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METABOLITE PROFILES IN STARVED *DIPOREIA* SPP. USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) BASED METABOLOMICS

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ABSTRACT

The holarctic amphipod *Diporeia* spp. was historically the most abundant benthic macroinvertebrate in the offshore region of the Laurentian Great Lakes basin. However, since the 1990's, the numbers of *Diporeia* have declined precipitously throughout the region. Competition for food with introduced dreissenid mussels may be partly to blame for this decline. Thus, a better understanding of how *Diporeia* responds and adjust to starvation is needed. For this purpose, we used liquid chromatography (LC) coupled with time-of-flight mass spectrometry (TOF-MS) to study the metabolite profiles of *Diporeia* during starvation. *Diporeia* were collected from Lake Michigan, brought to the laboratory and starved for up to 60 days. During the starvation period, metabolite levels were determined at 12-day intervals and compared to those of day 0. Principal component and cluster analyses revealed differential abundance of metabolite profiles across groups. Significantly down-regulated metabolites included polyunsaturated fatty acids, phospholipids, and amino acids and their derivatives. Overall, starved organisms relied predominantly on glycerophospolipid metabolism and protein based catabolism for energy production. This research demonstrates that LC-MS based metabolomics can be used to assess physiological status and has shown that unique metabolite profiles are distinguishable over several weeks of starvation in this freshwater amphipod. More importantly, these unique metabolites could be used to gain insights into the underlying cause(s) of *Diporeia*'s decline in the Laurentian Great Lakes.

KEY WORDS: *Diporeia*, Great Lakes environment, lipids, liquid chromatography, mass spectrometry, metabolomics, starvation

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INTRODUCTION

Diporeia spp., holarctic amphipods, were historically the most abundant benthic macroinvertebrate (more than 70% of the total benthic biomass) in the offshore region of the Laurentian Great Lakes of North America (Cook and Johnson, 1974; Nalepa, 1989). Due to their high abundance and rich lipid content, *Diporeia* have been an important energy source for a number of fish species, including lake whitefish (*Coregonus clupeaformis*), deepwater sculpin (*Myoxocephalus thompsoni*), and alewife (*Alosa pseudoharengus*). However, since the 1990s, coincident with the introduction and spread of dreissenid mussels (zebra, *Dreissena polymorpha*; and quagga, *D. bugensis*), populations of *Diporeia* have declined in all of the Great Lakes except Lake Superior (Nalepa et al., 1998, 2005; Dermott, 2001; Lozano et al., 2001).

Diporeia are benthic detritivores and consume settling organic material, such as diatoms, from the lake bottom (Marzolf, 1965; Johnson, 1987). Filter feeding of the water column by dreissenids has substantially decreased the amount of settling organic material to lake sediments (Vanderploeg et al., 2002; Nalepa et al., 2006). Thus, it is plausible that dreissenids are competing with *Diporeia* for the same food source, and that reduced food availability has contributed to *Diporeia* decline via starvation (Brett and Muller-Navarra, 1997; Nalepa et al., 2005; Watkins et al., 2007; Sundelin et al., 2008).

Little is known about the physiology of *Diporeia* during starvation. Previous studies have found no significant changes in total body lipids of *Diporeia* after being starved for a month (Gauvin et al., 1989). Starvation of an European amphipod species, *Monoporeia affinis*, led to predominantly lipid-based energy metabolism (Lehtonen, 1994). However, to our knowledge, no other published studies have evaluated how amphipod metabolic profiles respond to starvation. It is likely that the metabolic expression pattern of starved amphipods will differ from those of fed animals. Research focused on the effects of prolonged food deprivation in *Diporeia* is important because it will provide useful information on how starvation affects energy metabolism and other

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physiological processes. Moreover, development of a "starvation specific metabolic profile" may be useful in the health assessment of feral individuals from areas experiencing declines.

Metabolomics refers to the identification and quantification of metabolites within a cell, organ, or organism (Lin et al., 2006; Goodacre, 2007; Bundy et al., 2009). Unlike the "targeted approach" of earlier studies, metabolomics today is a "discovery"-driven science applying a shotgun approach for a holistic evaluation of hundreds of metabolites, i.e., without a priori selection, responding to differential environmental stimuli (Nicholson et al., 2002; Dunn and Ellis, 2005). Various platforms for metabolite identification have been developed including, Nuclear Magnetic Resonance (NMR) spectroscopy, Mass Spectrometry-based Gas Chromatography (MS-GC) and Liquid Chromatography (LC) (Sepúlveda et al., 2011). Compared to standard genomics or proteomics approaches, metabolomics offers a reduced complexity in interpretation of biological data because many metabolites share structural similarity and functional role across different taxa (Bino et al., 2004; Dunn and Ellis, 2005; Wishart, 2005).

Because of its high sensitivity and throughput potential, LC-MS is becoming a popular choice in metabolomics research. Indeed, a number of researchers have implemented LC-MS for metabolomic studies in yeast (Allen et al., 2003), plants (Tolstikov et al., 2003; De Vos et al., 2007), mice (Griffin, 2006), and aquatic invertebrates including *Diporeia* (Ralston-Hooper et al., 2008, 2011).

Environmental metabolomics can be used to study the impacts of environmental stressors in natural populations. In fish, for example, metabolomics has been successfully applied to 1) investigate effects of pollutants (Samuelsson and Larsson, 2008); 2) understand tumor development (Stentiford et al., 2005); 3) estimate the prevalence of pathogens (Solanky et al., 2005); and 4) study stress response (Lin et al., 2006). Invertebrates have also been used in studies evaluating changes in metabolite profiles after exposure to pollutants (Bundy et al., 2009; Ralston-Hooper et al., 2011). However, while the scope of applications is quite broad, the use of metabolomics to investigate invertebrate responses to environmental stressors has remained rather limited, in particular for non-model organisms like *Diporeia*.

In this paper, we test the use of LC-MS based metabolomics to study the physiological changes in *Diporeia* during starvation. We hypothesize that prolonged starvation of *Diporeia* will result in a decrease in the abundance of molecules derived from lipid- and protein-based metabolism.

Materials and Methods

Study Animals

Diporeia were collected in June, 2008 at site C-5 located in southern Lake Michigan (157 m depth; 42′49.00″N, 86′50.00″W) (Nalepa et al., 2008). Organisms were collected from the sediment using a Ponar grab (0.23 × 0.23 m opening with 500 μm) followed by washing the sediments through a screen with 0.5 mm mesh openings. Animals were then placed in 1 L Nalgene bottles filled with pre-chilled (4°C) lake water. Live specimens were transported back to the laboratory inside coolers on wet ice at 4°C. Intact sediment from the same site was placed in coolers and kept cool during transport. Upon arrival, random samples of animals were flash-frozen for later analysis of metabolomic profiles (these were the "Day 0" organisms, see below) and the remaining animals were allowed to acclimate at 4°C for 48 h before the start of the experiments.

Study Design

Organisms from day 36 were lost during sample analyses. A total of 10 live animals were flash frozen in liquid nitrogen prior to the initiation of the starvation trial, i.e., after being held for 48 hours. This group was designated as "Day 0" and served as the control, i.e., provided baseline metabolite comparisons, for the study. The experimental setup consisted of six replicates of 1 L Pyrex© glass beakers containing 50 g (2 cm) of autoclaved Lake Michigan sediment and 700 mL reconstituted moderately hard water (RHW) (Ralston-Hooper et al., 2008). The acclimated animals were randomly placed in each replicate (10 individuals per replicate). The entire experiment was housed inside a walk-in cooler (at 4°C) and in perennial darkness to simulate deep lake bottom conditions. Throughout the length of the study (60 days (d)) no food was provided. Sediment was sieved to remove any large debris and then autoclaved to remove any organic material that could serve as food source. Every 12 d, one live, active adult was randomly collected from each replicate for a total of 4-6 animals per time period and flash frozen for subsequent metabolomic analyses.

Sample Preparation

All reagents used were of analytical grade (Sigma Aldrich, St. Louis, MO, USA, and Regis 122 Technologies, Morton Grove, IL, USA). Single organisms (wet weight ranging from 0.0167 g to 0.0055 g) were prepared separately for metabolic profiling. Frozen specimens were thawed slowly at 4°C and placed in 300 μL chilled methanol and 150 μL molecular grade water (Milli Q). Specimens were then homogenized for 20 s using a 7 mm × 95 mm sawtooth stainless steel generator probe (Omni International, Marietta, GA, USA) and the homogenate was placed in a sonicator bath for 3 min to ensure uniform mixing. Next, 450 μL chilled chloroform was added to the solution and vortexed for 2 min before placing it on wet ice for an additional 10 min. The chilled solution was centrifuged at 3000 *g* for 20 min to separate the two phases (methanol and chloroform). The phases were separated, transferred to new vials and dried at 45°C using a Savant SPD 131DDA SpeedVac concentrator (Thermo Electron Corporation Milford, MA, USA). The dried chloroform extracted sample pellet (non-polar metabolites) was resuspended in a mobile phase solution (50% water, 25% methanol, 25% acetonitrile and 0.1% piperidine) and transferred to auto-sampling vials for LC/TOF-MS analysis.

Instrumental Conditions

Non-polar extracts were analyzed using reverse-phase liquid chromatography. An Agilent 1100 (Agilent technologies, Santa Clara, CA, USA) platform with a zorbax-C8 column (2.1 × 150 mm, 5 μm) (Agilent technologies, Santa Clara, CA, USA) was used for the separation. Each sample (10 μL) was loaded in the column with a flow rate of

300 μL/min. Two separate mobile phases, A (water + 0.1% piperidine) and B (50 : 50 v/v acetonitrile : methanol + 0.1% piperidine) were passed though the column according to the following gradient system: at time 0, 50% B for 5 min, gradually peaked up to 95% B for 20 min, maintained at 95% B for 20 min, gradually downgraded to 50% B for 5 min, and finally retained that stage for 10 min. Using piperidine in the mobile phase has helped increase the sensitivity limits of phospholipids in negative electrospray ionization (ESI) mode (Shui et al., 2007). After separation, the non-polar metabolites were subjected to MS analysis using TOF in negative electrospray ionization mode (Agilent G6200 series LC/MSD TOF). The instrumental parameters were set as follows: capillary voltage 3500 V, flow rate of desolvation gas 9.0 L/h, desolvation temperature 350°C, nebulizer pressure 40 psi, fragmentor voltage 175 V, skimmer voltage 65 V, and 250 V of octapole RF (Resonant Frequency). The effective mass range scanned was 100-1800 (da) with a scan rate of 1 spectrum/second. Agilent Masshunter (version 1.03) was used for collection and processing of sample spectra.

Data Processing

Processing of LC-MS spectra consisted of peak identification, alignment, merging of common peaks and data normalization using two in-house software packages, XMASS© and XAlign© (Zhang et al., 2005). Constant mean normalization was applied assuming equal intensity of total ion current (TIC) across all the samples compared. Based on the mass-over-charge ratio (m/z values), the potential identity of individual metabolites was identified by searching the Human Metabolome Database (HMDB) in negative ion mode (Wishart et al., 2007). A subset of metabolites was further validated by comparing their retention times with commercial standards. These included: glycerophosphocholine (Sigma Aldrich, St. Louis, MO, USA), L-methionine (ChemService, West Chester, PA, USA), Phosphatidylcholine (PC), and Lysophosphatidylcholine (LysoPC) (Avanti Polar Lipids, Alabaster, AL, USA).

Statistical Analysis

Normalized LC-MS data were analyzed using R® (version: v. 2.9.2, R Foundation for Statistical Computing, Vienna, Austria). Unsupervised classification techniques such as Principal Component Analysis (PCA) and different types (Ward, DIANA) of hierarchical cluster analyses were also performed to discover any underlying structure in the data. The Ward-based clustering is a form of agglomerative method which takes into account the variance structure of the data set and assigns each sample to a cluster. Conversely, DIANA is a divisive method of clustering that iteratively splits a large cluster with all the samples into two clusters based on their dissimilarity until there is only a single sample left in each terminal leaf. We also performed two-sample t-tests to compare metabolite abundance between different lengths of starvation. To control the error rate in the detection of significant peaks during simultaneous testing of multiple hypotheses, a false discovery rate (FDR) adjusted p-value criteria (<0.05) was implemented (Benjamini and Hochberg, 1995). Volcano plots were used to present the overall expression pattern of *Diporeia*'s metabolome for each group. Each point in the volcano plot represents a single metabolite with the y- and x-axis representing the p-value and fold change of that metabolite, respectively. Based on adjusted p-values (<0.05), a group of metabolites was selected as most significantly altered during starvation.

Results

Results of Statistical Analyses

Principal component and unsupervised hierarchical cluster analyses revealed distinctly different levels of metabolites across control and experimental groups (Figs. 1 and 2). In the PCA analysis, the first two principal components contributed 64% of the variation in the data. The loadings of the principal components revealed no overwhelming contribution of any single metabolite to the principal components. Trimethyllysine and 12,13-epoxy-11-oxo-trans-9 octadecenoic acid (12(13)Ep-9-KODE, a derivative of linoleic acid metabolism) contributed the most on the loadings of the first and second principal component, respectively. Interestingly, the metabolome expression profile of organisms varied temporally throughout the length of food deprivation, and unsupervised clustering results varied slightly between the two methods (Fig. 2) with both methods showing a high degree of consistency. In both analyses, the metabolic profiles of freshly collected animals (day 0) were less similar relative to starved ones regardless of length of starvation (12-60 d). In these unsupervised analyses, the "metabolic profile" of organisms varied temporally during the period of food deprivation.

Metabolites whose level varied with the length of starvation are depicted in Fig. 3 in the form of volcano plots. Within each starvation treatment, a group of 16 metabolites were down-regulated (~2-8-fold) and one metabolite was

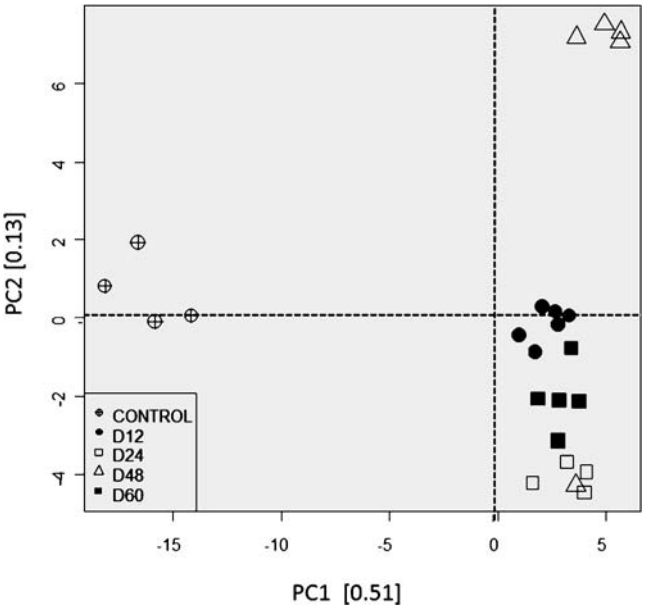

Fig. 1. PCA plot indicating temporal changes in the metabolome profile of *Diporeia* under starvation. Each point represents the projection of the metabolome levels of a single organism along principle components one and two. [Value] indicates the proportion of total variation explained by each principle component.

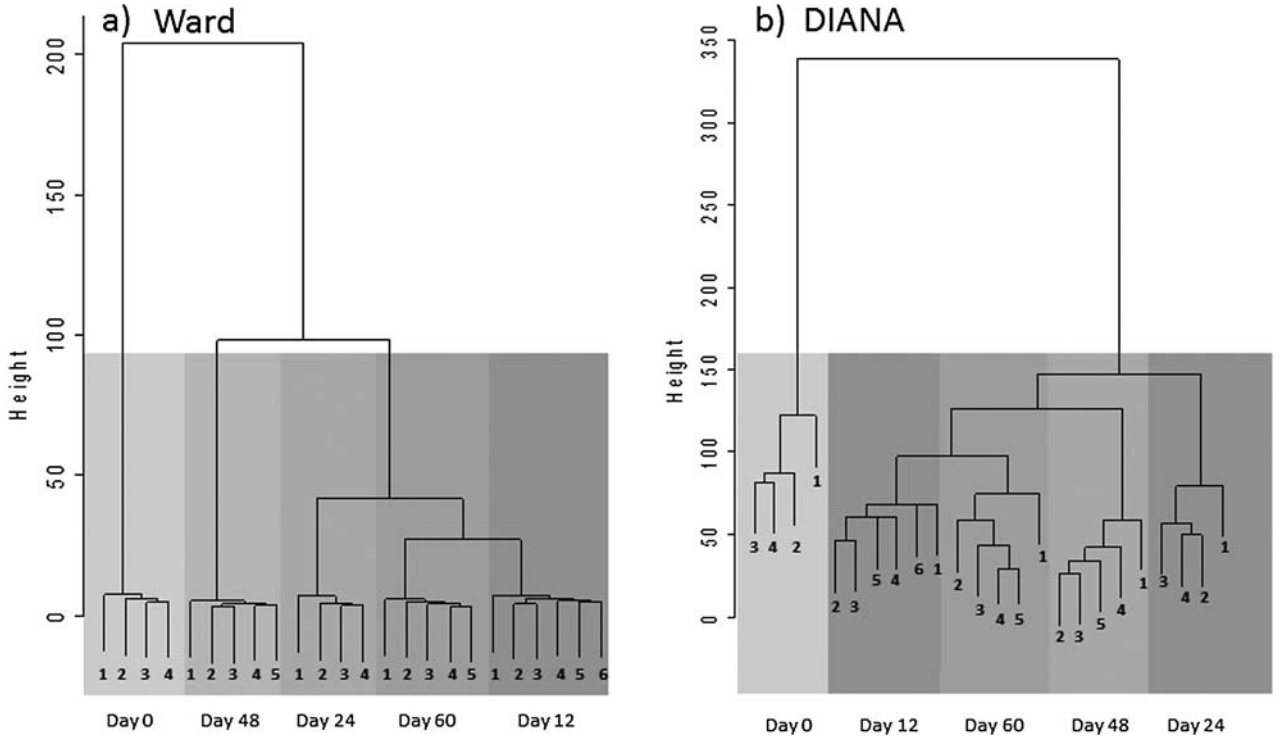

Fig. 2. Unsupervised classification. A, Hierarchical ward; B, Divisive cluster analysis (DIANA) of metabolite profiles of *Diporeia* starved for different lengths of time. Height on y-axis represents similarity between clusters.

up-regulated (~8-fold) compared to baseline metabolite levels ($t_{(8)} = 34.15$; $p < 0.0001$, Fig. 3).

Metabolite Identification

A list of metabolites identified, based on the HMDB LC-MS repository database, and which significantly changed throughout this study is presented in Table 1. A more detailed graphical depiction of temporal changes in specific metabolites is shown in Fig. 4. A number of fatty acids, lipids, and amino acids were found to be down-regulated during starvation. The degree by which metabolites changed over time varied, with some metabolites being completely absent in starved animals. Only one metabolite (N6,N6,N6-Trimethyl-L-lysine) was up-regulated during starvation. Further analyses were performed using the canonical pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) that revealed that the metabolism of histidine and glycerophospholipid were the major biochemical pathways affected during starvation of *Diporeia* (Fig. 5).

Discussion

As *Diporeia* were starved over a 60-day period, the expression of 16 metabolites were consistently altered, primarily through down-regulation (Table 1). Down-regulated metabolites fell into three major classes: fatty acids, lipids, and amino acids. In the following section, we will discuss the potential implications of such a change in metabolite profiles on *Diporeia*'s health and long-term survival. From these analyses, a "starvation metabolite fingerprint" could also be developed and used to assess the physiological condition of feral organisms.

One of the main classes of metabolites found to be down-regulated during starvation were the polyunsaturated fatty acids (PUFAs). Linoleic, eicosapentaenoic (EPA), and docosapentaenoic (DPA) acids declined as early as 12 d following the onset of starvation (Table 1, Fig. 4C). The abundance of PUFAs has also been reported to decrease in other starved invertebrates, such as the Pacific oyster (*Crassostrea gigas*), and our findings corroborate their important role in energy metabolism during the onset of nutritional stress (Langdon and Waldock, 1981). In addition to their role in juvenile somatic growth (Muller-Navarra, 1995), PUFAs are essential for a range of other physiological functions. In poikilotherms, PUFAs have been proposed to act as "physiological antifreeze" by maintaining the fluidity of cell membranes even at low temperatures, which is critical for survival (Singer and Nicolson, 1972; Pruitt, 1990; Hazel, 1995). They are responsible for maintaining and regulating ligand-based cellular interactions (Brett and Muller-Navarra, 1997) and aid in the synthesis of eicosanoids, which are involved in immune system functioning via the production of prostaglandins and leukotrienes (Smith and Borgeat, 1985; Blomquist et al., 1991).

In invertebrates, PUFAs help control water balance and ion flux (Stanley-Samuelson, 1994a, b), and play significant roles in survival and reproduction by controlling molting cycles, fecundity, and attracting sperm during fertilization (Kanazawa et al., 1977, 1979; Millamena et al., 1988; D'abramo and Sheen, 1993; Xu et al., 1993; Rees et al., 1994; Kubagawa et al., 2006; Branicky et al., 2010). PUFAs are especially important for crustaceans which commonly encounter periods of starvation with negative impacts on

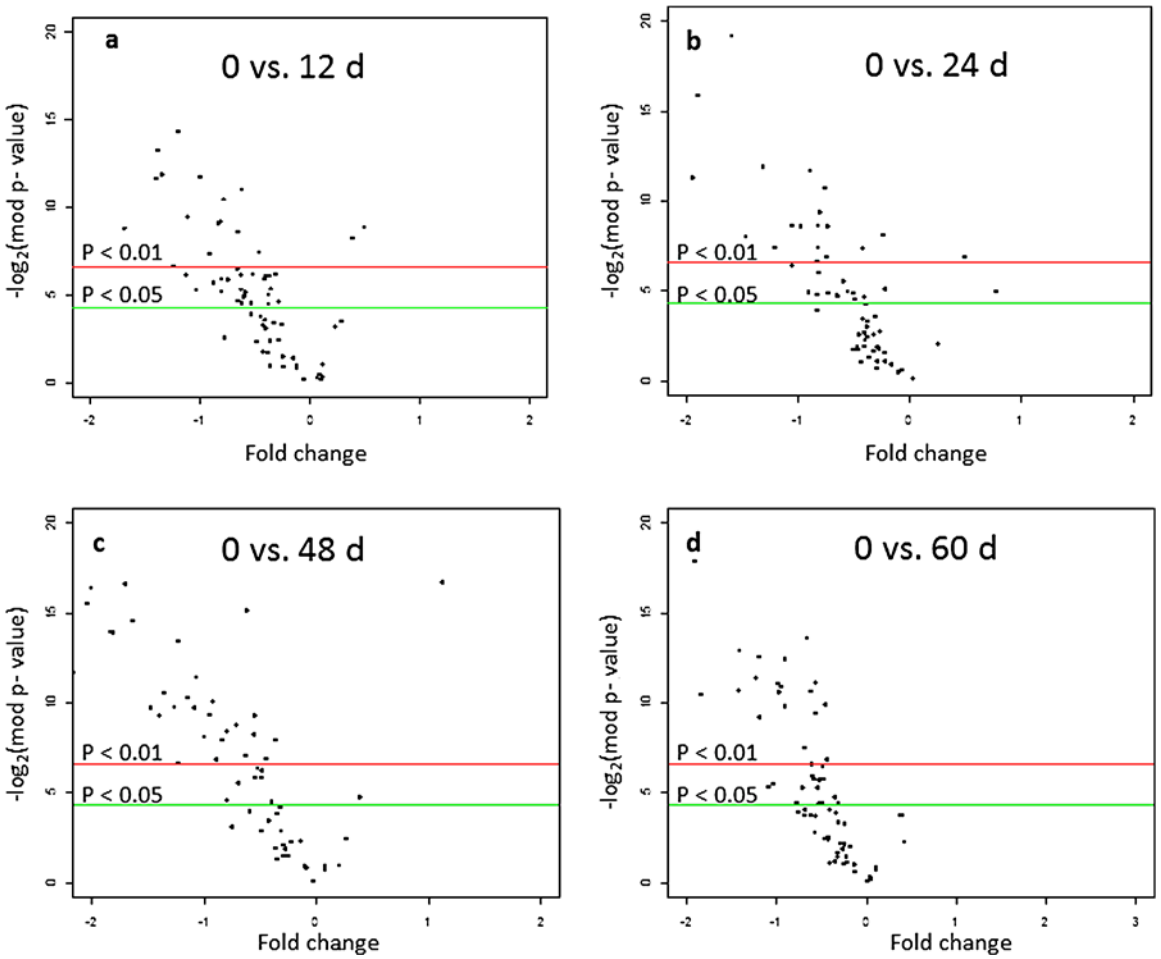

Fig. 3. Volcano plots from two-sample t-tests comparing 0 vs.: A, 12 d; B, 24 d; C, 48 d; D, 60 d. Each point represents a metabolite. Points above the top (green) or bottom (red) line are significantly different at the $p < 0.05$ and $p < 0.01$ levels, respectively. This figure is published in colour in the online edition of this journal, which can be accessed via http://www.brill.nl/jcb

growth and survival (Bychek et al., 2005). Since crustaceans have a limited capacity for de novo biosynthesis of lipids, they depend on external diet sources to replenish their lipid reserves. For example, linoleic acid, EPA and DPA are mostly derived from dietary sources (Harrison, 1990; Ravid et al., 1999; Wouters et al., 2001).

In the absence of food, these reserves are depleted. During prolonged starvation, crustaceans switch to multiple energy sources such as glycogen, followed by rapid utilization of glycerides and degradation of proteins and structural lipids leading to general depletion of lipid reserves (Schafer, 1968; Cuzon et al., 1980; Cherel et al., 1992). *Diporeia* "normally" undergo periods of food scarcity since most food inputs occur in the spring when isothermal conditions allow the diatom bloom to rapidly settle to the lake bottom. During the rest of the year, benthic food inputs are minimal and lipid reserves are depleted in *Diporeia* (Gardner et al., 1985). Our findings of declined abundance of PUFAs signify the onset of nutritional stress induced by starvation in *Diporeia*.

Phospholipids (PL) were observed to significantly decrease in starved *Diporeia* as well. Phospholipids contribute to membrane structure, signal transduction, and absorption and transportation of lipid molecules. Phosphatidylcholine is one such important PL, which forms lipoprotein precursors aiding in the synthesis of cholesterol (both high- and low-density lipoprotein forms) in shrimp (Sánchez-Paz et al., 2006). In crustaceans, cholesterol is crucial for oocyte maturation, larval development, and control of molting cycle (Lee and Puppione, 1978; Hertrampf and Meyer, 1991; Coutteau et al., 1997; Gonzalez-Felix et al., 2002; Yepiz-Plascencia et al., 2002). Therefore, it can be concluded that the persistent low levels of PUFAs and PLs in starved *Diporeia* can negatively impact several physiological functions, including energy production, immune function, osmoregulation and reproduction.

Ceramide, a precursor for the production of other sphingolipids (Merrill, 2002; Futerman and Hannun, 2004) was also down-regulated in starved *Diporeia* (Fig. 4B). However, compared to some of the previous metabolites discussed, ceramide levels did not decline as abruptly and starved animals were able to maintain measurable concentrations during the length of the study.

Sphingolipids are known to influence multiple cellular processes such as cell division, cell differentiation, and apoptosis (Hannun et al., 2001; Holthuis et al., 2001). Sphingolipids separate the cellular environment into micro domains, act as secondary messengers (Kim et al., 1991; Simons and Toomre, 2000; Allen et al., 2006; Morales et al.,

Table 1. Summary of metabolites that significantly changed over the starvation study. [a] Human Metabolome DataBase (HMDB) accession number. [b] Metabolite name based on m/z values searched against the HMDB. [c] Error term based on HMDB matches = ((observed mass − exact mass)/exact) ∗ 1 000 000. [d] Potential HMDB matches were placed in three categories: 1 (high probability): error falls within 1-50 ppm range and metabolites are biologically relevant; 2 (medium probability): error falls within 51-100 ppm range and metabolites are biologically relevant; and 3 (low probability): error falls within 101-200 ppm range and metabolites are biologically relevant. [e] p-value < 0.05 (Benjamini Hochberg corrected). [f] Calculated mean fold change in relation to the control group.

HMDB ID[a]	Metabolite name[b]	Error[c] (ppm)	Category[d]	p-value (BH adjusted)[e]	Mean expression and direction of change[f]	
Fatty acids						
HMDB00673	Linoleic acid	14.37	1	0.002	2.2	↓
HMDB03073	Gamma-Linolenic acid	13.27	1	<0.0001	2.2	↓
HMDB13623	12(13)Ep-9-KODE	63.94	2	0.0006	6.6	↓
HMDB06455	Arachidonyl carnitine	128.8	3	<0.0001	2.1	↓
HMDB01999	Eicosapentaenoic acid (EPA)	46.04	2	0.003	6.8	↓
HMDB13123	4,7,10,13,16-Docosapentaenoic acid	1.940	1	<0.0001	1.9	↓
HMDB11188	Triacylglycerol (TG)	45.78	1	<0.0001	2.1	↓
HMDB07560	Diacylglycerol (DG)	42.32	1	0.018	5.3	↓
Lipids						
HMDB13422	Phosphatidylcholine (PC)	60.69	2	<0.0001	2.1	↓
HMDB10406	Lysophosphatidylcholine (LysoPC)	53.22	2	0.01	2.3	↓
HMDB04950	Ceramide	28.05	1	0.007	5.5	↓
HMDB00086	Glycerophosphocholine	18.48	1	<0.0001	2.5	↓
HMDB13534	Phosphatidylglycerolphosphate (PGP)	4.417	1	0.02	7.5	↓
HMDB09914	Phosphatidylinositol (PI)	13.70	1	0.009	7.5	↓
HMDB10570	Phosphatidylglycerol (PG)	12.67	1	0.0009	7.1	↓
Amino acids & derivatives						
HMDB06801	2-Oxo-3-hydroxy-4-phosphobutanoic acid	128.8	3	0.04	5.3	↓
HMDB02005	Methionine sulfoxide	68.10	2	0.01	2.1	↓
HMDB00716	L-Pipecolic acid	17.84	1	0.004	5.8	↓
HMDB001943	Anserine	11.15	1	<0.0001	2.1	↓
HMDB00816	Phosphoglycolic acid	160.9	3	<0.0001	2.3	↓
HMDB01325	N6,N6,N6-Trimethyl-L-lysine	46.79	2	<0.0001	6.5	↑

2007) and are important components of specialized structures such as "lipid rafts," crucial for intracellular trafficking. In addition, they can influence essential functions such as calcium homeostasis, protein sorting and endocytosis (Isshiki and Anderson, 1999; Smart et al., 1999). In *Drosophila*, depressed sphingolipid levels have been related to enhanced cellular degeneration and oxidative stress-induced aging (Rao et al., 2007). Limited caloric intake resulting from starvation can cause a drop in sphingolipid production and thereby severely affect these cellular functions in *Diporeia*.

Among amino acid derived metabolites, methionine and histidine derivatives (methionine sulfoxide and anserine), and byproducts of lysine degradation (L-pipecolic acid and N6,N6,N6-Trimethyl-L-lysine) were detected during starvation. Methionine and anserine declined relatively quickly (by day 12) and never returned to pre-starvation levels during the rest of the experimental period (Fig. 4A). Methionine is required in limited amounts for the synthesis of carotenoids (Dall and Smith, 1987). Histidine-derived anserine compounds are also involved in maintaining the intracellular pH balance of skeletal muscles in fish (Abe, 1983; Abe and Okuma, 1991; Smutna et al., 2002). Anserine has antioxidant properties that help minimize the effect of aging and protein oxidation (Kohen et al., 1988; Decker, 1995). The persistent down regulation of methionine and histidine derivatives in starved *Diporeia* may result from rapid depletion of protein reserves and increased oxidative damage from protein catabolism.

Lysine is the precursor of carnitine compounds and fatty acid biosynthesis (Dall and Smith, 1987). Molting in crustaceans forces them to experience temporary periods of starvation during which they utilize tissue amino acid reserves. Lysine and methionine are among the amino acids stored as metabolic reserves for that purpose. A number of studies have reached similar conclusions about the essential role of these amino acid reserves in crustacean physiology (Cowey and Forster, 1971; Shewbart et al., 1973; Lasser and Allen, 1976; Miyajima et al., 1976). For example, in Kuruma shrimp (*Penaeus japonicas*) a methionine supplement is crucial for maintaining high growth (Kitabayashi et al., 1971). In another study, a 5-d starvation period resulted in decreased methionine concentrations in giant fresh water prawn (*Macrobrachium rosenbergii*) (Fair and Sick, 1982).

The amino acid derivative, N6,N6,N6-Trimethyl-L-lysine, was the only metabolite that was up-regulated (higher abundance) in the present study (close to 7-fold in starved animals compared to controls, see Table 1 and Fig. 4A). This metabolite functions as an active coenzyme in fatty acid oxidation. To meet their energy demands, *Diporeia* utilize

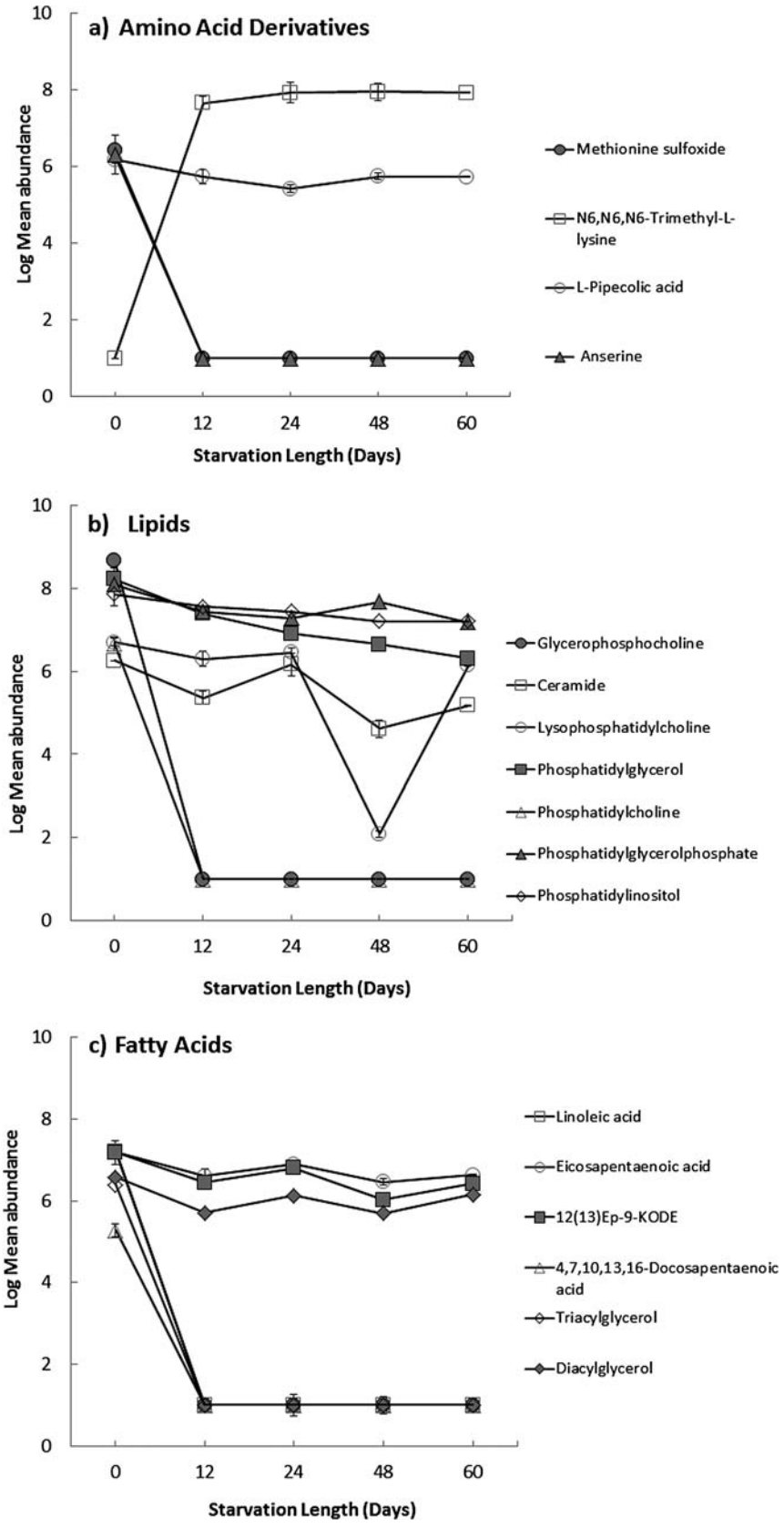

Fig. 4. Temporal changes in mean abundance levels of *Diporeia* metabolites across different starvation groups, by chemical class. A, Amino acid derivatives; B, Lipids; C, Fatty acids.

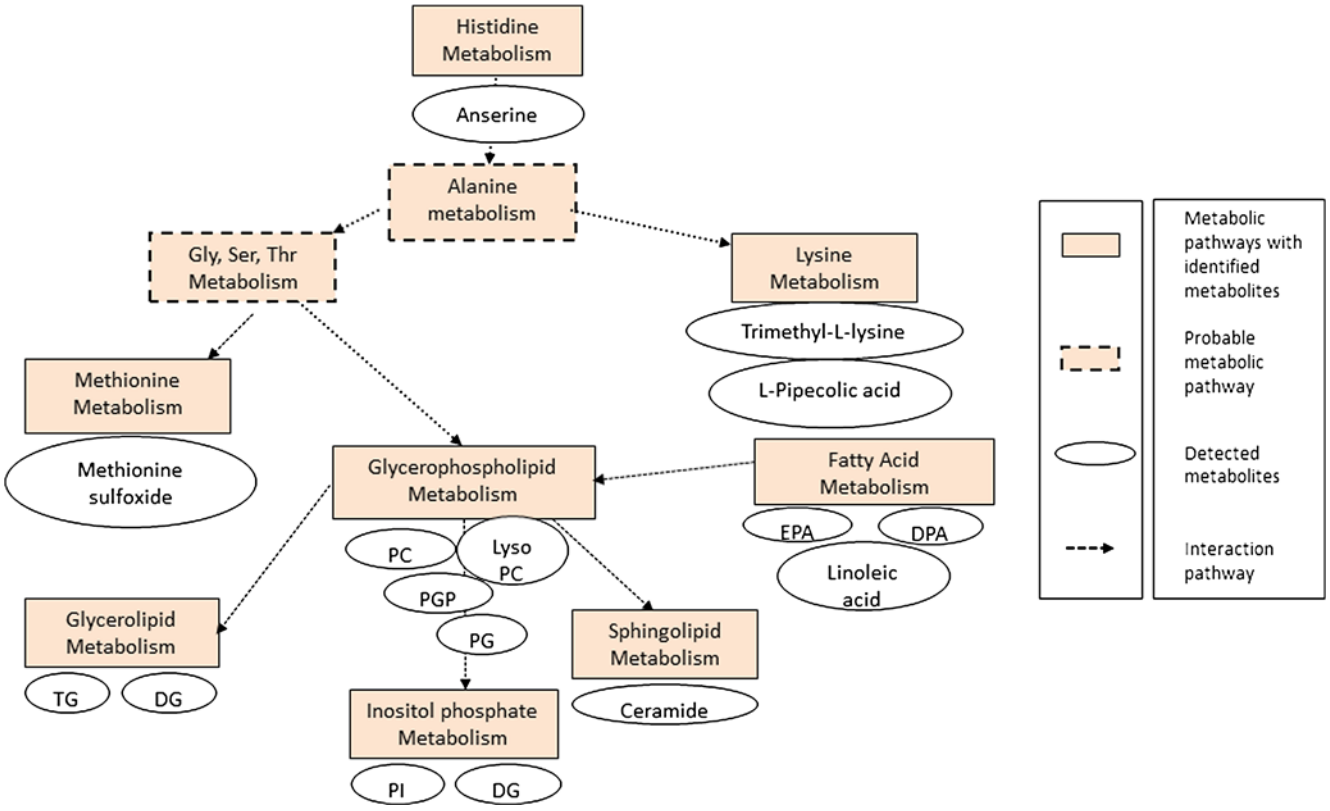

Fig. 5. Metabolic pathways affected during starvation constructed using canonical biochemical pathways from KEGG. DG = diacylglycerols; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; Gly = glycerine; LysoPC = lysophosphatidylcholine; PC = phosphatidylcholine; PG = phosphatidylglycerol; PGP = phosphatidylglycerolphosphate; PI = phosphatidylinositol; Ser = serine; TG = triacylglycerols; Thr = threonine.

lipid oxidation pathways, so any active coenzymes (such as trimethyllysine) related to lipid metabolism should also be present in high abundance during starvation. Thus, elevated levels of trimethyllysine indicate increased lipid oxidation in *Diporeia* during starvation.

One limitation of this research is that most, if not all, currently available metabolite databases have little information regarding metabolome of invertebrates in general and crustaceans in particular. This limited availability of information can hinder the interpretability of metabolomics data from non-conventional organisms. Another limitation of the current study is that the "starvation metabolite profile" generated might be indistinguishable between a lack of food due to time of year or competition with dreissenids.

Nevertheless, a significant outcome of this research was the identification of a number of metabolites that could be used as bioindicator of "starvation" or nutritional stress of *Diporeia* in the wild. Of the 16 metabolites that were down-regulated, 7 were completely absent after day 12 of starvation. These metabolites included two amino acid derivatives (methionine sulfoxide and anserine); two lipids (glycerophosphocholine and phosphatidylcholine); and three fatty acids (linoleic acid, DPA, and diaglycerol).

In summary, our studies have shown that in starved *Diporeia*, fatty acids, lipids and amino acids experienced steady declines resulting in altered histidine, glycerophospholipid, and sphingolipid metabolic pathways (Fig. 5). Increased protein and lipid catabolism will incur adaptive stress responses leading to cellular disintegration, increased oxidative stress, minimal energy production, impaired reproductive function, limited growth, and ultimately death if conditions of low to no food persist. A "metabolite profile" of starvation was observed that could be applied to feral studies that aim to evaluate *Diporeia*'s health condition in populations that are stable and in various stages of decline.

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