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Research article

Mussel mass mortality in the Clinch River, USA: metabolomics detects affected pathways and biomarkers of stress

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Biologists monitoring freshwater mussel (order Unionida) populations rely on behavioral, often subjective, signs to identify moribund ("sick") or stressed mussels, such as gaping valves and slow response to probing, and they lack clinical indicators to support a diagnosis. As part of a multi-year study to investigate causes of reoccurring mortality of pheasantshell (*Ortmanniana pectorosa;* synonym *Actinonaias pectorosa*) in the Clinch River, Virginia and Tennessee, USA, we analyzed the hemolymph metabolome of a subset of mussels from the 2018 sampling period. Mussels at the mortality sites were diagnosed in the field as affected (case) or unaffected (control) based on behavioral and physical signs. Hemolymph was collected in the field by non-lethal methods from the anterior adductor muscle for analysis. We used ultra-high-performance liquid chromatography with quadrupole time-of-flight mass spectroscopy to detect targeted and untargeted metabolites in hemolymph and compared metabolomic profiles by field assessment of clinical status. Targeted biomarker analysis found 13 metabolites associated with field assessments of clinical status. Of these, increased gamma-linolenic acid and *N*-methyl-l-alanine were most indicative of case mussels, while adenine and inosine were the best indicators of control mussels. Five pathways in the targeted analysis differed by clinical status; two of these, purine metabolism and glycerophospholipid metabolism, were also indicated in the untargeted analysis. In the untargeted nalysis, 22 metabolic pathways were associated with clinical status. Many of the impacted pathways in the case group were catabolic processes, such as degradation of amino acids and fatty acids. Hierarchical clustering analysis matched clinical status in 72% (18 of 25) of mussels, with control mussels more frequently (5 of 16) not matching clinical status. Our study demonstrated that metabolomic analysis of hemolymph is suitable for assessing mussel condition and complements field-based indicators of health.

Key words: hemolymph, metabolites, mussel die-off, pheasantshell, Unionida

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Introduction

Freshwater mussels (Order Unionida) are vital sentinels of aquatic ecosystem health. As infaunal suspension feeders, unionids stabilize substrate, remove particulates and contaminants, interconvert nutrients and transfer energy to other trophic levels (Vaughn, 2010, 2018). North America has the most diverse unionid fauna in the world, with 298 recognized species (Williams *et al.*, 2017), the greatest diversity of which occurs in the southeast USA (Haag, 2012). Freshwater mussels are one of the most critically imperiled faunal groups, both globally and in the USA. (Lydeard *et al.*, 2004; Régnier *et al.*, 2009). In North America, 70% of species are considered endangered, threatened or vulnerable, and 29 species have become extinct in the past 100 years (Haag and Williams, 2014); in 2021, eight additional species were proposed for removal from the US Federal Lists of Endangered Species due to extinction (86 FR 54298, 2021). Widespread habitat degradation (including pollution, siltation, river channelization and impoundment) is cited as the primary cause of extinction over the past century (Downing *et al.*, 2010; Haag, 2019), coupled with more recent introductions of invasive molluscs, *Corbicula* and *Dreissena* spp. (Strayer *et al.*, 1999; McMahon and Bogan, 2001; Ferreira-Rodríguez *et al.*, 2018). Still, the causes of most mussel population declines are ill-defined (Downing *et al.*, 2010).

Since the 1970s, episodic mass mussel mortality events have been documented in relatively pristine systems with no direct specific environmental changes or events, such as contaminants or drought (Haag, 2019). Investigations into causal agents of these mortality events have been limited in scope and without definitive answers (Neves, 1986; Starliper *et al.*, 2011; Waller and Cope, 2019). Complicating these efforts is the lack of diagnostic tools for objective assessment of mussel health and discovery of causal factors of mortality. Field biologists monitoring freshwater mussel populations rely on behavioral, often subjective, symptoms to identify "sick" or stressed mussels, such as gaping valves or an extended foot with a slow response to stimulus. Field assessment of "sick" or moribund mussels is seldom accompanied by clinical findings to confirm or support the diagnosis, primarily because health assessment tools are lacking.

We investigated a reoccurring mussel mortality event in the Clinch River in southwest Virginia and northeast Tennessee, USA, that primarily affected pheasantshell (*Ortmanniana pectorosa;* synonym*: Actinonaias pectorosa)*. The Clinch River is a biodiversity hotspot with 46 extant species of freshwater mussels (Jones *et al.*, 2014), 20 of which are on the US Fish and Wildlife Service Lists of Endangered Species. Although mussel richness and abundance in the upper river in Virginia steadily fell from 1979 to 2014, mussel densities in the Tennessee portion of the upper Clinch River increased from 1979 to 2014 (Jones *et al.*, 2018). Pheasantshell (*O. pectorosa)* was historically abundant in the Clinch River system (Jones *et al.*, 2014), but beginning in 2016, field biologists began reporting a disproportionate number of moribund and dead individuals of this species in the shoals near the Virginia and Tennessee state line. Mortality reoccurred in the fall months of 2017 to 2019, resulting in a 50–90% decline of pheasantshell population at monitoring sites in the lower river (Richard *et al.*, 2020). We conducted a multi-year study of mussels in the Clinch River to determine the role of infectious agents in pheasantshell mortality. We applied metagenomic analysis of hemolymph and compared unaffected (control) and affected (case) mussels in 2017 and 2018 to detect potential infectious agents associated with mussel mortality (Leis *et al.*, 2019; Richard *et al.*, 2020, 2021; Knowles *et al.*, 2022). Infectious agents associated with the mortality period in the Clinch River included a novel virus, Clinch densovirus 1, (Richard *et al.*, 2020), and the bacteria *Yokenella regensburgei* and *Aeromonas salmonicida* (Leis *et al.*, 2019; Richard *et al.*, 2021). Bacterial metagenomic analysis also found fewer sequence variants and greater bacterial load in case compared to control mussels (Richard *et al.*, 2021). Currently, the primary cause of mortality remains unclear. As part of the investigation, we analyzed hemolymph metabolites of a subset of pheasantshell that were sampled in 2018 from four sites in the Clinch River. We compared metabolomic profiles of mussels that were field diagnosed as control and case and investigated how closely hemolymph metabolomes matched these field-based clinical assessments and thus might enhance non-lethal assessments of mussel health.

Metabolomics is the detection and analysis of biologically relevant molecules, and the metabolome is the total set of

metabolites in a biological system (Wishart *et al.*, 2007; Zamboni *et al.*, 2015). Metabolomic profiles can be used to characterize the health status of individual mussels, and pathway analysis of metabolites can reveal mechanisms of disease (Liu *et al.*, 2013; Nguyen *et al.*, 2018, 2019). Metabolomics has been used in marine bivalve studies of disease (de Lorgeril *et al.*, 2018; Nguyen *et al.*, 2019), aquaculture conditions (Young *et al.*, 2015; Hao *et al.*, 2018; Tian *et al.*, 2021), vitamin supplementation (Yang *et al.*, 2019) and environmental stressors (Cappello *et al.*, 2017; Dumas *et al.*, 2020). Hemolymph is the circulatory fluid of bivalves and has multiple functions including immunity, gas exchange, osmoregulation, nutrient distribution and waste elimination (Machałowski and Jesionowski, 2020). The composition and concentration of hemolymph constituents reflect not only the metabolic products of various tissues but also those of infectious agents and other symbionts. Additional metabolites may be obtained from water and diet (phytoplankton, bacteria). Within this complex metabolome, those metabolites that have low variability and occur in most of the population can indicate stable condition of individual mussels, whereas shifts in levels of these metabolites from the status quo could signal stress. Metabolomic studies of freshwater mussels are limited in number and scope, particularly of hemolymph. Hemolymph chemistry values were determined using an automated clinical chemistry analyzer for *Elliptio complanata* from 19 populations to establish reference ranges for eight constituents (Gustafson *et al.*, 2005). Whereas proton nuclear magnetic resonance (1H NMR) spectroscopy yielded poor detection of hemolymph metabolites (Hurley-Sanders *et al.*, 2016), gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) detected shifts in hemolymph metabolites in *Amblema plicata* in response to captivity, food limitation (Roznere *et al.*, 2014) and relocation (Roznere *et al.*, 2017).

Here we apply LC–MS as a platform for targeted and untargeted metabolomics to characterize the metabolic profile of unaffected (control) and affected (case) mussels (based on field assessments of clinical status) and identify case-associated metabolites and pathways. Whether the infectious agents previously identified (e.g. densovirus, *Y. regensburgei*) in pheasantshell are the primary cause of mortality or indicative of environmental stressors that trigger mussel disease, defined as any deviation from normal function and structure, we expect metabolic differences between case and control mussels. Using both targeted and untargeted approaches to metabolite detection extended the scope of our analysis beyond that possible using a single approach. The targeted metabolomic results have higher confidence in the identification of specific metabolites, but the number of targeted metabolites is low, making affected pathway analysis difficult. The untargeted analysis relies on the MetaboAnalyst algorithm to identify potential metabolites based on molecular weight. The untargeted analysis detected many more metabolites than the targeted analysis and assigned those metabolites to specific pathways.

Materials and Methods

Field sampling

We sampled 30 pheasantshell from the Clinch River, including nine cases (from September and October of 2018) and 21 controls (from August, September and October 2018) at four sites (Fig. 1; Supplementary Table S1). Mussel samples were collected, and clinical status assessed as described by Richard *et al.* (2020). Briefly, during sampling, mussels were assigned to the control (apparently healthy, unaffected) group if they were firmly buried in the substrate, responded rapidly to tactile stimuli by valve closure or foot withdrawal and closed their valves strongly. Mussels were assigned to the case (moribund, affected) group if they were on the surface of the substrate and gaping, responded slowly to tactile stimuli and closed their valves weakly or held their valves slightly open. We sampled in August, before mortality was observed, and again in September and October when active mortality was occurring.

To collect hemolymph, we gently opened the valves of each mussel with a sterile pediatric nasal speculum, disinfected the outer surface of the anterior adductor muscle with 70% isopropyl alcohol and extracted up to 1.0 ml of hemolymph (depending on the size of the mussel) from the anterior adductor muscle sinus using a 1-ml tuberculin syringe. We placed hemolymph in sterile tubes on dry ice in the field and then stored samples at −80°C until further analysis. We marked mussels with glue-on shellfish tags (Hallprint, Hindmarsh Valley, Australia) to prevent re-sampling during successive sampling events and then returned mussels to the shoals from which they were collected.

Tissue sample preparation

Hemolymph samples were processed in groups of 24 to limit processing time (Hernandes *et al.*, 2017). After thawing, we removed 100 μl of hemolymph with 200 μl of cold methanol containing CUDA (CAS # 479413-68-8, 12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid; Cayman Chemical, Ann Arbor, MI) as an internal standard. The samples were then vortexed for 30 s and centrifuged at 18 787*g* for 7 min at 4°C. We used a CentriVap micro-Infrared Vacuum Concentrator (Labconco, Kansas City, MO) at 1700 revolutions per minute (rpm) and 35°C to evaporate the methanol and increased temperature to 47.5°C to evaporate residual water/methanol. The samples were reconstituted in 125 μl of cold methanol, vortexed for 30 s and centrifuged at 18 787*g* for 7 min at 4°C. A sample volume of 100 μl was used for analysis.

Metabolomic analysis

Multiple methods have been developed for the extraction, detection, identification and quantification of the metabolome (Liu and Locasale, 2017). We used a modified one-step extraction based on the modified Bligh–Dyer extraction method (Bligh and Dyer, 1959; Sitnikov *et al.*, 2016;

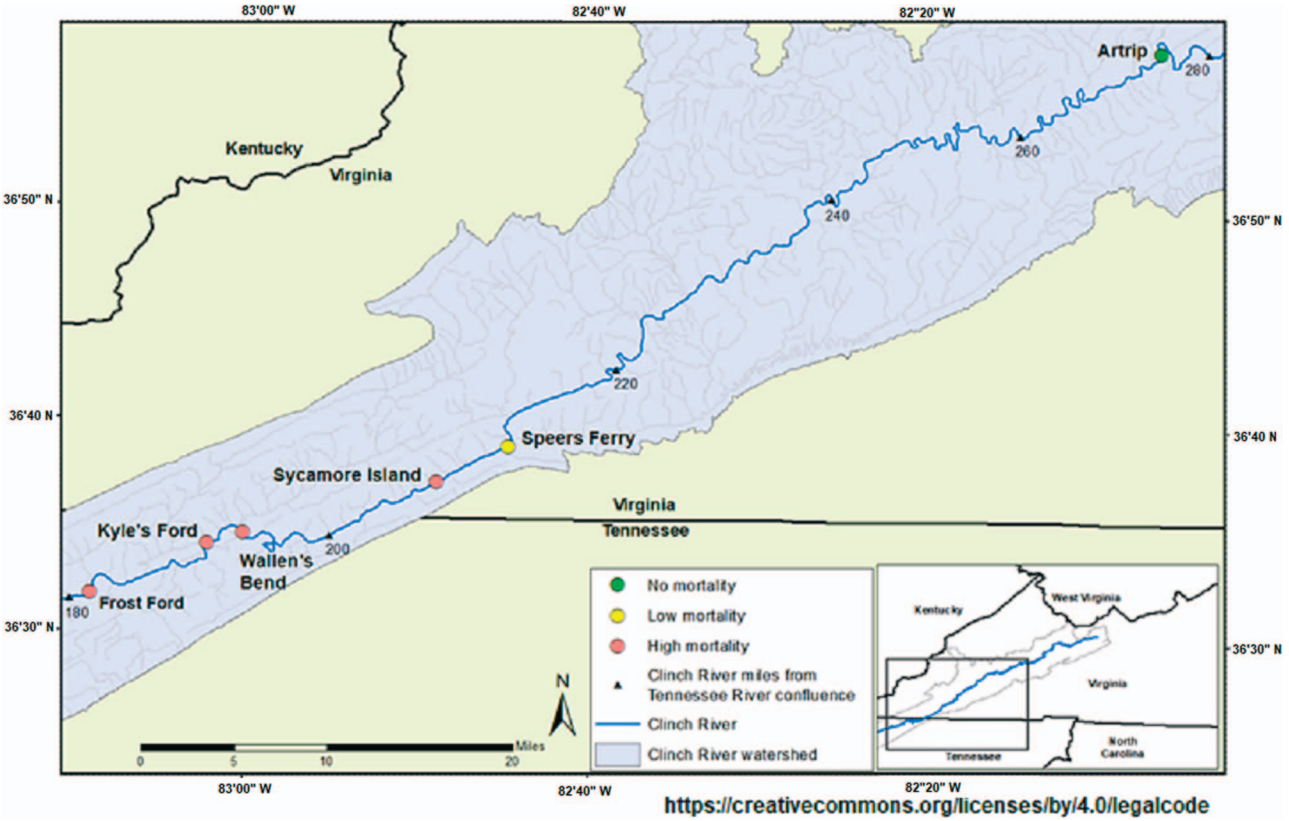

Figure 1: Sampling locations on the Clinch River, Tennessee and Virginia, USA (adapted from Richard *et al.*, 2020, original image published under a Creative Commons Attribution 4.0 International License).

Satomi *et al.*, 2017) and an ultra-high-performance liquid chromatography with quadrupole time-of-flight mass spectroscopy (HPLC-MS) to detect metabolomic signals in hemolymph (Putnam *et al.*, 2017). We used both targeted and untargeted metabolomic analysis. The targeted analysis compared the detected signal mass, spectrum and retention time with a database of known standards, whereas the untargeted analysis examined all detectable metabolomic signals, both known and unidentified metabolites. The analytical method, quality control and data processing are described in the supplemental data.

Data processing

We used the Mass Spectrometry Metabolite Library from IROA Technologies (Mass Spectrometry Metabolite Library, IROA Technologies, Sea Grit, NJ) to identify targeted metabolites. The IROA Library also allowed us to ensure there was consistent chromatographic retention and peak shape over all samples. Targeted metabolites were identified by the injection of standards. The detected IROA Library standards were used to create a mass spectrum library containing information about each standard: retention, signal, m/z and chemical adduct (Supplementary Table S2). We annotated metabolites in the targeted analysis using the Human Metabolomic Database (HMDB—https://hmdb.ca/, release version 4.0)(Wishart *et al.*, 2018) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp, release version 99.0, 99.1, and 100.0) (Hattori *et al.*, 2003; Kanehisa *et al.*, 2012). Data associated with the manuscript are available in the study by Putnam *et al.* (2022), "Metabolomic analysis of pheasantshell mussel (*Ortmanniana pectorosa*, order Unionida) from a mass mortality event in the Clinch River, Virginia and Tennessee, USA: U.S. Geological Survey data release", https://doi.org/10.5066/P9ZT9F3S.

Statistical analyses

We used a multi-tiered statistical approach to identify relationships between field assessments of clinical status (case vs. control). First, we used a principal component analysis (PCA) to identify natural groupings of mussels by field assessments of clinical status based on the underlying structure of the data. We also used a robust PCA analysis to identify orthogonal outliers, good and bad leverage points. Good leverage points follow the trends found in all mussels, whereas bad leverage points do the exact opposite (Hubert *et al.*, 2005; Alguraibawi *et al.*, 2015). Orthogonal outliers are outside

Table 1: Impacted untargeted pathways associated with field assessment based on gamma $P < 0.10$

Pathway	Total in pathway	Total detected	Significant hits	Gamma *P*
Glycerophospholipid metabolism[a]	156	24	12	>0.01
Fatty acid activation	74	23	9	>0.01
Phosphatidylinositol phosphate metabolism	59	5	3	0.01
Purine metabolism[a]	80	13	5	0.01
Ubiquinone biosynthesis	10	2	2	0.01
Androgen and estrogen biosynthesis and metabolism	95	10	4	0.01
Saturated fatty acids beta-oxidation	36	7	3	0.02
Methionine and cysteine metabolism	94	7	3	0.02
Prostaglandin formation from di-homo gamma-linoleic acid	11	3	2	0.02
Glycosphingolipid metabolism	67	20	6	0.02
Fatty acid oxidation	35	8	3	0.02
Butanoate metabolism	34	8	3	0.02
Glycine, serine, alanine and threonine metabolism	88	13	4	0.03
Linoleate metabolism	46	18	5	0.03
Caffeine metabolism	1	5	2	0.04
Urea cycle/amino group metabolism	85	21	5	0.05
Omega-3 fatty acid metabolism	39	6	2	0.05
Phytanic acid peroxisomal oxidation	34	6	2	0.05
Glycolysis and gluconeogenesis	49	7	2	0.07
Di-unsaturated fatty acid beta-oxidation	26	7	2	0.07
Carnitine shuttle	72	33	7	0.07
Hexose phosphorylation	20	8	2	0.08

The gamma *P* cutoff of 0.1 was used to ensure weakly related pathways are retained for further investigation.

[a]Also significant in target pathway analysis.

the normal plane of the PCA. The PCA analysis created a biplot of the samples based on the cosine similarity between two dimensional vectors in three dimensions. In addition, 95% confidence ellipses were added showing the Gaussian distribution of the normally distributed mussel metabolites. Second, we used volcano plots to detect important metabolites in a univariate analysis. The volcano plot analysis was completed by calculating the average abundance of each metabolite grouped into either a field assessment of case or control. The volcano plot analysis used the combined targeted and untargeted metabolomic data to show the influence of the field assessment. The *P* value was also calculated for each metabolite based on the field assessment. The fold change (FC) was calculated by taking the $\log_2$ of the average abundance for each metabolite for either the case or control mussels and then subtracting the control value from the case value. The volcano plot displays $\log_2$ FC for upregulated metabolites of the case mussels on the positive *x*-axis, and for the control mussels on the negative *x*-axis. Those metabolites with FC >1.0 and *t* test *P* value <0.1 were considered important for distinguishing case and control mussels. Metabolites with a *P* value between 0.05 and 0.10 show a weak relationship and when linked to FC >1.0 may offer further insight into differences between field assessment groups (Fan *et al.*, 2020).

We used MetaboAnalyst (MetaboAnalyst, 2021) for the biomarker, targeted and untargeted pathway analysis and heat map analysis (Chong *et al.*, 2019; Pang *et al.*, 2021). All data used in the MetaboAnalyst platform had outliers filtered by calculating the relative standard deviation; data were normalized by the median, log transformed and scaled by Pareto scaling. All *P* values reported from MetaboAnalyst are adjusted *P* values. The biomarker analysis used area under the curve (AUC), FC of metabolite abundance and the false discovery rate *t* test between the field assessment (case vs. control) to rank metabolites as potential biomarkers. The IROA standard metabolites were assigned to targeted metabolic pathways using MetaboAnalyst. The untargeted

pathway analysis in MetaboAnalyst uses an algorithm to identify metabolites based on known pathway and network data. The algorithm conducts multiple permutations to identify possible matches. These permutations are then used to create a gamma-distributed *P* value (Chen *et al.*, 2014). The pathway analysis also uses an enrichment factor analysis to measure the number of pathway metabolites identified compared to expected values. We used the heat map tool to visualize the concentrations of the top 25 significant untargeted metabolites. Individual mussels were grouped in a hierarchical cluster by metabolite concentration. *t* Test values were used to compare mean values of the metabolites.

Results

The PCA results showed that two mussels were outside the normal metabolomic distribution (Supplementary Fig. S1), but both were retained because they showed variation within a population. One case mussel (Kyle's Ford, October sample) influenced the shape of the ellipse for the normal distribution of the case mussels' metabolites and was retained because it belonged to the affected classification.

The robust PCA analysis indicated additional mussels were either good leverage points (strong indicators of morbidity signs) or orthogonal outliers—unrelated signs (Supplementary Fig. S2). No bad leverage points were detected—mussels with metabolite distributions well outside the normal distribution. Three mussels were identified as good leverage points, meaning that they followed the trends of the normal observations. Two mussels were identified as case mussels and a third mussel was a control. These mussels may show the natural variability within the population. Four mussels were orthogonal outliers—one from a location well upstream from the mortality. Another orthogonal outlier was the mussel with the highest viral load and lowest field condition. A third orthogonal outlier was the only mussel collected at Wallen Bend. The fourth orthogonal outlier was a case mussel that may have been misdiagnosed, based on low viral loads, or have been within the natural variability of the population sampled.

We removed the five August mussels because of the small number of samples. Inclusion of August samples would increase the complexity of the dataset and lead to higher potential misclassification (Smith *et al.*, 2014) without providing identification of differences between field diagnosis and temporal changes. The focus of this manuscript was the relationship between case and control and did not focus on the seasonality between a short period (August to October 2018).

Targeted analysis

We detected 92 targeted metabolites (Supplementary Table S2). Volcano plot analysis (Fig. 3) indicated 17 targeted metabolites that were associated with field assessments of clinical status based on the adjusted *P* value (<0.1) and FC (≥ 1). We identified five metabolic pathways in our targeted analysis, which differed between case and control mussels based on the enrichment factor and *P* value. These included arginine/proline metabolism, purine metabolism, tryptophan metabolism, glycerophospholipid metabolism and arginine biosynthesis. Of these, purine metabolism and glycerophospholipid metabolism were also detected in the untargeted pathway analysis (Table 1). The targeted biomarker analysis found 13 metabolites that were associated with field assessment based on AUC, adjusted *P* value and FC (Table 2). Of these, increased gamma-linolenic acid (GLA) and *N*-methyl-L-alanine were most indicative of case mussels (Fig. 2A and B), while adenine and inosine were the best indicators of control mussels (Fig. 2C and D).

Untargeted analysis

The volcano plot analysis was used to visualize metabolites indicative of field assessment (Fig. 3). We detected significant differences in 94 metabolites between case and control mussels. Twenty-two metabolic pathways were associated with field assessment (Table 1). Many of the impacted pathways were catabolic processes, such as degradation of amino acids and fatty acids. Eleven pathways were related to lipid and fatty acid metabolism. The untargeted biomarker analysis identified 22 metabolites as potential biomarkers (AUC >0.7, FC $\geq |1.5|$, $P \leq 0.05$; Supplementary Table S3). We used a more stringent FC and adjusted *P* value requirement for the untargeted biomarkers to select those metabolites with the greater potential to indicate disease.

Overall, the heat map showed a distinct separation of case and control mussels based on concentrations of 25 significant metabolites (Fig. 4). Seven of 25 (28%) mussels differed in overall metabolite concentrations between their classification of case or control. Two case mussels were categorized as controls and separated as a pair from other case mussels. Five control mussels were grouped with case mussels. Three of these clustered together and were collected from the same location in October. We created a heat map to verify the metabolites related to case vs. control were also not related to a temporal change between September and October (Supplementary Fig. S3). The Supplementary Fig. S3 heat map shows how metabolites are related to the temporal change during the sampling period.

Discussion

Targeted analysis identified 13 biomarkers, based on AUC, adjusted *P* value and FC criteria, and five pathways that differed between case and control groups (Table 2). Of the 13 biomarkers, inosine and adenosine, both biomarkers in the purine metabolism pathway, were the most robust indicators of the control group. Purine metabolism and the associated metabolites inosine, adenosine and adenine were less abundant in case mussels. Purine metabolism was indicated as significant in both the targeted and untargeted pathway

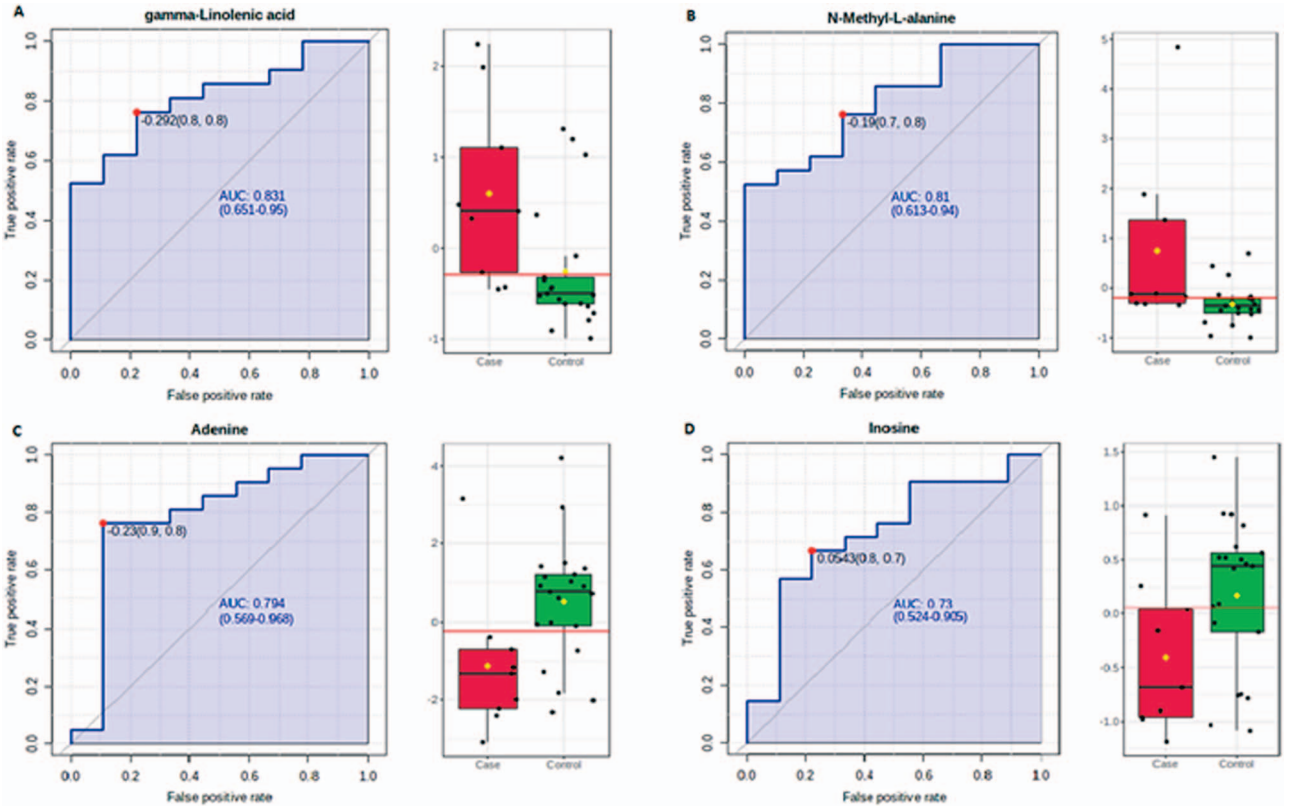

Figure 2: Four targeted metabolites identified as potential biomarkers based on AUC. GLA (A) and *N*-methyl-l-alanine (B) abundances were relatively greater in case vs. control mussels. Adenine (C) and inosine (D) abundances were slightly greater in control versus case mussels.

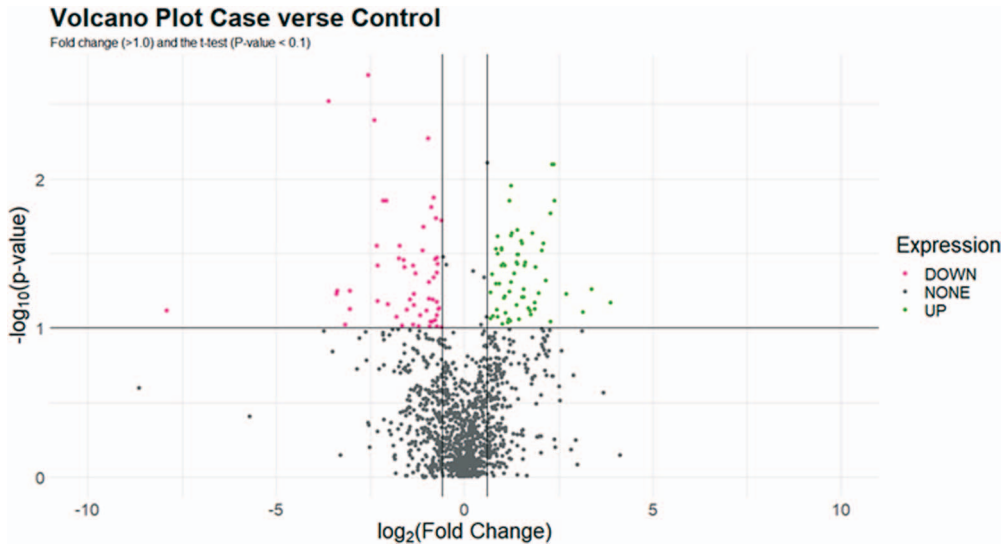

Figure 3: Volcano plots of both targeted and untargeted metabolites in hemolymph of pheasantshell (*O. pectorosa)* diagnosed as case or control. All metabolites were used in the volcano plot. Points in the upper left quadrant are metabolites with lower abundance in control mussels. Points in the upper right quadrant are metabolites with higher abundance in case mussels Metabolite differences between case and control mussels were deemed significant when FC (>1.0) and the *t* test ($P < 0.1$).

Table 2: Targeted metabolites identified as potential biomarkers

Metabolite name	HMDB ID	KEGG ID	Exact mass	Retention time (min)	AUC	log_2 FC	*P*
GLA	HMDB0003073	C06426	278.22	3.78	0.831	2.869	0.007
Methyl jasmonate	HMDB0036583	C11512	224.142	2.16	0.840	−1.922	0.003
Gamma-valerolactone	HMDB0029185	C02240	100.0522	2.96	0.771	−1.388	0.020
Hexylamine	HMDB0032323	C08306	101.121	5.49	0.750	−4.970	0.040
Betaine	HMDB0000043	C00719	117.0785	0.52	0.750	1.927	0.037
l-Homocystine	HMDB0000676	C01817	268.0557	0.47	0.772	1.149	0.085
Inosine	HMDB0000195	C00294	268.081	0.57	0.729	−1.731	0.054
l-Isoleucine	HMDB0000172	C00407	131.0944	0.52	0.729	−1.379	0.073
Adenosine	HMDB0000050	C00212	267.0975	0.59	0.722	−2.143	0.030
l-Palmitoylcarnitine	HMDB0000222	C02990	399.2375	1.41	0.715	1.557	0.054
Stachyose	HMDB0003553	C01613	666.4989	2.31	0.715	1.501	0.063
Caprylic acid	HMDB0000482	C06423	144.1147	3.52	0.708	−1.158	0.082
l-Valine	HMDB0000883	C00183	117.0791	0.48	0.701	1.928	0.087

The criteria for inclusion as a potential biomarker included AUC >0.7, $P < 0.1$, and log_2 FC >1.00. The *P* value cut off was selected to ensure weakly related biomarkers are retained for further investigation. Positive FC values indicate higher metabolite level in case mussels, while a negative FC value indicates higher metabolite level in the control mussels. HMDB ID in Human Metabolomic Database (https://hmdb.ca/) and KEGG ID in Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp).

analyses. Both adenine and inosine were categorized as "stable" metabolites in mussels in unperturbed Indiana streams (Waller *et al.*, 2023). Inosine and other nucleotide metabolites decreased during captivity of *A. plicata* (Roznere *et al.*, 2014). The shifts in purine pathways that were observed in our study and by Roznere et al. (2014) support the potential use of nucleotide metabolites as biomarkers of mussel health.

GLA was the biomarker most associated with case mussels. The untargeted analysis also found that the pathway for prostaglandin formation from di-homo gamma-linoleic acid was impacted. GLA is an omega-6 fatty acid and plays a role in maintaining membrane fluidity and in immune responses (Brett and Müller-Navarra, 1997; Filimonova *et al.*, 2016). GLA modulates immune function through conversion to di-homo-GLA and synthesis of prostaglandins, a class of eicosanoids. Eicosanoids are signaling molecules that can play a role in immune response to infectious agents (bacteria, fungus, virus) and inflammation processes. The higher presence of GLAs in case mussels may be indicative of inflammation or a mounting immune response, both of which underlie the potential significance of the GLA biomarker. GLA is also an abundant fatty acid in some cyanobacteria (Napolitano, 1999; Gugger *et al.*, 2002). Studies on zooplankton *Branchinella kugenumaensis* (Yang *et al.*, 2016) and golden mussel, *Limnoperna fortunei* (Mytilidae) (Hernando *et al.*, 2021), found GLA was a potential biomarker for *Microcystis aeruginosa* as a food source in primary consumers. The source of GLA in our mussels did not appear to be related to cyanobacteria ingestion. Metagenomic analysis of hemolymph from the same mussels used in the present study found cyanobacteria were associated with controls and were absent from moribund mussels (Richard *et al.*, 2021).

Other biomarkers that were elevated in case mussels included betaine, L-homocystine, palmitoylcarnitine and L-valine. Each of these is also part of one or more pathways that were significant in the untargeted pathway analysis. Betaine is an osmolyte and found in pathways for glycine, serine and threonine metabolism and glycerophospholipid metabolism. L-Homocystine is a metabolite of cysteine and methionine metabolism. Palmitoylcarnitine is a long-chain fatty acid ester of carnitine and functions to transfer fatty acids into the mitochondria for oxidation (i.e. carnitine shuttle pathway). These biomarkers and associated pathways indicate increased activity of catabolic pathways in case mussels, a potential response to a stressor.

The biomarker analysis also identified several metabolites that may be exogenous, (e.g. methyl jasmonate, stachyose and hexylamine). These metabolites may point to a xenobiotic exposure (Liu *et al.*, 2021), microbial activity (Aldridge and Rhee, 2014), food sources (Erban *et al.*, 2019) or potential misidentification of a similar compound (Alseekh *et al.*, 2021). For example, methyl jasmonate, which is derived from linolenic acid, could have been mistaken for a breakdown product of GLA. Alternatively, methyl jasmonate is a metabolite of microorganisms including bacteria discovered in sponges (Bibi *et al.*, 2020), a bacterium isolated from halophyte *Prosopis strombulifera* (Piccoli *et al.*, 2011), and fungus

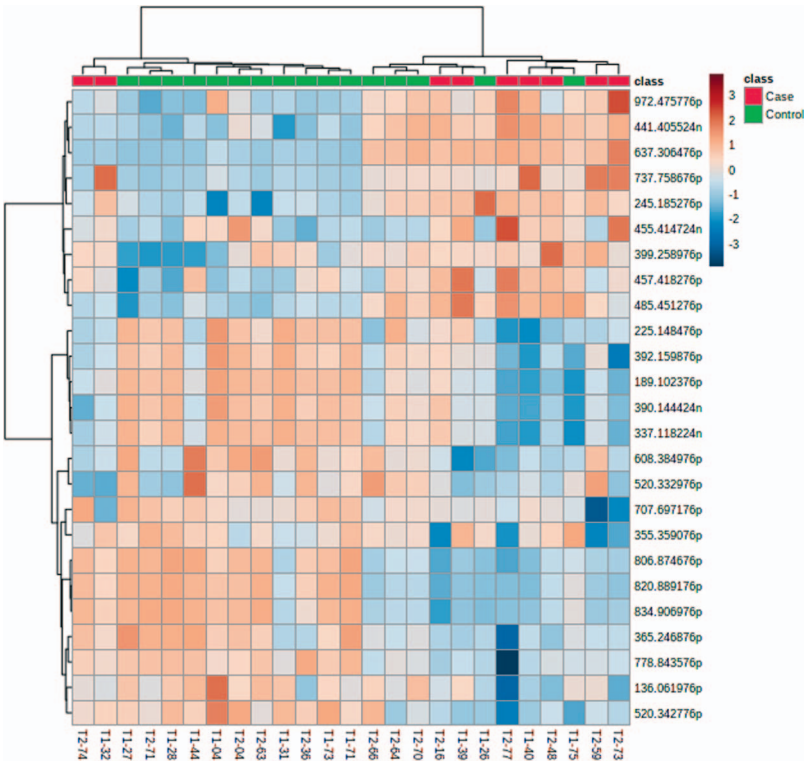

Figure 4: Heat map of 25 significant metabolites and hierarchical cluster of case and control pheasantshell (*O. pectorosa)* mussels by metabolite concentration. The horizontal axis is the unique mussel identification T1 = September, T2 = October sample. The vertical axis is the mass and mode of detection (p = positive, n = negative).

(Piccoli *et al.*, 2011; Eng *et al.*, 2021). Further work would be required to confirm the identification of these metabolites and to determine their biological significance in unionids. Even if unidentified, these biomarkers may have value for future health classification analyses.

Targeted pathway analysis found arginine biosynthesis and arginine/proline metabolism were different between case and control mussels. The majority of arginine in mollusk tissue is in the phosphagen form as phosphoarginine and is released to generate ATP, similar to creatine phosphate in mammals, in the initial stages of anaerobic metabolism (Bishop *et al.*, 1983). Arginine metabolism produces proline and polyamines, such as acetylputrescine (Supplementary Table S2), the latter of which was lower in abundance in case mussels (Supplementary Table S2). Roznere *et al.* (2014) reported decreased levels of polyamine metabolites when mussels were brought into captivity. Polyamines are important for cell division and growth, and decreased levels may indicate that resources are diverted away from these processes. The major role of arginine in anaerobic metabolism and the association with field assessment make it noteworthy for further investigation as a biomarker of health.

We used a traditional adjusted *P* value (<0.05), AUC and FC values in the untargeted biomarker analysis and found 22 additional unidentified metabolites that could be indicators of health classification (Supplementary Table S3). Although we did not identify these specific metabolites, the untargeted biomarkers were useful in construction and comparison of metabolomic profiles and pathway analysis of case and control mussels. Those specific metabolites that show the most potential as biomarkers can be identified using the exact molecular weight and retention time.

The pathway analysis distinguished trends and relationships between all detected metabolite signals. It also provided a broader, holistic view of the mussel's health status and metabolic changes. The untargeted pathway analysis found multiple pathways that differed by field-based clinical assessment. Generally, pathways associated with cases were catabolic and oxidative pathways (Table 1). Furthermore, 11 pathways related to lipid and fatty acid metabolism were greater in case mussels. As lipids are key sources of energy, the number of these pathways that were detected as affected in the untargeted analysis indicates case mussels were consuming energy resources at a higher rate than control mussels. A key outcome of the untargeted pathway analysis was identifying pathways to investigate in future targeted analyses. For example, glycerophospholipid and ubiquinone pathways differed between case and control mussels, and metabolites within these pathways may be selected for future targeted analysis.

Both are important for formation and maintenance of cell membranes. Glycerophospholipids are a major component of cell membranes and are critical for membrane stability as well as cell proliferation (Hu *et al.*, 2022). Glycerophospholipid metabolism may be altered in an immune or inflammatory response. Ubiquinone (coenzyme Q) has multiple roles in cellular metabolism and is an antioxidant important for maintaining membrane integrity (Turunen *et al.*, 2004). Determining shifts in specific metabolites within these pathways may provide insight on the mechanism of stress or disease that is contributing to mussel mortality.

Hierarchical clustering showed separation of case and control mussels but also indicated discrepancies with current methods to categorize mussel clinical status in the field. Heat map classification supported field assessment of 72% (18 of 25) of mussels in our study; however, 5 of 16 control mussels clustered with case mussels, while 2 of the 9 case mussels clustered with controls. The health status of a mussel in the field is most often based on behavioral response indicators, such as gaping valves, extended foot and slow response to probing, which vary widely and are highly subjective. For example, females that are gravid often move to the substrate surface and may be agape (Haag, 2012) and slow to respond, all behavioral signs of stress. In contrast, mussels may bury and die in place without outward signs of stress. Ten mussels in our study were gravid females, seven in the control group and three in the case group. Of these, three grouped with the other group in the cluster analysis (two control and one case, 30% misclassified), similar to the percentage for all mussels. Pheasantshell spawn in late summer and females become gravid starting in August and September (Ortmann, 1921). Gravid females may allocate energy substrates to larval development (Gustafson *et al.*, 2005; Hornbach *et al.*, 2019), resulting in a different metabolic profile relative to non-gravid females and adults. However, metabolomic analysis of two lampsiline species from three stable streams found considerable overlap in metabolite levels between gravid and non-gravid females, and sex-related differences were less important than site and species (Waller *et al.*, 2023).

Metabolite composition may not shift strongly in early, asymptomatic stages of a disease (Wagner *et al.*, 2015). Mussels that were field assessed as controls during active mortality may have been in this early stage and not yet showing physical or behavioral signs of disease. Increased sampling intensity and higher resolution field-based measures of clinical status may identify/detect a gradient of metabolite alterations that corresponds to the progression of disease.

Although few studies have followed seasonal changes in metabolites, we expect temporal variability in metabolite composition and pathways (Melvin *et al.*, 2018; Whipple *et al.*, 2021). We initially found differences between August and October control mussels in pathways for fatty acid biosynthesis and amino acid metabolism that may reflect shifts in diet. However, our sample size in August was limited and precluded further evaluation of temporal changes. Furthermore, viruses are also known to promote fatty acid synthesis and glycolytic metabolism, suggesting that these altered pathways could possibly be the result of viral infection (Sanchez and Lagunoff, 2015; Thaker *et al.*, 2019). Notably, several viruses were identified from moribund mussels during the autumnal mortality event (Richard *et al.*, 2020).

Metabolites in hemolymph are more transient and occur in lower concentration than those in other tissues. For example, metabolites in hemolymph were less detectable than in digestive gland, foot, adductor muscle, gill and mantle tissue using $^{1\text{ (not superscripted)}}$H NMR (Hurley-Sanders *et al.*, 2016). However, other researchers have successfully detected a range of metabolites in hemolymph using HPLC–MS and GC–MS analyses (e.g. Roznere *et al.*, 2014 and 2017; Nguyen *et al.*, 2019; Nguyen and Alfaro, 2020). Despite the limitations of hemolymph metabolite analysis, hemolymph collection is generally non-lethal and less invasive than foot or mantle biopsy, a key consideration when working with imperiled fauna. In addition, hemolymph is relatively sterile compared to other tissues and potentially less contaminated by exogenous metabolites. Overall, we found that hemolymph analysis uncovered metabolomic differences that supported field-based clinical assessments of most mussels in our study.

Conclusions

Determining the health status of freshwater mussels *in situ* is especially challenging as no defined and verified clinical signs of disease have been established, resulting in health assessments that are based on subjective behavioral observations. As used herein, metabolomics was a real-time quantitative assessment tool to supplement field diagnosis of mussel mortality. A definitive cause of mussel mortality in the Clinch River has not been verified, but both targeted and untargeted analysis detected metabolic differences among case and control mussels. The targeted biomarker analysis found 13 metabolites associated with field assessments of clinical status. Of these, increased GLA and *N*-methyl-L-alanine were most indicative of case mussels, while adenine and inosine were the best indicators of control mussels. Five pathways in the targeted analysis differed by clinical status; two of these, purine metabolism and glycerophospholipid metabolism, were also indicated in the untargeted analysis. In the untargeted analysis, 22 metabolic pathways were associated with clinical status.

Metabolomic analysis of hemolymph presents a relatively non-lethal, minimally invasive method to discern organismal status, which could be incorporated into monitoring programs for imperiled mussel populations. However, routine use of metabolomics is hindered by the limited database on freshwater mussel metabolism. Only a few studies have investigated metabolic shifts in response to specific stressors in freshwater mussels [e.g. captivity/starvation (Roznere *et al.*, 2014); relocation (Roznere *et al.*, 2017)]. To identify potential causes of disease, controlled studies that expose

mussels to different stressors and then characterize biomarkers and metabolomic pathway shifts associated with the stressor will be important. Temporal sampling of hemolymph metabolite levels within and across mussel populations will also be important for characterizing seasonal variation in metabolism. We investigated metabolite levels in the mussel species most affected by mortality in the Clinch River in 2018 (i.e. pheasantshell). Additional sampling of unaffected species may help discern differences in species resistance to stressors. Further understanding of interspecies variation, or similarity, in metabolism may lead to use of common species as surrogates for rare and imperiled mussel species. Conservation efforts for freshwater mussels have increased around the globe and would benefit from a variety of tools to assess the real-time health status of individual mussels to supplement long-term assessment tools (growth, recruitment), especially the ones that are non-lethal and can portend a stressor before mortality ensues.

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Author Contributions

J.C.R., E.L., D.L.W., S.K., J.G.P. and T.L.G. designed the study; J.C.R., R.A. and T.L.G. performed field work; J.G.P. and J.N.S. performed laboratory analysis; J.G.P., J.N.S. and D.L.W. analyzed and interpreted the data; all authors contributed to writing, reviewing and improving the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest. Any use of trade, firm or product names is for descriptive purposes only and does not imply endorsement by the US Government or the authors. The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the US Fish and Wildlife Service.

Ethics and Animal Welfare Statement

We obtained biological samples in accordance with all federal, state and local laws and policies.

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Data Availability

Data associated with the manuscript are available in the study by Putnam *et al.* (2022), "Metabolomic analysis of pheasantshell mussel (*O. pectorosa*; Order Unionida) from a mass mortality event in the Clinch River, Virginia and Tennessee, USA: U.S. Geological Survey data release", https://doi.org/10.5066/P9ZT9F3S.

Supplementary Material

Supplementary material is available at *Conservation Physiology* online.

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