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Metabolomics reveal physiological changes in mayfly larvae (*Neocloeon triangulifer*) at ecological upper thermal limits

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Abstract

Aquatic insects play critical roles in freshwater ecosystems and temperature is a fundamental driver of species performance and distributions. However, the physiological mechanisms that determine the thermal performance of species remain unclear. Here we used a metabolomics approach to gain insights into physiological changes associated with a short-term, sublethal thermal challenge in the mayfly *Neocloeon triangulifer* (Ephemeroptera: Baetidae). Larvae were subjected to a thermal ramp (from 22 to 30 °C at a rate of 1°C/h) and metabolomics analysis (both Nuclear Magnetic Resonance (NMR) Spectroscopy and Gas Chromatography coupled Time-of-Flight Mass Spectrometry (GC-TOF-MS)) indicated that processes related to energetics (sugar metabolism) and membrane stabilization primarily differentiated heat treated larvae from controls. Limited evidence of anaerobic metabolism was observed in the heat treated larvae at 30°C, a temperature that is chronically lethal to larvae.

Graphical Abstract

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Competing interests

The authors declare no competing financial interests in this work.

Author contributions

WP, JDS and SS conducted the metabolomics studies and data analyses, and wrote the methods section. HC and DBB conceived of the research and wrote the paper.

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Keywords

Aquatic insects; mayfly; thermal limits; metabolomics; temperature

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Introduction

Mayflies are aquatic insects that play important roles in freshwater food webs and are commonly used as ecological indicators in freshwater ecosystems. Despite their ecological importance and use in monitoring programs, mayfly physiology remains poorly studied because relative few species are amenable to laboratory rearing. *Neocloeon triangulifer* is a parthenogenetic species that is emerging as a useful laboratory model (Weaver et al., 2015) for understanding responses to environmental stressors such as contaminants (Conley et al., 2009; Conley et al., 2011; Xie and Buchwalter, 2011; Kim et al., 2012; Conley et al., 2013; Kunz et al., 2013; Conley et al., 2014; Wesner et al., 2014; Lopez et al., 2016), salinity stress (Johnson et al., 2014; Soucek and Dickinson, 2015; Struewing et al., 2015) and temperature (Sweeney and Vannote, 1984).

Many human activities including those that result in global climate change are threatening the freshwater environment (Carpenter et al., 1992; Durance and Ormerod, 2007; Vörösmarty et al., 2010; Issak and Rieman, 2013). Temperature is a fundamental driver of species performance (survivorship, metabolism, larval growth, timing of adult emergence and reproduction output) and distributions. Mayflies, like other insects generally follow the temperature size rule (TSR) (Atkinson, 1994), with longer development time, larger body size and more reproduction output at colder temperature and shorter development time, smaller body size and less reproduction output at warmer temperature (Sweeney and Vannote, 1984). The tradeoffs between development time and body size/individual reproduction output help determine niches and limits of a species. Unfortunately, thermal optima and ecological limits have not been experimentally characterized for many aquatic insects and the physiological mechanisms underlying these thermal effects remain unclear.

In their seminal work nearly 4 decades ago, Vannote and Sweeney (1980) articulated a bioenergetics perspective (Von Bertalanffy, 1960) for understanding thermal limits of aquatic insect species based largely on life history observations. They proposed that warming results in assimilated energy shifts from growth to maintenance, resulting in smaller body size and less reproduction output. More recently, the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis has been proposed as an explanation for thermal performance in all ectotherms (Pörtner, 2001; Pörtner, 2002; Pörtner et al., 2006; Pörtner, 2010). This perspective proposes that under acute thermal exposure, aerobic metabolism no longer supports energy demand beyond critical thermal limits (T_{crit}) due to the mismatch between oxygen demand and supply. However, compensated anaerobic metabolism postpones the death of the animals. The OCLTT hypothesis, however, has been questioned by scholars about its global applicability (Klok et al., 2004, Stevens et al., 2010, McCue and De Los Santos, 2013; Verberk et al., 2016a) – particularly in air breathing species (Klok et al., 2004, Stevens et al., 2010).

Recent work with aquatic insects has generally supported the OCLTT, however these studies strongly depended on exposing species to acute thermal challenge until they reach the critical thermal maximum (Ct_{max}) (Verberk and Bilton, 2011; Verberk and Bilton, 2013a; Verberk and Bilton, 2015). Ct_{max} is the point where an animal loses the ability to escape conditions that will lead to its death (Lutterschmidt and Hutchison, 1997), but Ct_{max} values reported for aquatic insects (e.g, Verberk et al., 2016b) do not generally occur in nature. While many studies show oxygen can modulate Ct_{max} , it can be easily assumed that sub-lethal thermal responses are also modulated by oxygen. However, Verberk et al (2016b) pointed out the importance to study whether oxygen availability affects both lethal and nonlethal thermal challenges. Kim et al (accepted) suggested that in *N. triangulifer* larvae, oxygen limitation may indeed play a significant role but only when larvae were warmed beyond their ecologically relevant thermal limit (30°C) up to their Ct_{max} (40°C) temperature. Since there is a wide gap between the ecological relevant thermal limit and the unrealistic Ct_{max} the physiological processes that occur under Ct_{max} conditions may not reflect processes that are important in limiting successful development, metamorphosis and reproduction of aquatic insects in nature. Therefore, studying the biochemical changes at the ecological relevant thermal limit rather than Ct_{max} may be better to help link physiological processes to life history outcomes such as development and reproduction.

In the last decade, metabolomic analyses have been widely used to study insect physiology (Snart et al., 2015). This approach offers physiological insights by comparing changes in the presence and abundance of numerous compounds in tissues or whole organisms. However, the metabolic changes and pathways being affected under short term sub-lethal thermal challenge in aquatic insects have not been largely explored. In this study, we ask the question whether pathways related to energy or anaerobic metabolism are affected at ecologically relevant thermal limit. We design the experiment to ramp mayfly *Neocloeon triangulifer* (Ephemeroptera: Baetidae) larvae at a rate of 1°C/hour from 22°C (a temperature associated with excellent survival and reproductive output) to 30 °C (a temperature that is chronically lethal to larvae based on life history survivorship data (Kim et al., accepted). We use both NMR and GC-TOF-MS techniques to observe and compare metabolite changes from samples taken at 22 °C and 30 °C

Material and methods

Mayfly husbandry for metabolomics studies

The parthenogenetic mayfly *N. triangulifer* (WCC-2 clone) were reared on the laboratory benchtop at room temperature (21–22 °C) using food (natural WCC periphyton) provided by Stroud Water Research Center (SWRC; Avondale, PA). American Society for Testing and Materials (ASTM) artificial soft water (ASW) was reconstituted from American Chemical Society (ACS) grade salts and deionized water as follows: 48 mg L⁻¹ NaHCO₃, 30 mg L⁻¹ CaSO₄•H₂O, 30 mg L⁻¹ MgSO₄, and 2 mg L⁻¹ KCl; pH 7.6 ± 0.4. For this study, equally sized mature larvae were deprived of food overnight and subjected to a gradual rise in temperature at a rate of 1 °C/h from 22 to 30 °C. Kim et al (accepted) showed gene expression studies with the same rate of ramping conditions but pushed the larvae further to 34°C. We decided to use the same ramping rate because we can refer gene expression to

metabolic changes. We collected samples at 30 °C because we are interested in exploring metabolic changes at the ecological thermal limit in this mayfly species. Control larvae were held at 22 °C for the duration of the experiment and all larvae had fasted for identical lengths of time. Thermally challenged larvae were struggling at 30°C, however there were no mortalities. Although starvation may have an effect on metabolic change, those that appear in both groups will eliminate when mapping the changes. Only the metabolites that were affected by thermal stress will stand out. At the end of the experiment, larvae were divided into three replicate groups per treatment (control and heat-challenged) and all were flash frozen in liquid nitrogen. A replicate consisted of 12–13 whole larvae combined to achieve a total sample biomass of 50 mg fresh weight.

¹H NMR metabolomics methodology

Sample preparation, data acquisition, and statistics were performed as previously described (Sumner et al., 2010; Banerjee et al., 2012; Church et al., 2014). Aliquots of 50–300mg of insect larvae were mixed with degassed 1:1 acetonitrile: water solution (50 mg tissue/mL), homogenized, and then centrifuged at 4 °C for 5 minutes at 14000 rcf. A volume of the homogenate corresponding to 40 mg insect larvae were taken and dried completely by vacuum centrifuge and were reconstituted by adding 630 µL of D₂O (Aldrich) and 70 µL of Chenomx ISTD solution (Chenomx, Edmonton, Alberta, Canada). The samples were vortexed and centrifuged at 14000rcf for 2 min and a 650 µL aliquot of sample was transferred into 5mm NMR tubes. ¹H NMR spectra of insect extract samples were acquired on a Bruker Avance III 950 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using a 1D NOESY pre-saturation pulse sequence [noesypr1d, Beckonert et al., 2007] and ambient temperature of 25 °C, with a 2 s relaxation delay, 100 ms mixing time, and water suppression during the relaxation and mixing periods. Spectra were zero filled, and Fourier transformed after exponential multiplication with line broadening factor of 0.5. Phase and baseline of the spectra were manually corrected for each spectrum. Spectra were referenced internally to the DSS signal at δ = 0 ppm. The quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers. NMR bins (0.10–9.60 ppm) were made using ACD NMR Processor 12.0 software (ACD Labs Inc., Toronto, Canada) after excluding DSS, water (4.70–4.80 ppm), and Imidazole (7.25–7.35 ppm) using intelligent bucket integration with a 0.04 ppm bucket width with 50% looseness. Integrals of each of the bins were normalized to total integral of each of the spectrum.

GC-TOF-MS metabolomics methodology

A 300 µL aliquot of the solution used for the NMR analysis was exchanged with water and then dried down by vacuum centrifuge. The dried samples were derivatized for GC-TOF-MS analysis by the two-step derivatization method and analyzed on Leco Pegasus 4D time-of-flight mass spectrometer (TOF-MS) (Fiehn et al., 2008; Kind et al., 2009). Compounds were separated and characterized on a Restek Rxi-5Sil MS capillary column (30 m × .25 mm × .25 µm with an additional 10m integrated guard column). Acquisition parameters were similar to those established by Fiehn et al (2008). Briefly, 0.5 µl of sample was injected into the GC (at 250 °C injector temperature) in splitless mode with 25 seconds splitless time. An Agilent 6890 gas chromatograph (Santa Clara, CA) was used with a 30 m long, 0.25 mm i.d.

Rxi5Sil-MS column with 0.25 μm 5% diphenyl film and an additional 10 m integrated guard column (Restek, Bellefonte PA). Chromatography was performed at a constant flow of 1 ml/min, ramping the oven temperature from 50 $^{\circ}\text{C}$ to 330 $^{\circ}\text{C}$ with 20 $^{\circ}\text{C}/\text{min}$ ramp rate. Mass spectrometry was performed using a Leco Pegasus 4D TOF-MS with a 280 $^{\circ}\text{C}$ transfer line temperature, electron ionization at -70 V and an ion source temperature of 250 $^{\circ}\text{C}$. Mass spectra were acquired from m/z 85–500 at 20 spectra s^{-1} and 1850 V detector voltage.

Metabolomic data analysis

Following GC-TOF-MS data acquisition, data files were processed for deconvolution by ChromatTOF software (Leco, St. Joseph, MI) and transferred to BinBase for peak retention index calculations, spectral identification, and generation of a table of peak identifications and intensities (Skogerson et al., 2011). Data were normalized to the sum intensities of all known metabolites and used for further multivariate and other statistical analyses. Normalized NMR and GC-TOF-MS data were used for multivariate data analysis using SIMCA 13.0 (Umetrics, Sweden). Data were mean centered and Pareto scaled and principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed. Variable influence on projections (VIP) plots in OPLS-DA was used for identifying the NMR bins or metabolites (GC-TOF-MS) that were important for the separation of the study groups. The NMR bins or metabolites (GC-TOF-MS) that had a VIP ≥ 1.0 with a jack-knife confidence interval that did not include 0 were determined to be important for differentiating the study groups. The VIP statistic summarizes the importance of the NMR bin or the metabolites (GC-TOF-MS) in differentiating the phenotypic groups (Eriksson et al., 2013). All models used a 7-fold cross-validation to assess the predictive variation of the model (Q^2) (Eriksson et al., 2013). Important NMR bins were library matched to metabolites using Chenomx NMR Suite 7.5 Professional software (Chenomx, Edmonton, Alberta, Canada). Possible pathways involved in sub-lethal thermal challenge were evaluated by the pathway analysis using MetaboAnalyst (<http://www.metaboanalyst.ca/>).

Results and discussion

Multivariate data analysis (OPLS-DA) suggests that metabolites found in mayfly larvae subjected to a gradual and continuous 8 $^{\circ}\text{C}$ thermal rise in temperature from 22 to 30 $^{\circ}\text{C}$ at a rate of 1 $^{\circ}\text{C}/\text{h}$ were clearly differentiated from control larvae held at 22 $^{\circ}\text{C}$ (Fig. 1). The metabolites (GC-TOF-MS and NMR) that are deemed to be important for differentiating temperature challenged larvae are shown in Table 1. Results of GC-TOF-MS analysis identified a total of 24 metabolites that differentiated from thermally challenged group to control group. Among these identified metabolites, the abundance of 7 metabolites increased and 17 metabolites decreased in the thermally challenged group (Table 1) when compared to the control group. Results from NMR metabolomics showed an increase of 12 metabolites and a decrease in 22 metabolites in the thermally challenged group (Table 1) when compared to the control group. These metabolites can be categorized but not limited to groups of sugars and polyols, lipids and hydrocarbons, amino acids, and DNA/RNA synthesis/degradation. Note that some metabolites (e.g. Leucine, Valine) feature both as increasing and decreasing metabolites. We used whole larvae from this study and it may be a result of

different biochemical processes undergoing in different tissue. It is also difficult to definitively assign an NMR chemical shift and a MS m/z value to a specific metabolite. Therefore the contradicting metabolites are likely due to the lack of correlation in between the two analytical methods. However, it is necessary to conduct and combine both NMR and mass spectral data because of the diverse and complementary information it provides (Marshall and Powers, 2017). We tend interpret metabolites that either increased or decreased and not discuss in depth of the contradicting metabolites.

Metabolites that decreased in response to thermal challenge (Table 1) include those related to glucose/energy metabolism (glucose, glucose 6 phosphate, gluconic acid and gluconic acid lactone) suggesting that exposure to thermal rise appears to strongly influence energetic pathways and reduce available glucose stores. We found malic acid, an intermediate metabolite in the TCA cycle increased in response to thermal challenge. The disruption of TCA cycle under thermal challenge was also found in other published research (Michaud et al., 2008; Verberk et al., 2013b). This perturbation indicates glycolysis as an important energy source under thermal challenge for maintenance as oxidative phosphorylation from TCA cycle was contributing less energy. However, decreased pantothenic acid, a precursor for coenzyme A (CoA), suggests pathways other than glycolysis (i.e. beta oxidation) were also involved under sub-lethal thermal challenge for energy maintenance. This is supported by the changes of lipids and lipid transport-related metabolites (myristic acid, phosphocholine, ethanolamine and carnitine) found in thermally challenged larvae. These lipid and lipid transport-related metabolites are involved in energy storage, energy production as well as membrane stability. Therefore, the results suggest mayfly larvae under sublethal thermal challenge rely on at least 2 different energy sources (glycolysis and beta-oxidation) to generate enough energy for maintenance. Thus, we speculate that once glucose stores in *N. triangulifer* are depleted the larvae will be forced to shift to alternative energy sources such as lipids and amino acids.

Additionally, we found several metabolite changes (spermidine, adenosine, malate, aspartate and uridine) that indicate lower energy status in thermally challenged larvae. Spermidine is crucial for many roles including cell proliferation, differentiation, survival and DNA stability (Eisenberg et al., 2009, Bacharach, 2010). The increase of spermidine suggests protection from oxidative damage (increase in xanthine, decrease in b-alanine and taurine). Studies show that spermidine alleviates oxidative stress in insect *Bombyx mori* (Yerra et al., 2016) and increases stress resistance in yeast (heat and hydrogen peroxide), flies (paraquat and hydrogen peroxide) and mouse (hydrogen peroxide) (Rider et al., 2007; Eisenberg et al., 2009; Minois et al., 2012). Adenosine is involved in energy transfer and signal transduction. Sacktor and Cochran (1957) found that in addition to hydrolysis of ATP to ADP, the hydrolysis of UTP to release the stored chemical energy involved splitting all three phosphates, resulting in free uridine. The lower energy status found in thermally challenged larvae can be results of either increased maintenance costs or lower total energy budget due to disruption of TCA cycle. It is possible that this compromised energetic status contributes to the TSR. Larvae reared at higher temperatures either have lower energy budget to start with, or will experience a relatively larger cost of maintenance. However, in order to truly link the physiological processes to life history outcomes, it will be necessary to conduct similar metabolomics studies after longer term, chronic thermal exposures.

Although the OCLTT hypothesis has been generally supported in aquatic insects, Kim et al (accepted) tested the hypothesis by measuring oxygen consumption and gene expression in *N. triangulifer*. Kim et al suggested that oxygen limitation may indeed play a significant role but only when *N. triangulifer* larvae were warmed beyond their ecologically relevant thermal limit (30°C) up to their $C_{t_{max}}$ temperature. Although we found increased lactate in our thermally challenged larvae, we note that we used whole mayfly larvae for metabolomics studies. The increased lactate may indicate that not all tissues received adequate oxygen supply and some anaerobic metabolism was occurring in the larvae. Takahashi and coworkers (Takahashi et al., 2000) showed that rat lung fibroblasts cultured under hypoxia elevates hydroxyproline, an indicator of prolyl hydroxylation. We found no elevation of hydroxyproline in our heat treated mayfly larvae, and Kim et al (accepted) showed no increase in expression of the prolyl hydroxylase gene EGL-9 in response to thermal stress (though it was responsive to hypoxia). This suggests that despite the increase of lactate, other evidence of systemic hypoxia was not observed.

The MetaboAnalyst pathway analysis (Fig. 2) is a qualitative assessment used to identify metabolic pathway disruption (though it does not predict whether a pathway is up or down regulated). From these analysis, the top pathways impacted by thermal challenge were biosynthetic pathways for valine, isoleucine, and leucine (L-threonine, L-isoleucine, L-leucine, L-valine), histidine metabolism (L-histidine), phenylalanine metabolism (L-phenylalanine), glycine, serine, and threonine metabolism (glycine, L-serine, L-threonine, sarcosine, glyceric acid), phenylalanine, tyrosine, and tryptophan biosynthesis (L-phenylalanine), taurine and hypotaurine metabolism (taurine), beta-alanine metabolism (beta-alanine, L-aspartic acid, spermidine), methane metabolism (L-serine, glycine), alanine, aspartate, and glutamate metabolism (L-glutamine, L-aspartic acid, L-alanine), glycerolipid metabolism (glycerol, glyceric acid), arginine and proline metabolism (L-arginine, L-proline, hydroxyproline, spermidine, L-aspartic acid, L-glutamine).

Most of these pathways are in the amino acid metabolism category and many of the products eventually enter and become involved in energy metabolism (glycolysis/TCA cycle), i.e. serine, which was found less abundant after thermal challenge, is synthesized via a pyruvate or phosphoglycerate precursor in glycolysis.

Collectively, the metabolomics data show that the physiological status of mayfly larvae is indeed affected by short-term, sub lethal thermal challenge. These data indicated that thermal challenge greatly influences energetic pathways (including reduced available glucose stores), the need to stabilize membranes and only show limited evidence of anaerobic metabolism. The future research goal is to study metabolomics in mayflies reared long term (from eggs to close to the end of larval stage) across different static temperature to identify potential biomarkers of chronic thermal stress.

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Summary Statement

Metabolomic analysis of mayfly larvae (*N. triangulifer*) subjected to short-term, sublethal thermal challenge indicates that an ecologically relevant thermal challenge greatly influences energetic pathways.

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Highlights

- Effects of short-term, sublethal thermal challenge on *Neocloeon triangulifer* were examined.
- Metabolomics data indicate thermal challenge greatly influences energetic pathways.
- Limited evidence of anaerobic metabolism were found in thermally challenged mayflies.

Fig. 1. Scores plots of OPLS-DA of binned NMR data (left panel) and GC-TOF-MS data (right panel)

The score plots presents the predicted scores vs orthogonal scores with x axis showing variation between groups and y axis showing variation within group. Both plots indicate the metabolomics profiles of the heat treated larvae ($n = 3$) differ from control larvae ($n = 3$).

Model Statistics for NMR data (left panel) [$R^2X = 0.97$; $R^2Y = 1.00$; $Q^2 = 0.99$]; Model Statistics for GC-TOF-MS data (right panel) [$R^2X = 0.83$; $R^2Y = 1.00$; $Q^2 = 1.00$].

Figure 2. Pathway analysis of short-term thermal challenged mayfly larvae

The $-\log(p\text{-value})$ represents the enrichment score. The impact score (0–1) indicates the pathway topological importance of the metabolites. The color and size of each circle is based on p values and pathway impact values, respectively. Darker color and larger circle suggest smaller p values and higher pathway impact values. a. Valine, isoleucine, and leucine biosynthesis. b. Histidine metabolism. c. Phenylalanine metabolism. d. Glycine, serine, and threonine metabolism. e. Phenylalanine, tyrosine, and tryptophan biosynthesis. f. Taurine and hypotaurine metabolism. g. beta-Alanine metabolism. h. Methane metabolism. i. Alanine, aspartate, and glutamate metabolism. j. Glycerolipid metabolism. k. Arginine and

proline metabolism. l. Aminoacyl-tRNA biosynthesis. m. Galactose metabolism. o. Starch and sucrose metabolism. n. Purine metabolism. p. Glyoxylate and dicarboxylate metabolism.

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Table 1

List of metabolites affected by thermal challenge

GCMS metabolites that were identified by the supervised multivariate analysis (OPLS-DA) discriminated heat treated mayfly larvae from control larvae as being important for differentiating heat treated larvae metabolomics profiles from control larvae profiles. The NMR bins were identified by the supervised multivariate analysis (OPLS-DA) through Chemomx library matching as being important for differentiating heat treated larvae metabolomics profiles from control larvae profiles. Bins can contain more than one metabolite. Positive fold change means that the bin intensity or the intensity of the GCMS metabolite was higher in the thermally stressed group than in the control group.

Decreased Metabolites	VIP	Fold Change	method	Increased Metabolites	VIP	Fold Change	method
O-Phosphocholine, Arginine, Betaine, Carnitine, Histidine, Taurine, TMAO	3.8	-1.1	NMR	Lipoproteins (4 bins)	1.1-3.5	1.1	NMR
Carnitine, Proline, Taurine	3.2	-1.2	NMR	Lysine (2 bins)	2.0-2.8	1.0-1.1	NMR
Isoleucine, Leucine	3.0	-1.1	NMR	Arginine, Leucine, Lysine	2.2	1.1	NMR
Serine	2.1	-1.0	NMR	2-Hydroxyvalerate, Unknown	1.9	1.1	NMR
Glycine	2.0	-1.1	NMR	3-Hydroxykinurenine, Lactate, Proline	1.9	1.1	NMR
L-Aspartic acid	1.9	-2.0	GC-MS	Lipoproteins, 2-Hydroxyvalerate	1.8	1.0	NMR
Glucose-6-phosphate	1.7-1.9	-1.7	GC-MS	Xanthine	1.7	1.3	GC-MS
N-Acetyl-L-leucine	1.9	-1.3	GC-MS	Adenosine	1.5	3.4	GC-MS
Gluconic acid	1.9	-1.3	GC-MS	N-Acetyltyrosine	1.5	1.1	NMR
Glycerol	1.9	-1.1	NMR	Threonine	1.5	1.0	NMR
Arabitol	1.8	-1.7	GC-MS	Malic acid	1.4	1.3	GC-MS
Allo-inositol	1.8	-1.4	GC-MS	Adenine	1.3	1.3	GC-MS
Mannitol	1.8	-1.4	GC-MS	Heptadecanoic acid	1.3	1.2	GC-MS
Glucose	1.1-1.8	-1.3 (-1.1)	GC-MS	2-Hydroxyglutarate	1.3	1.1	NMR
Pantothenic acid	1.8	-1.3	GC-MS	Valine	1.3	1.0	NMR
Ethanolamine	1.7	-1.4	GC-MS	Spermidine	1.2	1.3	GC-MS
Myristic acid	1.6	-1.3	GC-MS	N-Acetyltyrosine, Inosine	1.2	1.0	NMR
trans-4-Hydroxyproline	1.6	-1.3	GC-MS	Oleic acid	1.1	1.6	GC-MS
Sugars	1.6	-1.3	NMR	N-Acetyltyrosine	1.0	1.0	NMR
Glycerol, Valine	1.6	-1.1	NMR				
Gluconic acid lactone	1.5	-1.4	GC-MS				
Glyceric acid	1.5	-1.3	GC-MS				

Decreased Metabolites	VIP	Fold Change	method	Increased Metabolites	VIP	Fold Change	method
Phenylalanine	1.5	-1.1	NMR				
β -alanine	1.4	-1.3	GC-MS				
Lipids, Sarcosine	1.4	-1.1	NMR				
Nicotinic acid	1.3	-1.4	GC-MS				
Alanine, Arginine, Glutamine	1.3	-1.1	NMR				
Uridine	1.2	-1.4	GC-MS				
Proline	1.2	-1.1	NMR				
3-Hydroxykinurenine	1.0	-1.2	NMR				