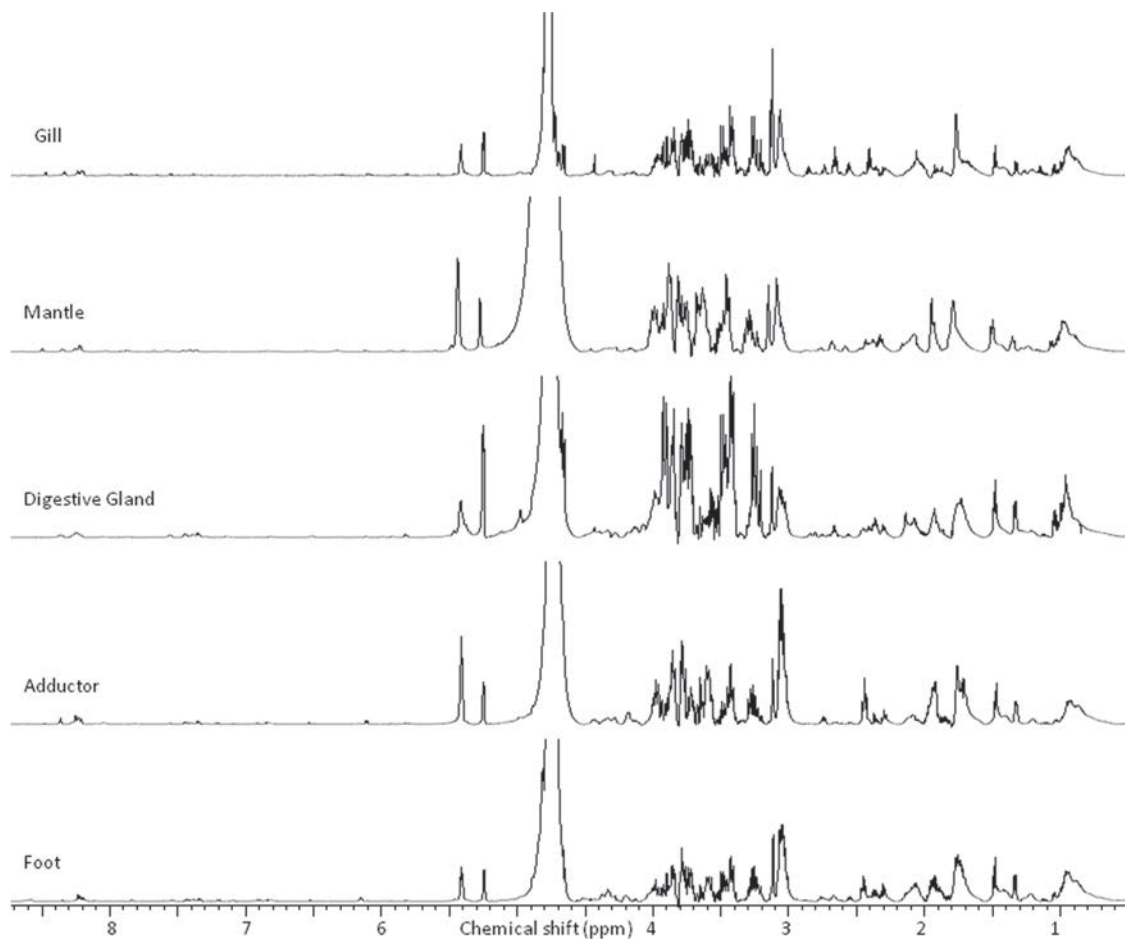


Figure 1: Representative ¹H nuclear magnetic resonance spectra of each tissue from one individual.

Haemolymph samples yielded poor spectra with rare peaks above background noise and were not included in further analyses. Numerous small-molecular-weight polar metabolites were identified (Table 2).

Principal components analysis showed differentiation between tissue types (Fig. 2). Variability along principal component 1 (PC1) partly 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, reflecting the physiological function of these tissues. Variability in principal component 2 (PC2) partly separated gill tissue associated with glutamate and several unidentified peaks from muscle tissues associated with amines and amino acid intermediates (Fig. 3).

Tissue-associated variability was responsible for the greatest variation between samples when all samples were analysed simultaneously. However, within tissue type, two variations were noted. A site effect for digestive gland samples was suggested by the variability along PC1 of glucose, maltose and glycogen, which separated the New Hope Creek digestive gland from the Eno River samples (Fig. 4). Mantle tissue displayed a possible sex-related 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 0.85–0.93 ppm ($-\text{CH}_3$) range and at 2.05 ppm (likely to be $\text{CH}_2\text{C}=\text{O}$ or $\text{CH}_2\text{C}=\text{C}$ in structure; Fig. 5). The two mantle samples that grouped with the gill tissue samples in the overall principal components analysis map (Fig. 2) were both female. Little to no site- or sex-associated difference was observed in other tissues.

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; Tables 3 and 4). No measurements from either the Eno River or the New Hope Creek were above US Environmental Protection Agency criteria for drinking water (www.epa.gov).

Discussion

This study serves as a preliminary investigation of the use of ^1H NMR spectroscopy-based metabolomic techniques for the study of freshwater mussels. We sampled a small number of *E. complanata*, a freshwater mussel common in North Carolina, from two watersheds in Orange County (NC, USA) to identify the predominant polar endogenous metabolites in several tissues as a first step in evaluating the application of NMR spectroscopy as a tool to study freshwater mussel metabolism. Our study yielded identifiable metabolite spectra for six of the seven tissues sampled. Tissue-based differences were identified and gave preliminary information about possible sex- and diet-related differences in metabolic profile.

Haemolymph collection and analysis has been shown to identify physiological parameters and circulating haemocytes

effectively in freshwater mussels (Gustafson *et al.*, 2005b; Burkhard *et al.*, 2009) and has been shown to reflect physiological change in other mollusc species (Cotou *et al.*, 2013; Bianchi *et al.*, 2014). In our study, haemolymph samples extracted from the anterior adductor muscle of mussels did not yield useful spectra. Although the maximal amount of haemolymph was aspirated from the adductor sinus of each individual, we observed few visible peaks above baseline noise, potentially because of low concentrations of detectable metabolites in the extracted biofluid. Roznere *et al.* (2014) developed viable profiles of metabolic pathways in haemolymph extracted from the adductor sinus in food-limited and non-food-limited *Amblema plicata* (Threeridge mussel) using mass spectrometry. Their ability to observe differences in haemolymph metabolites may have reflected the greater sensitivity of mass spectrometry or differences in sample preparation and metabolite extraction procedures. Perhaps, larger volumes of haemolymph from larger individuals or pooling of samples would have yielded useful spectra.

All identified metabolites were found in all tissues other than haemolymph, with each tissue having a characteristic pattern of metabolites (Figs 1 and 2). Many of the metabolites found in *E. complanata* have been identified in American oyster (*Crassostrea virginica*) tissues using proton NMR, but 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 may reflect evolutionary adaptations of the mussel to the low osmolarity of their freshwater environment (Dietz and Branton, 1975). Second, *E. complanata* tissues contained predominant peaks of the polyamines putrescine and cadaverine not identified in oyster tissue (Tikunov *et al.*, 2010). The physiological significance of these polyamines in the sampled freshwater mussels is unknown; however, putrescine and cadaverine are products of amino acid metabolism, and these biogenic amines have been documented in fish and other aquatic species (Hu *et al.*, 2012).

In this study, despite statistically significant differences ($P < 0.001$) in temperature, conductivity, pH, turbidity and dissolved oxygen, tissue type influenced sample grouping more than river site in the principal components analysis of all spectra (Table 4 and Fig. 2). This suggests that the water quality and food resources of both rivers fell within a range that allows normal metabolic function for *E. complanata* or that the observed statistically significant variation in water quality was not biologically significant. 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 of *E. complanata* or other unionids (Matteson, 1948; Aldridge *et al.*, 1987; Griffiths and Cyr, 2006; Kessler *et al.*, 2007; Cummings and Graf, 2010). Our findings suggest that the upstream areas of the study sites are functionally very similar despite statistical differences in water parameters. Both sites were located in wooded areas, upstream from a road bridge,

Table 2: Suggested major metabolites and peak assignments

Metabolites	Chemical shift and peak shape (ppm)*	Reference
Amino acids		
Alanine	1.46(d), 3.76(m)	[0] [1] [2]
Arginine	1.68(m), 1.90(m), 3.23(m), 3.74(m)	[0] [1] [3]
Glutamate	2.08(m), 2.34(m), 3.74(t)	[0] [1] [3] [6] [10]
Glycine	3.54(s)	[0] [1] [3]
Isoleucine	0.92(t), 1.00(d), 1.26(m), 1.44(m), 1.96(m), 3.66(d)	[0] [1] [3] [4]
Leucine	0.94(d), 0.96(d), 1.66(m), 3.71(t)	[0] [1] [3] [4]
Serine	3.84(m), 3.96(m)	[0] [1] [4] [10]
Taurine	3.25(t), 3.42(t)	[1] [5]
Valine	0.98(d), 1.03(d), 2.25(m), 3.59(d)	[0] [1] [3] [4]
Energy-related substances		
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)	[0] [1] [4] [10]
Glycogen	3.40(m), 3.60(m), 3.80(m), 3.96(br.s.), 5.40(br.s.)	[1] [2] [10]
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] [10]
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]
Ketoglutarate	2.43(t), 3.00(t)	[0] [3] [6]
Pyruvate	2.46(s)	[2]
Lactate	1.32(d), 4.10(q)	[0] [2] [10]
Osmolytes and organic acids		
Betaine	3.25(s), 3.89(s)	[1] [4]
β -Alanine	2.55(t), 3.18(t)	[0] [1]
γ -Aminobutyric acid	1.89(m), 2.28(t), 3.00(t)	[0] [9] [10]
Krebs cycle intermediates		
Succinate	2.41(s)	[1] [7]
Fumarate	6.51(s)	[0] [7]
Fatty acid metabolism		
Malonate	3.11(s)	[7]
Carnitine	2.43(dd), 3.21(s), 3.42(m), 4.56(2)	[0] [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 (0.8–0.93; multiple overlapping peaks)	
Unknown peak 2	2.05(br.s)	
Unknown peak 3	2.39(t)	
Unknown peak 4	2.64(t)	
Unknown peak 5	2.84(m)	
Unknown peak 6	3.19(s)	
Unknown peak 7	3.24(m)	
Unknown peak 8	3.63(br.s)	

Abbreviations: br.s, broad singlet; d, doublet; dd, doublet of doublets; m, multiplet; s, singlet; t, triplet. [0] Leonard *et al.* (2013); [1] Tikunov *et al.* (2010); [2] Gäde 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] Gagné *et al.* (2007); and [10] Leonard *et al.* (2014). *Chemical shifts were identified using the Human Metabolome Database (HMDB), Biologic Magnetic Resonance Data Bank (BMRB) and Chenomx NMR Suite 7.6.

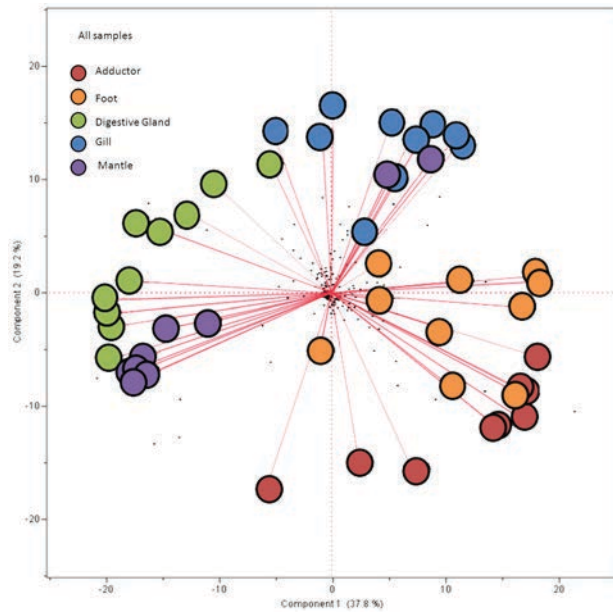


Figure 2: Two-component principal components map of all samples from both rivers. Each tissue separated from the other, indicating a characteristic metabolic profile for each tissue. The adductor and foot muscle profiles overlapped, indicating a relationship between the two muscle tissues. The two mantle samples that grouped with the gill samples were both from females; this grouping may be due to seasonal reproductive changes to the mantle tissue.

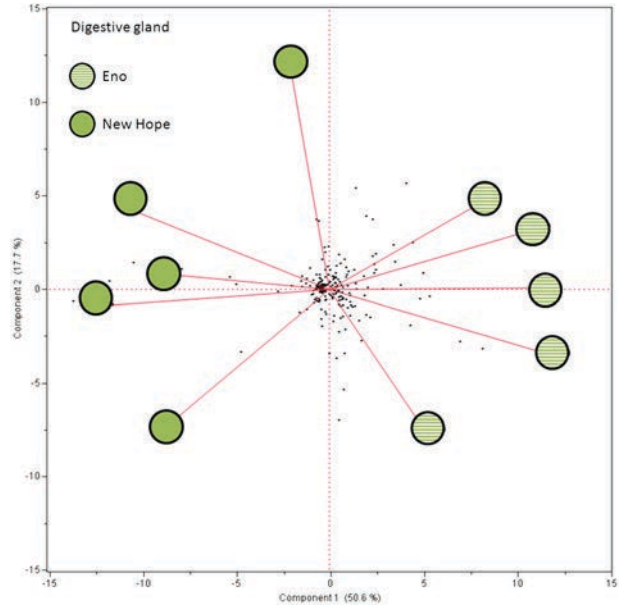


Figure 4: Tissue-specific two-component principal components map of digestive gland samples. Eno River samples (Eno) group separately from New Hope Creek samples (New Hope), suggesting location-specific effects on the digestive gland metabolome.

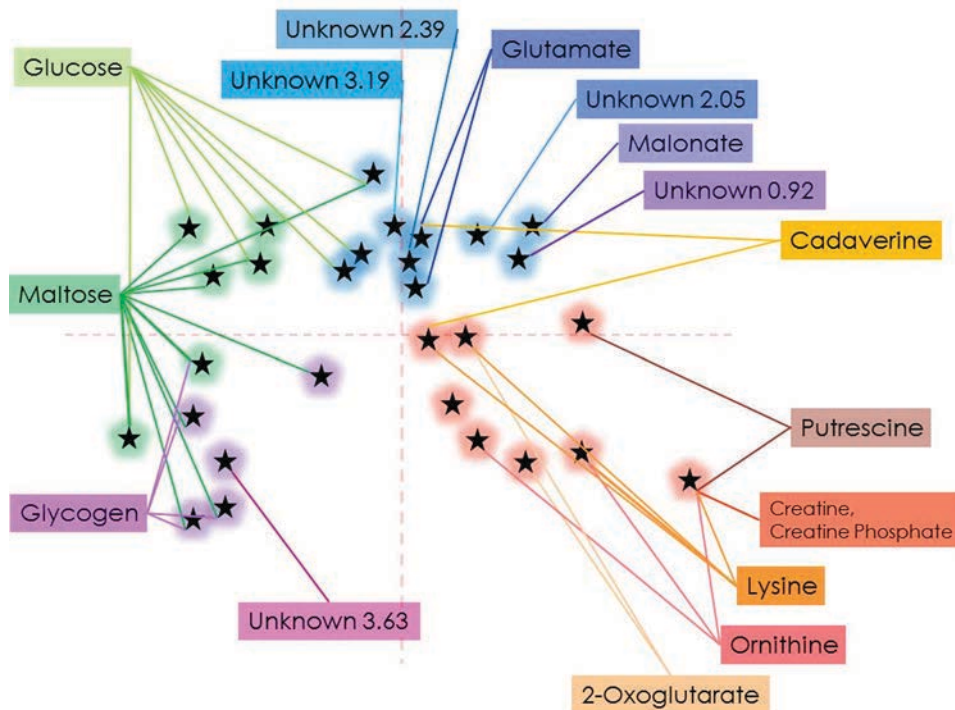


Figure 3: Loading plot of all tissue samples from both rivers. Blue stars correspond to the scores within the gill grouping; green, digestive gland; purple, mantle; and red, combined adductor and foot muscle.

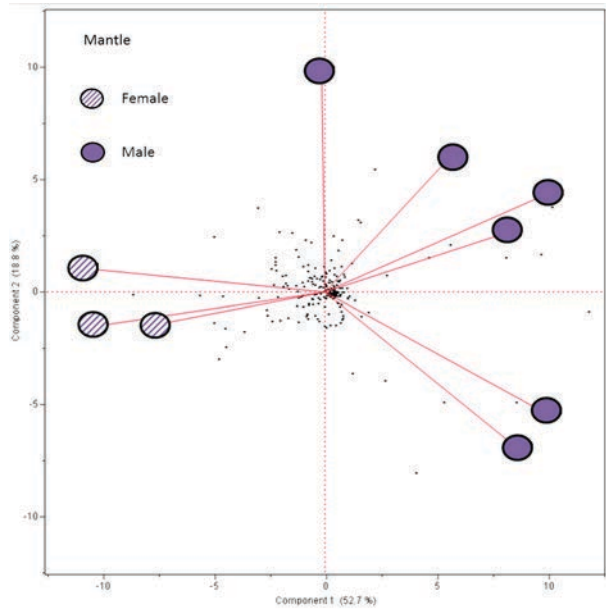


Figure 5: Tissue-specific two-component principal components map of mantle samples. Samples from males group separately from samples from females, suggesting gender-specific effects on the mantle metabolome.

and the land use of the upstream watershed is similar, although the Eno River site has more downstream development than the New Hope Creek site. To identify geographical effects on the metabolism of *E. complanata*, more study sites and a larger number of animals from each site would need to be studied.

The principal components groupings by tissue in our study are likely to reflect differences in physiological function. 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 one we examined where a metabolomic difference by river location was noted based on single-tissue principal components analysis. This separation was driven by differences in the relative concentrations of maltose in particular, and glucose and glycogen (Fig. 4). Site-associated variation in nutritional status or differences in the gastrointestinal microbiome could be responsible for these findings. Bacterioplankton and bacterial sediment populations vary greatly in different streams (Crump and Hobbie, 2005; Bucci *et al.*, 2014). Investigations incorporating gut depuration techniques and larger numbers of individuals would be useful to elucidate the basis of site-related variations in the mussel digestive gland metabolome.

In this study, *E. 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

Table 3: Water-quality parameters from the Eno River and New Hope Creek

	Eno River						New Hope Creek					
	Temperature (°C)	Conductivity (mS/cm)	pH	Turbidity (NTU)	ODOsat (%)	ODO (mg/l)	Temperature (°C)	Conductivity (mS/cm)	pH	Turbidity (NTU)	ODOsat (%)	ODO (mg/l)
Mean	18.25	0.078	7.04	5.26	84.83	8.01	16.76	7.31	14.65	96.64	9.44	
Maximum	24.7	0.189	7.22	14.9	101.1	8.86	21.2	7.62	152.2	114.2	11.9	
Median	18.3	0.077	7.03	5.3	84.4	8.02	16.2	7.31	10.6	99.35	9.68	
Minimum	14.2	0.009	6.52	0	81.1	7.04	13.3	7.16	0	83	7.69	
SD	2.95	0.007	0.07	4.32	2.4	0.49	2.56	0.04	17.08	6.87	1.1	
n	572	572	572	572	572	572	572	572	572	572	572	

Abbreviation: ODOsat, optical dissolved oxygen saturation; ODO, optical derived oxygen.

Table 4: Parameters and averages measured by YSI 6920 every 15 min over the span of 1 week between rivers

	<i>n</i>	Eno River mean	New Hope Creek mean	<i>P</i> -value
Temperature (°C)	572	18.25	16.76	<0.001
Conductivity (mS/cm)	572	0.08	0.09	<0.001
pH	572	7.04	7.31	<0.001
Turbidity (NTU)	572	0.14	4.55	<0.001
ODOsat (%)	572	84.83	96.63	<0.001
ODO (mg/l)	572	8.02	9.44	<0.001

Abbreviation: ODOsat, optical dissolved oxygen saturation; ODO, optical derived oxygen. All parameters were statistically significantly different between the two rivers at a value of $P < 0.001$ (Welch's two-sample *t*-test).

as putrescine, which is derived from ornithine, and cadaverine produced from lysine, have been found in many animal and plant tissues and appear to have multiple functions in cellular metabolism, including promotion of cell proliferation and induction of apoptosis (Tabor and Tabor, 1976; Jänne *et al.*, 2004). The prominence of putrescine in freshwater mussels deserves further investigation in order to gain a better understanding of the role of polyamines in their physiology.

Freshwater bivalve gills function in respiration, in 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 0–6 ppm range of spectra from mussel gills are consistent with methyl groups ($-\text{CH}_3$) and alkenes (C_nH_{2n}) and suggest that gill tissue may have relatively increased levels of lipoproteins or steroid hormones (Fan and Lane, 2008; Leonard *et al.*, 2013, 2014). These metabolites are better studied using less polar extraction methods, and the concentrations of protons key to their specific identification were insufficient in our samples to distinguish from similar protons from the low-molecular weight compounds in the same part of the spectrum (Brown *et al.*, 2008).

Unionid mantle tissue, like gill 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). Mantle samples in our study were characterized primarily by carbohydrate metabolites, which reflects the role of the mantle as a site of carbohydrate storage for bivalves, as was seen in digestive gland (Patterson *et al.*, 1999; Ojea *et al.*, 2004; Tikunov *et al.*, 2010). It was interesting that two of the mantle samples from the Eno River grouped with the gill samples from the same river based on principal components analysis. This may be related to the influence of macromolecular lipids or steroid hormones related to reproductive status, because both of these samples were from females (De Zwann and Wijsman, 1976; Cavaletto and Gardner, 1999). When only mantle tissue was evaluated in a principal components analysis, all three samples from females separated from the samples from males, regardless of location

(Fig. 5). The three female mussels separated from the male mussels along PC1 based on higher glycogen, glucose and maltose concentrations associated with the male mussels and the relative differences in putrescine, cadaverine and lysine peaks along with an unidentified compound (Unknown 1), suspected to be lipoprotein overlap in females. Earlier studies have noted a gender-related division by principal components analysis in the mantle metabolome in *Mytilus galloprovincialis* (Hines *et al.*, 2007). Other than lysine, the metabolites associated with the *Mytilus* gender separation were different from those identified in our study of *E. complanata*. It is possible that these differences are related to the vastly different natural habitats of the two species. Longitudinal studies designed to include more balanced sex ratios of mussels are needed to assess seasonal changes and characterize the impact of reproductive status on the metabolome of freshwater mussels.

Conclusion

Using proton NMR, we observed relative concentration differences in multiple metabolites across different tissues of *E. complanata*. This supports the potential for more extensive studies using these techniques to characterize the metabolomic responses of different unionid tissues to environmental changes. Our studies represent an initial step toward understanding which prominent metabolites characterize freshwater mussel tissue and how metabolomic studies may inform efforts to understand freshwater mussel physiology relevant to health and disease.

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