Polar Biol (2018) 41:433–449
https://doi.org/10.1007/s00300-017-2200-6

ORIGINAL PAPER

Multivariate statistical analysis of metabolomics profiles in tissues of polar bears (*Ursus maritimus*) from the Southern and Western Hudson Bay subpopulations

A. D. Morris[1,2] · R. J. Letcher[1,2] · M. Dyck[3] · B. Chandramouli[4] · A. T. Fisk[5] · J. Cosgrove[4]

Received: 16 February 2017 / Revised: 2 August 2017 / Accepted: 3 August 2017 / Published online: 29 August 2017

Abstract Polar bears (*Ursus maritimus*) are apex predators of the Arctic, which exposes them to an array of natural and anthropogenic stress factors. Metabolomics analysis profiles endogenous metabolites that reflect the response of biological systems to stimuli, and the effects of multiple stressors can be assessed from an integrated perspective. A targeted, quantitative, liquid chromatography–mass spectrometry-based metabolomics platform [219 metabolites including amino acids, biogenic amines, acylcarnitines, phosphatidylcholines (PCs), sphingomyelins, hexoses (Hex), and fatty acids (FAs)] was applied to the muscle and liver of polar bears from the Southern and Western Hudson Bay (Canada) subpopulations (SHB and WHB, respectively). Multivariate statistics were then applied to establish whether bears were discriminated by sex and/or subpopulation. Five metabolites identified by variable importance projection (VIP) discriminated the hepatic profiles of SHB males and females (Hex, arginine, glutamine, one PC, one sphingomyelin), while fifteen metabolites (primarily PCs along with leucine) contrasted the livers of males from SHB and WHB. Metabolite profiles in the muscle of male and female bears could not be differentiated; however, the muscles of SHB and WHB males were discriminated primarily by PCs and FAs. Stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were variably related to metabolites; $\delta^{13}C$ was correlated with some VIP metabolite concentrations, particularly in comparisons of male bears from SHB and WHB, suggesting an influence of dietary differences. However, $\delta^{15}N$ and age exhibited few, relatively weak correlations with metabolites. The metabolite profiles discriminating the sexes and subpopulations may have utility for future assessments regarding the effects of specific stressors on the physiology of Hudson Bay polar bears.

Keywords Metabolomics · Multivariate analysis · Partial least squares discriminant analysis (PLS-DA) · Polar bears (*Ursus maritimus*) · Hudson Bay

Introduction

Metabolite profiling (or metabolomics) is a field of bioinformatics that measures endogenous, low-molecular weight metabolites and their intermediates that reflect the dynamic response of biological systems in cells, tissues, or organisms to various biological and environmental conditions (Dunn and Ellis 2005; Clarke and Haselden 2008; Cai and Li 2016). These metabolites are the result of gene expression or protein activity, and are chemically diverse compounds that include amino acids (AAs), lipids, sugars, nucleic acids, and fatty acids (FAs). These analyte profiles can be used to assess the effects of a number of endogenous

Electronic supplementary material The online version of this article (doi:10.1007/s00300-017-2200-6) contains supplementary material, which is available to authorized users.

✉ A. D. Morris
adam.morris@canada.ca; adam.morris.phd@gmail.com

[1] Ecotoxicology and Wildlife Health Division, National Wildlife Research Centre, Environment and Climate Change Canada, Ottawa, ON, Canada

[2] Department of Chemistry, Carleton University, Ottawa, ON, Canada

[3] Department of Environment, Government of Nunavut, Igloolik, NU, Canada

[4] AXYS Analytical Services, Sidney, BC, Canada

[5] Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON, Canada

and extrinsic variables on living things and can provide information about the physiology of an organism in relation to its environment (Idle and Gonzalez 2007; Bundy et al. 2009).

Environmental metabolomics uses targeted or untargeted analytical methods in conjunction with multivariate statistics to identify covarying metabolites that drive statistical discrimination between groups (Lin et al. 2006). Variability between these groups can be due to inherent physiological differences or changes in physiological processes after exposure to stimuli (Lin et al. 2006; Bundy et al. 2009). The generation of metabolomic profiles is most often achieved using analytical methods based on gas or liquid chromatography (GC and LC, respectively) with tandem mass spectrometry (MS/MS) or nuclear magnetic resonance spectroscopy (^{1}H-NMR) (Lin et al. 2006). After measurement and application of multivariate statistics, it has been shown that differentiation can be made between experimental and natural treatment groups, the basal biology and physiology in non-model species can be revealed, and the influence and effects of environmental and anthropogenic stressors can be better understood (Schrimpe-Rutledge et al. 2016).

Metabolite profiling has been successfully applied to identify potential biomarkers (specific metabolites or a "fingerprint" of several metabolites) that relate to exposure to specific stressors including seasonal change (Sadler et al. 2014), pharmaceuticals (Samuelsson et al. 2011), anthropogenic contaminants (Aliferis and Chrysayi-Tokousbalides 2011; O'Kane et al. 2013; Huang et al. 2016), heat or cold stress (Ellis et al. 2014), fasting (van Ginneken et al. 2007), migration (Benskin et al. 2014), and pathogenic infection (Ellis et al. 2014), among others. Despite the breadth of investigations, there are few metabolomics studies that have been strictly field based, and even fewer that relate to wildlife and megafauna, particularly in the Arctic. Field-based studies have also been shown to provide unique information that can be masked by laboratory acclimation, as was found in a study that investigated metabolomic responses to hypoxia in mantle tissue of mussels (*Mytilus galloprovincialis*) (Hines et al. 2007).

Published studies on ursids have been limited to an investigation into changes in the milk metabolome of captive giant pandas (*Ailuropoda melanoleuca*) throughout lactation (Zhang et al. 2015), differences in metabolites associated with energetics in different age classes of black bears (*Ursus americanus*) (Niemuth and Stoskopf 2014), and a non-quantitative investigation of differences in glucose, AAs, and lipids between subadults, adult male, and adult female polar bears from Norway (Størseth et al. 2009). Sex-specific differences were also identified in the plasma metabolome of humans and seem to have similar patterns in terms of discriminatory metabolites to those observed in polar bears (Bertram et al. 2009; Størseth et al. 2009).

Polar bears (*Ursus maritimus*) are an apex and pagophilic predator of the marine food web of the Arctic Basin (Derocher et al. 2004) and are a species of "Special Concern" in Canada (Committee on the Status of Endangered Wildlife in Canada COSEWIC 2008). Polar bears experience a number of stressors that affect their physiological state, including annual seasonal stress and dietary changes associated with temperatures and sea-ice (Derocher et al. 2004; Gormezano and Rockwell 2013b) dietary shifts to less energetically favorable sources during longer ice-free periods (Gormezano and Rockwell 2013a, b), increased competition due to the expanding ranges of other wildlife (Derocher et al. 2004; Environment and Climate Change Canada (ECCC) 2014), and biomagnification of anthropogenic contaminants (Letcher et al. 2010; Muir et al. 2013), among others. The position of the polar bear as an apex predator, their socioeconomic importance to the Inuit, and prominence as an international symbol of the Arctic make conserving the health of these animals a priority.

A targeted, quantitative LC–MS/MS-based metabolomics platform was recently applied in studies to investigate shifts in metabolite profiles during migration in sockeye salmon (*Oncorhynchus nerka*), or during exposure to xenobiotic compounds in zebrafish (*Danio rerio*) (Benskin et al. 2014; Huang et al. 2016). We applied this platform to the liver and muscle samples of polar bears from the Hudson Bay Region (Canada). Stable isotope analyses of carbon (δ^{13}C) and nitrogen (δ^{15}N) were also used to track dietary variability between the bears. Bears from the western Hudson Bay (WHB) and southern Hudson Bay (SHB) were selected for the study as Hudson Bay is a "hot spot" for climate change effects and persistent organic pollutants in Canadian polar bears due to the relatively low latitudes of their ranges (Derocher et al. 2004; Muir et al. 2006; Letcher et al. 2009, 2010; McKinney et al. 2011b).

The study objective was to describe metabolites within the established metabolomic platform that would be useful in the discrimination of these polar bears as a function of subpopulation and sex. This will generate baseline metabolomic data that can be used to assess future changes in the physiological state of bears in this population. Differences between sexes have been previously described in the metabolomic profiles in plasma of Norwegian polar bears (Størseth et al. 2009) as well as in humans (Bertram et al. 2009). Based on these results, we do expect some sex-related discrimination here, with the targeted method allowing for identification of the specific metabolites driving discrimination. Whether the closely oriented subpopulations of the SHB and WHB have metabolomic signatures that can be discriminated is currently unknown. However, relatively recent analyses suggest that both diet

and contaminant exposure (Thiemann et al. 2008; Gormezano and Rockwell 2013b; Morris et al. 2016), among other factors, do differ between the subpopulations, which may affect the physiology of the animals enough to discriminate their metabolite profiles here.

Materials and methods

Sampling

Under a validated Nunavut Wildlife Research Permit (NWRP), samples of legally harvested bears were taken from Hudson Bay based on established quotas. These tissue samples (liver and muscle) were obtained over the course of October 2013 to April 2014 (Table S1). The NWRPs were prepared and evaluated in collaboration with communities via the Nunavut Department of Environment. Nunavut Hunters and Trappers Organizations (HTOs) and HTO-associated hunters carried out the harvests from Arviat, Rankin Inlet, Whale Cove, and Sanikiluaq, harvesting bears by gunshot before dissection.

Samples were collected from polar bears from the WHB ($n_{Male} = 14$, $n_{Female} = 3$) and SHB ($n_{Male} = 15$, $n_{Female} = 9$) subpopulations (Fig. 1). Because of the small n value, the WHB females were not included in the present investigation but are included with all collection data in Table S1. The muscle and liver samples were consistently collected within about 1 h post mortem from the same sites for all bears under study. Liver samples were taken from the lower left lobe. The muscle samples were collected from the sternocleidomastoid muscle in the neck. Tissues were immediately wrapped in foil, sealed in sterile Whirl–Pak® bags (Nasco Inc., Fort Atkinson, MI), and frozen at −20 °C in the field. All samples were shipped to Ottawa (Canada), frozen at −20 °C, and then stored at −20 °C in ECCC's National Wildlife Specimen Bank in Ottawa until analysis. Since the bears were harvested over the course of October 2013 to April 2014, the samples were stored for between 15 and 21 months prior to metabolite analysis in July 2015. At the NWRC, subsamples of muscle (≈2 g) were frozen at −25 °C for $\delta^{13}C$ and $\delta^{15}N$ analyses at the University of Windsor (ON, Canada).

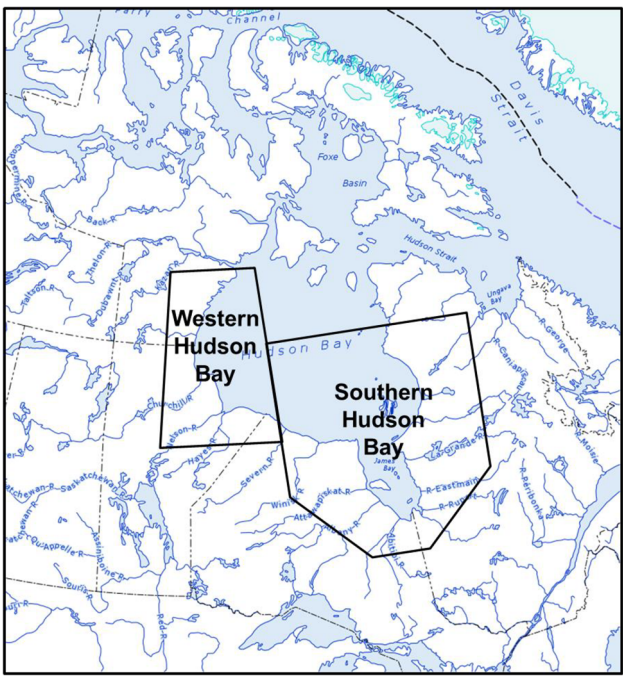

Fig. 1 Map illustrating the polar bear (*Ursus maritimus*) geographic ranges in the Southern and Western Hudson Bay. The ranges were appoximated from the polar bear management unit map availabe from Environment and Climate Change Canada (Environment and Climate Change Canada (ECCC) 2014)

Aging and stable isotope analysis

Age was determined by staining and counting cementum layers in canine teeth (Matson's Laboratory, Milltown, MT, USA) (Matson et al. 1993; Boertje et al. 2015). The age of one male bear from the WHB subpopulation (K14-39100) was not available, so its age was estimated via regression of the age of the other sampled male bears against *cis*-chlordane concentrations using the formula: Age = 14.432 − (8.247 × log [cis-chlordane]) = 8.65 (≈9); this approach was validated and applied by McKinney et al. (2011b) (ages in Table S1). Because the WHB females were not included in the detailed analyses, the missing age for bear K14-39093 was not estimated. Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) were measured in lipid-free muscle tissue by elemental combustion followed by analysis on a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA) using previously described methods at the Great Lakes Institute for Environmental Research (University of Windsor, Windsor, ON, Canada) (McMeans et al. 2009; McKinney et al. 2012).

Metabolite extraction and analysis

The metabolomics platform applied here was developed and optimized by SGS AXYS (Sidney, BC) with a suite of 219 metabolites measured in the muscle and liver. The analytes included 21 AAs, 22 biogenic amines (BgAs), 13 bile acids (liver only), hexoses (Hex), 18 FAs, 40 acylcarnitines (ACs), 89 phosphatidylcholines (PCs) [39 diacyl, 37 acyl-alkyl, 13 lysophosphatidylcholines (lysoPCs)], and 15 sphingomyelins (SMs). The total number of analytes decreased in each tissue based on quality assurance results and resulted in 156 metabolites in the final statistical models in both the liver and muscle, though these were not the same suite in both tissues (details below and in the

Online Resources, all metabolites and acronyms in Table S2).

Extraction from tissue samples and the identification and quantification of metabolites via high-performance liquid chromatography and tandem mass spectrometry (HPLC–MS/MS) or flow injection (FI)–MS/MS-based methods have been described in complete detail elsewhere (Benskin et al. 2014; Huang et al. 2016), and are presently summarized in the Online Resources. Briefly, AAs, BgAs, bile acids, Hex, and FAs in the metabolite-containing sample fractions were analyzed on an Agilent 1100 HPLC system coupled to an API 4000 triple quadrupole mass spectrometer (MS/MS; Applied Biosystems/Sciex, Concord, ON, Canada), while ACs, SMs, and PCs were analyzed by FI–MS/MS. Quantification was achieved using multi-point calibration isotope dilution/surrogate standard quantitation where possible (AAs, BgAs, FAs, bile acids, and Hex), or single-point internal standard quantitation (ACs, PCs, and SMs) (Table S2) (Benskin et al. 2014; Huang et al. 2016).

Quality analysis/quality control

Analyses of $\delta^{13}C$ and $\delta^{15}N$ were validated by comparison with (1) Internal reference standard (tilapia (*Oreochromis* spp.), made in house by Fisk laboratory), bovine liver 1577c (National Institute of Standards and Technology, Gaithersburg, MD, USA), USGS 41 (United States Geological Survey, (Reston, VA, USA), and Urea IVA 33802174 (IVA Analysentechnik GmbH & Co, Meerbusch, Germany). The standard deviations of repeated analyses ($n = 10$–22) of the four certified reference materials ranged from 0.10 to 0.21‰ for $\delta^{15}N$ and 0.060 to 0.18‰ for $\delta^{13}C$, indicating good precision in the analyses. The accuracy of the instrumental analysis was confirmed by measurements within 0.16‰ and 0.060‰ of their certified values for $\delta^{15}N$ and $\delta^{13}C$, respectively [United States Geological Survey (USGS) certified reference material USGS 40, $n = 22$].

The metabolomics analytical platform used in this study has been accredited through the International Organization for Standardization (ISO17025) (Benskin et al. 2014). Method blanks and certified internal reference materials (IRMs; *Oncorhynchus nerka* liver, SC6106) were extracted with each batch of samples ($n = 3$ for each batch of samples). IRMs indicated generally acceptable recoveries of the selected metabolites, ranging from 41 to 154% in the liver and 37 to 172% in the muscle, with some exceptions. Relative standard deviations (RSDs) for metabolites in IRM samples ranged from 6.9 to 37% in the liver and from 5.2 to 27% in the muscle, with most of the RSDs <10% (details in the Online Resources, and Table S3). Spike-recovery experiments for metabolites were performed during optimization of the method and have been reported previously ($n = 5$ each per low, mid-range, and high spiking levels) (Benskin et al. 2014).

When metabolite concentrations in blanks exceeded 30% of the batch-specific sample concentration, they were excluded from the dataset (see Online Resources for list of excluded metabolites). The remaining data were blank corrected to the arithmetic mean of the tissue-specific blanks to control for background contamination. The calibration standard containing the metabolites of interest (Table S2) was analyzed at a frequency of every 20 samples to confirm instrument stability, and instrument blanks (MeOH) were run after high-concentration calibration standards to assess and eliminate analyte carryover.

Limits of detection (LODs) for HPLC–MS/MS data were defined as either (1) the greater of the concentration at a sample/metabolite-specific signal-to-noise ratio of three, or (2) the mean concentration of the blank plus 3× the blank standard deviation. For the FI–MS/MS data, after deconvolution, peak areas below a threshold of 20,000 were considered non-detects with detection limits calculated specifically using the area of the lowest corresponding internal standard. In cases where the blank concentration added to 3× the blank standard deviation exceeded this calculated LOD, that value (blank + 3σ) was used as the LOD. Quantification limits (LOQs) were defined using the calibrated range for each compound. Values < LOQ (outside of this range) were quantifiable, but had greater uncertainty.

Data handling and statistics

The statistical power of the analyses was improved by removing metabolites when they had a tissue-specific detection frequency (<LOQ) of less than 50%. If a metabolite was detected in 50 to 100% of the samples for a given tissue, a concentration between zero and the sample-specific LOQ was randomly assigned in place of zero values for statistical analyses.

After blank correction and filtering the inconsistently detected metabolites, the data were used to build the partial least squares discriminant analysis (PLS-DA) models in both the muscle and liver using MetaboAnalyst 3.0 (www.metaboanalyst.ca) (Xie et al. 2015). PLS-DA models were used to compare the three groups together (WHB males, SHB males, and SHB females) and were also used to make pairwise comparisons (SHB males versus females, SHB males versus WHB males, and SHB females versus WHB males). Discussion focused on SHB female–male comparisons (to test for sex-related differences), and SHB–WHB male comparisons (for subpopulation-related differences). Models were validated using both tenfold cross validation (which generated accuracy, R^2, and Q^2 values and informed the optimal number of components for each

model), and permutation tests (2000 iterations testing the prediction accuracy of the model). Metabolite data were normalized by sum, cube root or log-transformed, and scaled (mean centered) to improve comparability of samples and variables and to improve the performance of the models and analysis.

The data for liver PLS-DA models were normalized by sum, cube root transformed, and mean centered, with three-group tenfold cross validation (CV) values of 0.73 (accuracy), 0.94 (R^2), and 0.76 (Q^2) indicating good predictability of the model. Testing the prediction accuracy of the model using permutation testing indicated good classification and performance by the model (2000 iterations tested, $p = 0.001$). Two-group validation scores in the liver were also strong. The SHB male–female model had the validation scores of 0.88 (accuracy), 0.80 (R^2), and 0.61 (Q^2), and the permutation p value was 0.043. The model for the SHB–WHB males also validated well (accuracy = 0.76, $R^2 = 0.84$, $Q^2 = 0.48$, permutation $p = 0.038$), as did the SHB female–WHB male model (accuracy = 0.87, $R^2 = 0.85$, $Q^2 = 0.68$, permutation $p = 0.011$).

The muscle data were normalized by sum, log-transformed, and were again scaled only by mean centering. The three-group, tenfold cross validation scores were smaller than those of the liver, but were still within acceptable ranges for accuracy of (0.57), R^2 (0.74), and Q^2 (0.51) and had a permutation $p = 0.009$. The two-group comparisons were the same as in the liver and had acceptable score for the SHB male versus WHB male model (accuracy = 0.83, $R^2 = 0.74$, $Q^2 = 0.45$, permutation $p = 0.039$) and the SHB females and WHB males (accuracy = 0.91, $R^2 = 0.86$, $Q^2 = 0.69$, permutation $p = 0.041$); however, the model for the SHB females and SHB males did not validate (indicative of insignificant variation between groups to discriminate them) (Skelton et al. 2013).

Variable importance projection (VIP) was then applied to identify the metabolites of significance in the projection of the PLS-DA scores using a weighted sum of squares analysis of the loadings (www.metaboanalyst.ca) (Xie et al. 2015). VIP metabolites with scores exceeding one were considered significant, and these were then cross referenced with significantly different metabolites identified by univariate tests [Kruskal–Wallis (KW) ANOVA on ranks with Dunn's post hoc tests or Mann–Whitney U tests]. The metabolites found to have VIP scores >1 and that were significantly different in the univariate tests were selected for further discussion.

Non-parametric univariate tests were performed on the non-normalized data, as were Spearman correlation analyses to test for significance between groups and relationships between variables, respectively. Emphasis in the discussion is placed on correlations with Spearman correlation coefficients (r_s^2) ≥ 0.25 ($r \geq 0.50$), indicating moderate–strong correlations (Asuero et al. 2006); those with $r_s^2 < 0.25$ are discussed briefly with caveats. ANOVA with Tukey's post hoc tests were applied to compare age, $\delta^{13}C$ and $\delta^{15}N$, and PLS-DA scores; however, in most cases a failure of the normality tests resulted in the use of KW ANOVAs with Dunn's tests. Statistically significant results were accepted at a type I error rate of (α) = 0.05 for these tests.

The Pathway Analysis tool in MetaboAnalyst 3.0 (www.metaboanalyst.ca) (Xie et al. 2015) was then applied to VIP-identified metabolites in the pairwise comparisons in order to ascertain which physiological processes were potentially impacted when compared between the groups (Xia and Wishart 2011). Pathway analysis used the rat model (*Rattus rattus*), and only pathways with Holm–Bonferroni adjusted p values exceeding 0.05, and impact scores greater than zero are discussed. VIP metabolite concentrations were labeled using their human metabolome database (HMDB) identification numbers before being uploaded and normalized (the normalization/transformation/scaling procedures were the same as for PLS-DA analysis) for pathway analysis. Note that the acyl-alkyl PCs and several other metabolites did not have Kyoto encyclopedia of genes and genomes (KEGG) identification numbers and hence could not be included in the background pathways. This had no impact on the overall result; it only affected the estimate of the total compounds screened that were present in each pathway (total compounds in Tables S5 and S10; see Online Resources for details).

Multivariate analyses were performed using MetaboAnalyst 3.0 (www.metaboanalyst.ca) (Xie et al. 2015). General statistical comparisons such as summary statistics, univariate tests, and correlation analyses were performed using SigmaPlot 11 (SYSTAT, Chicago, IL, USA).

Results

Age, stable isotopes, and diet

Although the range of the ages of the polar bears were quite different, the median ages were not statistically different (KW ANOVA on ranks, $H = 3.95$, $p = 0.139$) but were of the order SHB males (15 years) > WHB males (14 years) > SHB females (9 years) (Table 1). Dietary similarities among the SHB bears was inferred from their stable isotope signatures, as they were not significantly different for either $\delta^{13}C$ (SHB males = −19.6 ± 0.128‰, SHB females = −19.9 ± 0.177‰; KW ANOVA on ranks, $H = 14.9$, $p < 0.001$; Dunn's test $Q = 1.15$, $p > 0.05$) or $\delta^{15}N$ (SHB males = 18.2 ± 0.221‰ and SHB females = 17.8 ± 0.308‰; KW ANOVA on ranks,

Table 1 Age, stable isotope ratios of carbon ($\delta^{13}C$, $^{13}C/^{12}C$) and nitrogen ($\delta^{15}N$, $^{15}N/^{14}N$), total percent carbon, total percent nitrogen, and the carbon to nitrogen ratio (C:N) in muscle of polar bears from the Southern and Western Hudson Bay sub-populations

	Southern Hudson Bay		Western Hudson Bay
	Females	Males	Males
n	9	15	14
Age	5 (3–7)	6 (2–18)	7 (1–13)[a]
$\delta^{13}C$ (‰)	-19.9 ± 0.177^{A}	-19.6 ± 0.128^{A}	-19.0 ± 0.150^{B}
$\delta^{15}N$ (‰)	17.8 ± 0.308^{A}	18.2 ± 0.221^{AB}	18.9 ± 0.529^{B}
Percent carbon (%)	44.9 ± 0.217	44.6 ± 0.229	44.6 ± 0.19
Percent nitrogen (%)	14.1 ± 0.182	13.9 ± 0.0918	14.1 ± 0.124
C:N	3.18 ± 0.0402	3.20 ± 0.0216	3.17 ± 0.0313

Ages are medians and ranges, all other values are means ± standard errors. Values that share uppercase superscript letters (or have no letters) are not significantly different (KW ANOVA with Dunn's tests, $p > 0.05$)

[a] The age of one WHB bear was unavailable and was estimated using linear regression of the logarithm of *cis*-chlordane concentrations (wet weight) versus the age of the remaining male bears, as in McKinney et al. (2011a, b)

$H = 6.73$, $p = 0.035$; Dunn's test $Q = 2.48$, $p < 0.05$) (Table 1). However, the WHB male bears were significantly more ^{13}C enriched ($\delta^{13}C = -19.0 \pm 0.150$‰) than both sexes of SHB bears (KW ANOVA on ranks, $H = 14.9$, $p < 0.001$; Dunn's test $Q = 3.61$ and 2.85, $p < 0.05$), and significantly enriched in ^{15}N ($\delta^{15}N = 18.9 \pm 0.529$‰) relative to the SHB females only (KW ANOVAs on Ranks, $H = 6.73$, $p = 0.035$, Dunn's test $Q = 2.48$, $p < 0.05$).

Metabolomic profiling and modeling in the liver

The PLS-DA model for the three-group comparison (SHB males, SHB females, WHB males) of the hepatic metabolite profiles used four components that explained 58.9% of the total variance (Component 1 = 31.7%, Component 2 = 12.4%, Component 3 = 11.5%, Component 4 = 3.3%) (Fig. 2, loadings plots Fig. S1). The data treatment achieved good clustering and reasonably well-resolved discrimination of the three groups, with the greatest degree of separation observed between the scores of SHB female bears and the WHB male bears, while the scores of SHB males overlapped substantially with the other groups. The mean PLS-DA scores of both the SHB and WHB males were significantly different from those of SHB females across Component 1 (KW ANOVAs on ranks, $H = 19.5$, $p < 0.001$; Dunn's tests = SHB males versus SHB females, $Q = 2.83$, $p < 0.05$; WHB males versus SHB females $Q = 4.41$, $p < 0.05$), while the scores of the WHB differed only from those of the SHB female bears across Component 2 (KW ANOVAs on ranks, $H = 10.8$, $p = 0.004$; Dunn's test = WHB males versus SHB females, $Q = 3.23$, $p < 0.05$); no significant differences in scores across Component 3 or 4 were detected (KW ANOVAs on ranks, $H = 3.92$ and 1.47, $p = 0.14$ and 0.478, respectively).

A series of two-group comparisons were made to establish differences in the metabolite profiles that were related to sex and subpopulation. The PLS-DA model for the SHB male–female comparison was based on two components that explained 48.3% of the total variance (Component 1 = 34.6%, Component 2 = 13.7%) (Fig. 2). The model comparing SHB–WHB males was composed of three components explaining 53.5% of the variance (Component 1 = 24.4%, Component 2 = 17.3%, Component 3 = 11.8%). The metabolite profiles of SHB females and WHB males were well discriminated (Fig. S2), which was unsurprising given that both sex and location differ between them. In the other comparisons, the bears share physiological similarities due to sex (males), or they reside within the same range and likely have a similar diet and exposure to stressors that can influence metabolite profiles (SHB bears). The comparison of SHB females with WHB males will not be the focus of the discussion here, as differences related to any single factor are confounded by others (this comparison also yielded similar results to the three-group comparison). The discussion of the SHB female–WHB male data is largely restricted to the Online Resources for this reason.

Of the 156 metabolites that qualified for final statistical analyses in the liver, nine metabolites in the three-group comparison had significantly different concentrations between at least two groups and also had VIP scores exceeding one (relative concentrations are shown with VIP plots in Fig. 2, all VIPs are shown in Fig. S3, and all metabolite concentrations in the liver in Table S4). The discriminating metabolites were Hex, three unsaturated diacyl PCs (PC aa 34:2, PC aa 36:2, PC aa 38:3), a lysoPC (lysoPC C20:3), and long-chain polyunsaturated fatty acids (LC-PUFAs) including three

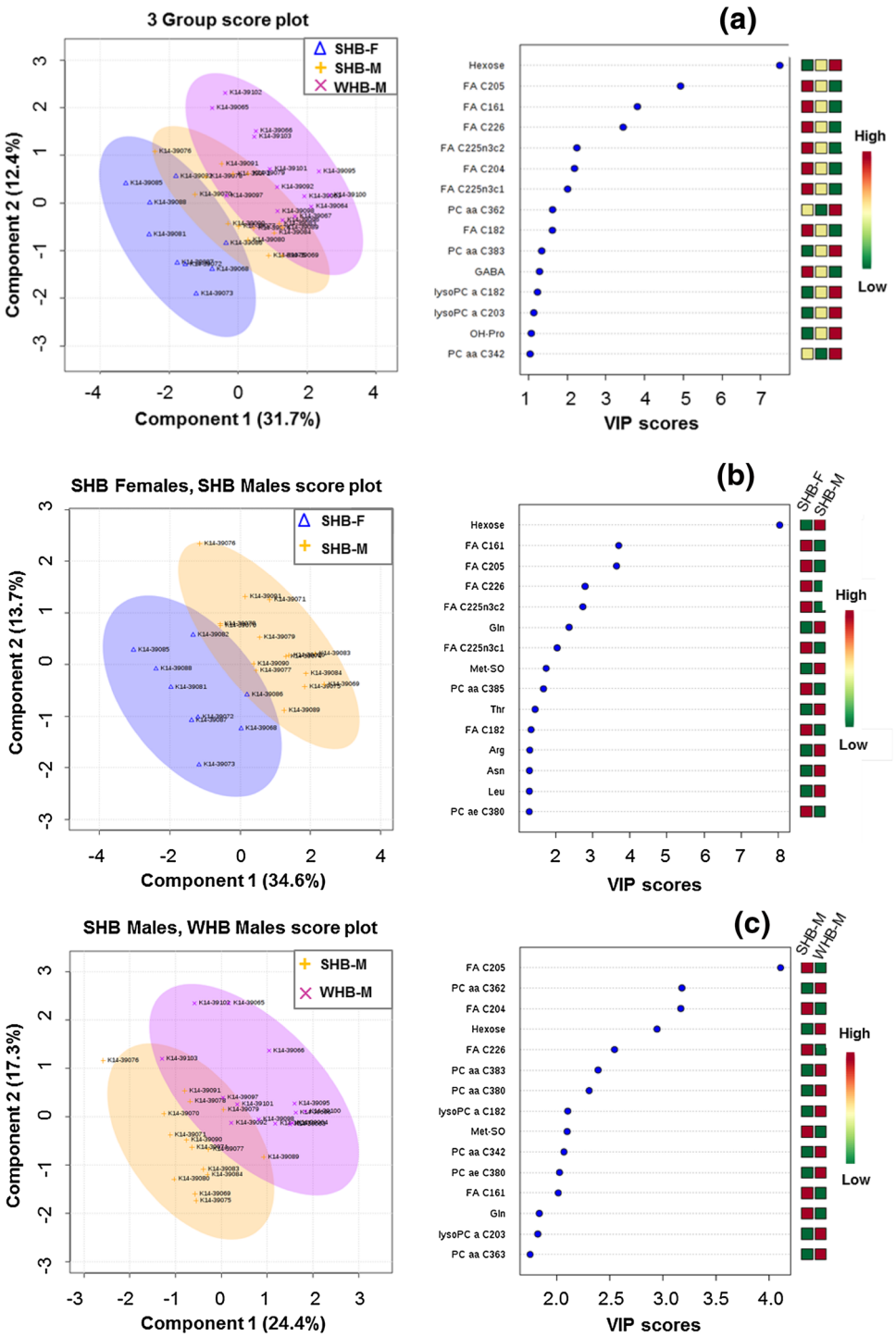

Fig. 2 PLS-DA score plots and corresponding VIP score plots for liver of polar bear (*Ursus maritimus*) from the Southern and Western Hudson Bay subpopulations of Canada in **a** the three-group comparison, **b** SHB females and SHB males, and **c** SHB males and WHB males. Only the top 15 VIP metabolites are shown (all of the VIP identified metabolites are shown in Figure S3). Numbers in parentheses on the axes of the score plots are the percentage of the variance explained by that component. The *red*, *beige* and *green* boxes on the *right side* of the VIP plots indicate the relative difference in concentrations between the groups. Sample sizes were SHB females = 9, SHB males = 15, and WHB males = 14. *SHB-F* Southern Hudson Bay females (*blue*), *SHB-M* Southern Hudson Bay males (*orange*), *WHB-M* Western Hudson Bay males (*purple*). Sample sizes were SHB females = 9, SHB males = 15, and WHB males = 14. Note that direct comparisons between the SHB females and WHB males are restricted to the Online Resources

omega-3 (n-3) compounds [eicosapentaenoic acid (FA 20:5), a docosapentaenoic acid isomer (FA 22:5 n3c1), and docosahexaenoic acid (DHA, FA 22:6)], and one omega-6 (n-6) PUFA (arachidonic acid, FA 20:4) (Table S2). The LC-PUFAs were the greatest in SHB females, while Hex, PCs, and lysoPC C20:3 were the greatest in WHB males, with concentrations in SHB males consistently intermediate to the concentrations of these metabolites in bears from the other locations (Fig. S3).

In the two-group comparisons, only five metabolites were identified by VIP that also varied significantly in the SHB female–SHB male model, and they shared only Hex with the three-group and WHB male–SHB female

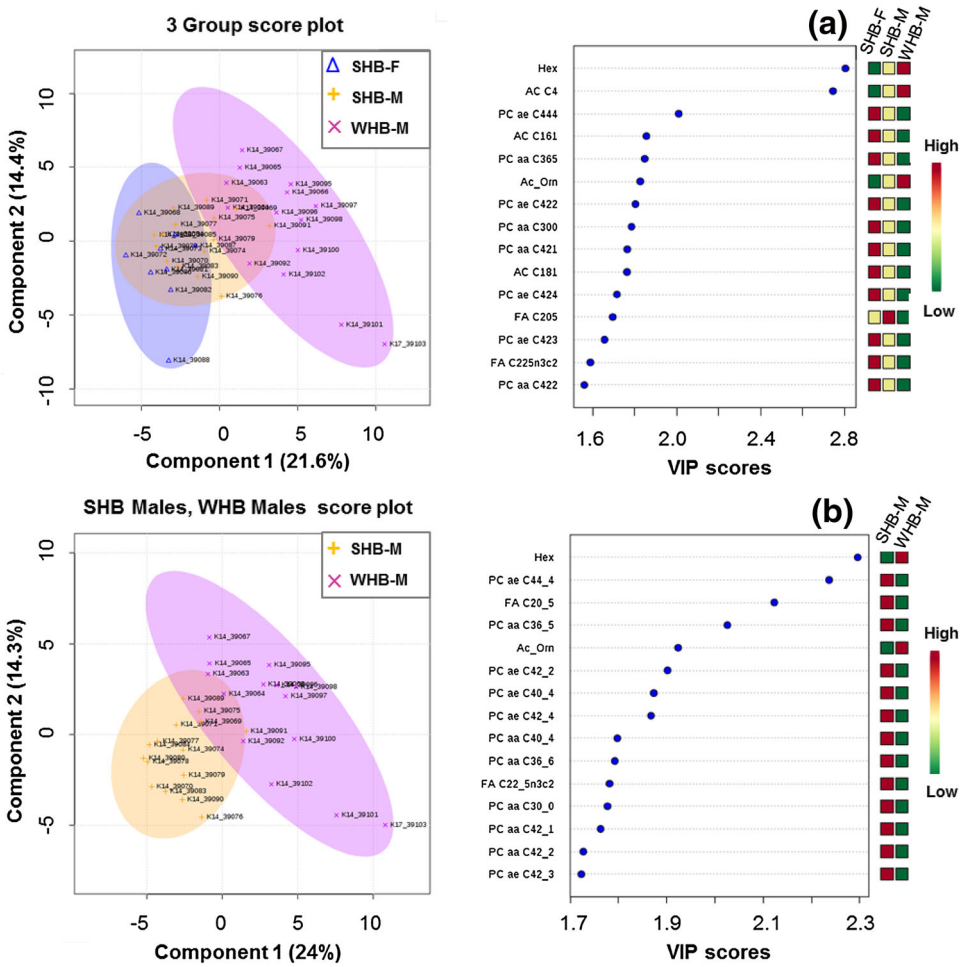

Fig. 3 PLS-DA score plots and corresponding VIP plots for muscle of polar bears (*Ursus maritimus*) for **a** the three-group comparison and **b** the comparison of SHB males with WHB males. Only the top 15 VIP metabolites are shown (all VIP metabolites for muscle are listed in Table S9). Numbers in parentheses on the axes of the score plots are the percentage of the variance explained by that component. The *red*, *beige* and *green* boxes on the *right side* of the VIP plots indicate the relative difference in concentrations between the groups. *SHB-F* Southern Hudson Bay females (*blue*), *SHB-M* Southern Hudson Bay males (*orange*), *WHB-M* Western Hudson Bay males (*purple*). Sample sizes were SHB females = 9, SHB males = 15, and WHB males = 14. Note that direct comparisons between the SHB females and WHB males are restricted to the Online Resources. No model could be validated for the comparison of SHB females and SHB males

comparisons as a discriminatory variable (Fig. S3). Unlike the three-group comparison, where groups were differentiated based primarily on FAs and membrane lipids, the VIPs for the SHB female and male comparison included two AAs [arginine (Arg) and glutamine (Gln)], a single diacyl PC that was unique to this comparison (PC aa C38:5), and a monounsaturated sphingomyelin (SM C24:1). The scores in the livers of the SHB and WHB males were discriminated based primarily on membrane lipids including diacyl (8) and acyl-alkyl (4) PCs, lysoPC a C20:3, and SM C24:0, as well as Leu—the only non-lipid, VIP compound for this comparison, and the only metabolite greater in the SHB males over the WHB bears.

Male bears from SHB and WHB had four of fifteen metabolites in common with the other models in which subpopulation was likely to be a significant contributing factor to the variation between groups (3-group comparison and the WHB males–SHB females; PC aa C34:2, PC aa C36:2, PC aa C38:3, lysoPC C20:3), but had no metabolites in common with the SHB male–female model. This unique discriminatory profile for the metabolites in male bears alone is indicative that sex was a contributing factor in the other comparisons as well as subpopulation. Likewise, the VIP-identified metabolites in the SHB male–female model were relatively unique (excluding Hex), providing a set of compounds in the liver that discriminate the bears from SHB based on gender.

Metabolomics profiling and modeling in muscle

The three-group, PLS-DA model of the metabolite profiles in the muscle was optimized using two components that explained 36% of the total variance (Component 1 = 21.6%, Component 2 = 14.4%) (Fig. 3, loadings are shown in Fig. S4). As in the liver, the profiles of the WHB males and SHB females clustered and separated well in the PLS-DA score plot (Fig. 3). However, the scores of the profiles for SHB males overlapped substantially with the 95% confidence range of both other groups of bears, particularly the SHB females (Fig. 3). The scores of the PLS-DA model showed that WHB had significantly different scores than males and females from the SHB across Component 1 (KW ANOVA on Ranks, $H = 26.7$,

$p < 0.001$; Dunn's tests = WHB males versus SHB females, $Q = 4.89$, $p < 0.05$; WHB males versus SHB males, $Q = 3.68$, $p < 0.05$); however, there was no significant variation across Component 2 between the groups (KW ANOVA on ranks, $H = 5.13$, $p = 0.077$).

No suitable PLS-DA model could be validated for the SHB male and female metabolite profiles in muscle tissue. Failure of PLS-DA models to validate is a result of limited variation in the concentrations between groups (Skelton et al. 2013), which was supported by the few significant differences in metabolite concentrations (Table S8), and the lack of significant differences in the PLS-DA scores between males and females from SHB. The data for the SHB males–WHB males resulted in a PLS-DA model that did discriminate the groups using two components and explained 38.3% of the variance in that dataset (Component 1 = 24%, Component 2 = 14.3%) (see Online Resources for SHB female–WHB male results).

The metabolites identified by VIP as significantly influencing the discrimination between the groups were different than the liver, though most were also lipid-related compounds. In the three-group assessment for muscle tissue, 40 metabolites had VIP scores exceeding one and were also significantly different between the groups (KW ANOVA on ranks with Dunn's test, $p < 0.05$) (Table S9). Of these, 30 metabolites (75%) were phosphatidylcholines, with 13 diacyl PCs and 17 acyl-alkyl PCs (18); the remaining metabolites identified by VIP were four ACs [AC C2, AC C4, AC C4-OH (C3-DC)], two FAs (FA 18:1, FA 20:5), two BgAs [acetyl ornithine (Ac-Orn), sarcosine], citrulline (Cit), and Hex. A smaller number of metabolites were identified by VIP analysis of the SHB and WHB males (37) and included Hex, Ac-Orn, one n-6 FA (FA 18:2), two n-3 FAs (FA 20:5, FA 22:5 n3c2), two short-chain ACs (AC C5, AC C5:1-DC), 12 diacyl PCs, 14 acyl-alkyl PCs, and lysoPC a 20:4 (Table S9).

The trends of concentrations of the metabolites varied considerably by class (i.e., sugars, AAs, FAs, etc.) in the three-group comparison, with Hex, AAs, and Ac-Orn being significantly greater in WHB male bears than SHB bears (Table S9). Fatty acids were significantly greater in SHB females than in WHB males, while the differences in males were based on structure, with the n-6 LC-PUFA (FA 18:2) being greater in the WHB, and the n-3 LC-PUFAs being greater in SHB males. Short-chain ACs were consistently greatest in WHB male bears compared with both sexes of SHB bears, while the lone long-chain AC identified by VIP (AC C18:1-OH) was greater in SHB females over WHB males. The diacyl PCs were greater in both sexes of SHB bears over WHB males, while acyl-alkyl PCs with fewer than 36 carbons were greater in WHB males than in either sex of SHB bears, and longer chain acyl-alkyl PCs were greater in SHB bears (Table S9). Again, as in the liver the confounding factors of sex and location make the interpretation of the results of the three-group comparison and that of the SHB females and WHB males limited. However, the profiles of ACs and PCs were different, and Cit, sarcosine, and FA 18:1 were part of the discriminatory metabolites when the females were included and may therefore be sex related.

Discussion

Age, stable isotopes, and diet

The significant differences in $\delta^{13}C$ and $\delta^{15}N$ were only found between the bears from the WHB and the SHB animals, and no differences were found between the SHB males and females (i.e., within the SHB) (Table 1). The differences in the $\delta^{13}C$ signatures indicate a difference in the carbon sources in the diet of the bears, while the differences in the $\delta^{15}N$ suggest that the WHB males are feeding on prey of a greater trophic level (and $\delta^{15}N$ signal) than the SHB females (and perhaps males, but to an insignificant degree) (Hobson and Welch 1992). Previous measurements of isotopes in SHB ($\delta^{15}N = 19.2‰$, $\delta^{13}C = -18.9‰$) and WHB bears ($\delta^{15}N = 18.9‰$, $\delta^{13}C = -18.8‰$) collected from 2007 to 2008 during a similar period (November–May) had less divergent isotopic signatures between the two subpopulations [difference (Δ) in isotope ratios = $\Delta\delta^{13}C = 0.090‰$; $\Delta\delta^{15}N = 0.34‰$] than those observed here ($\Delta\delta^{13}C = 0.60$–$0.90‰$, $\Delta\delta^{15}N = 0.70$–$1.1‰$), particularly for $\delta^{13}C$ (six- to tenfold differences; data are from the unadjusted isotope values in the Supporting Information of McKinney et al. 2011a). Also interesting to note is that these previous measurements showed slightly greater $\delta^{15}N$ signatures in the SHB over the WHB, while the opposite was true here (Table 1) (McKinney et al. 2011a).

The stable isotope values measured in both sexes of SHB bears in the present study were significantly more ^{13}C depleted ($\delta^{13}C$ = One-way ANOVA with Tukey's post hoc test, $F_{(2,\ 42)} = 25.5$; McKinney versus SHB females = $q = 8.96$ and $p < 0.001$; McKinney versus SHB males = $q = 7.54$, $p < 0.001$) and ^{15}N depleted ($\delta^{15}N$ = One-way ANOVA with Tukey's post hoc test, $F_{(2,\ 42)} = 12.7$, McKinney versus SHB females = $q = 6.30$ and $p < 0.001$; McKinney versus SHB males = $q = 5.33$, $p = 0.002$) than previous measurements from 2007 to 2008 (McKinney et al. 2011a). However, there were no significant differences between WHB male bears here and those reported by McKinney et al. (2011a) (two-tailed t tests, $t = 1.08$ and -0.0274; $p = 0.290$ and 0.978 for $\delta^{13}C$ and $\delta^{15}N$, respectively)

(McKinney et al. 2011a). Together, these time-point differences may indicate a measurable shift to a more ^{13}C- and ^{15}N-depleted diet in the SHB over the 6-year period (Rounick and Winterbourn 1986). This shift could be due to dietary changes that may include terrestrial or freshwater food sources (Rounick and Winterbourn 1986), or increased proportions of ^{13}C- and ^{15}N-depleted marine prey items (e.g., harbor seals (*Phoca vitulina*) rather than ringed seals) (McKinney et al. 2011a) in the SHB over the WHB.

This hypothesis is in contrast to previous studies on 2007–2008 harvested bears where harbor seals were found to be a more substantial portion of the WHB polar bear diet over SHB (Thiemann et al. 2008; McKinney et al. 2011b), and others that suggest increased terrestrial foraging in the WHB (Gormezano and Rockwell 2013a). Unfortunately, most of these assessments have not included the SHB animals for comparison. However, the estimates for prey species in the WHB are outdated and more recent data for the 2013–2014 time point are not available to compare the diet shift of SHB and WHB polar bears. Food chain composition as well as resource utilization in each polar bear subpopulation may now be quite different from previous years (Thiemann et al. 2008). Since the majority of the diet of polar bears is typically blubber, a difference in dietary sources between the SHB and WHB would likely affect lipids and lipid-related metabolites most prominently, which is supported by the results of the metabolomics analyses. Correlations of metabolites with δ^{13}C and δ^{15}N will be explored in the following sections as evidence of potential dietary influences over the discriminatory profile of metabolites.

Metabolomics analysis

Targeted and untargeted analyte approaches to metabolomics profile analysis have different advantages and disadvantages (Menni et al. 2017). The targeted metabolomics platform that is presently applied measures metabolites largely related to bioenergetics, which can be affected by a number of different natural or anthropogenic factors in the environment (Benskin et al. 2014; Huang et al. 2016, 2017). The targeted analyte approach offers a degree of specificity as detailed concentration profiles for analytes are generated relatively quickly with high precision and accuracy (Alonso et al. 2015). Untargeted analyte methods assess the overall variation in the metabolome of a given tissue or fluid and can thus capture unexpected changes and variation, but with less accuracy or precision than targeted methods (Bundy et al. 2009). The ability to identify unexpected shifts in a given metabolome is an advantage of untargeted methods (Menni et al. 2017).

The specificity and sensitivity of a targeted analyte approach was more valuable in the present study because we sought to describe differences in specific metabolites of closely oriented populations of polar bears and relate them where possible to functional effects and pathways (Bundy et al. 2009). Although beyond the scope of the present study, future studies will analyze these metabolomic data concurrently with detailed contaminant profiles to reveal covariance/correlation and other relationships between contaminants and these endogenous metabolites. Again, accurate concentrations are most useful for statistical modeling for those purposes. Admittedly, although the metabolites selected here were sufficient to discriminate the profiles of the bears in most cases, untargeted methods could reveal other interesting and discriminatory differences. These could be useful when developing other metabolomics platforms for these animals, but were beyond the objectives of the present study.

There are few studies that have specifically investigated field-based metabolomics, and even fewer that relate to biota and Arctic wildlife, or even large mammals. There is evidence that data from field-based studies can reveal shifts in metabolites that can be altered and masked in laboratory investigations as was observed in mussels exposed to hypoxia (Hines et al. 2007), giving unique value to field-based metabolomic investigations. Published studies of metabolomics in bears in the literature include measurements of the milk metabolome of captive giant pandas shifting throughout lactation (Zhang et al. 2015), and in wild black bears, a suite of hepatic metabolites associated with energetics and protein catabolism/synthesis differed between adults and subadults (Niemuth and Stoskopf 2014). To our knowledge, there is only one recent publication on Arctic biota using metabolic fingerprinting (Hansen et al. 2013). That study used a combination of metabolomics tools including ^{1}H-NMR and LC–MS to identify species-specific shifts in metabolites and metabolomic fingerprints in the three Arctic copepods *Calanus finmarchicus*, *Calanus glacialis*, and *Calanus hyperboreus*.

Sex-influenced differences in hepatic metabolite profiles

The discriminatory metabolites in the comparison of the male and female bears from SHB included Hex, Arg, Gln, PC aa C38:5, and SM C24:1. A previous investigation described gender-influenced differences in the metabolite profiles in plasma of polar bears from Svalbard, Norway, sampled in 2008 ($n = 70$) (Størseth et al. 2009). Qualitative assessment of nuclear magnetic resonances (^{1}H-NMR spectroscopy) identified a nearly identical pattern of metabolites to that observed in the present bear livers (Størseth et al. 2009). Hex (glucose only in Norwegian bears) and AA resonances were greater in male bears, while the lipid-related resonances were larger in female

polar bears (Størseth et al. 2009). In the same assessment, bears <5 years old had discernible but less discriminatory metabolite resonances than adult bears (Størseth et al. 2009). In the aforementioned ^{1}H-NMR assessment of hepatic metabolites in American black bears, it was found that the bears could be discriminated by age class based on AAs, glucose, FAs, carnitine, and other energy-related metabolites (Niemuth and Stoskopf 2014), supporting some of the observations in the Norwegian polar bears (Størseth et al. 2009).

In response to these previous results, we did also test for effects of age on the PLS-DA models, but there was insufficient variation to discriminate bears when they were subdivided by age class ($Q^2 < 0.20$, permutation $p > 0.05$). This may have been a function of sample size rather than actual statistical similarity between subadults and adults, as the subdivided groups were relatively small compared to those reported by Størseth et al. (2009) (see Table S1, bears ≤4 years of age were conservatively considered subadults of both sexes here (Committee on the Status of Endangered Wildlife in Canada COSEWIC 2008). Regardless, the combination of elevated Hex (glucose) and key AAs in males, in combination with greater concentrations or resonances of lipid-based metabolites in females, seems to be a consistent discriminatory pattern for sexes and possibly ages of multiple species of bear (Størseth et al. 2009; Niemuth and Stoskopf 2014). It is also worth noting that PC aa C38:5 was unique to this model and thus it may be useful for discrimination of the metabolite profiles in males and female polar bears.

Pathway analysis identified several potential physiological effects related to these metabolites; however, many of these did not have a significant impact on the results (Fig. 4; Table S5). The significant pathways identified in order of impact score included (VIP metabolites in each pathway are in parentheses) the following: (1) alanine, aspartate, and glutamate metabolism (Gln), (2) glycerophospholipid metabolism (PC aa C38:5), and (3) Arg and Pro metabolism (Gln, Arg). To a lesser extent, pathways including, (4) starch and sucrose metabolism (Hex) and (5) galactose metabolism (Hex) were significantly different between the male and females bears and had significant impact on the model (Fig. 4; Table S5). These pathways represent a wide range of physiological processes, including protein synthesis and catabolism (AAs), glycolysis (Hex), gluconeogenesis (Hex, AAs), and membrane homeostasis/cellular signalling/FA oxidation/triacyl glycerol synthesis (PC aa C38:5) (Stryer 1995; Buang et al. 2005).

The discriminatory metabolites identified by the VIP were variably correlated (Table S6a). Hex was weakly, positively correlated with Gln ($r_s^2 = 0.24$, $p = 0.016$, $n = 24$) and negatively correlated with PC aa C38:5 ($r_s^2 = 0.24$, $p = 0.016$, $n = 24$); Gln was also negatively correlated with PC aa C38:5 ($r_s^2 = 0.34$, $p = 0.003$, $n = 24$). Arg had a number of very weak ($r_s^2 < 0.20$) correlations, including a positive correlation with Gln ($r_s^2 = 0.19$, $p = 0.033$, $n = 24$), which may be due to their connection through nitrogen cycling (Stryer 1995; Bertolo and Burrin 2008). Nitrogen cycling affects Arg and Pro

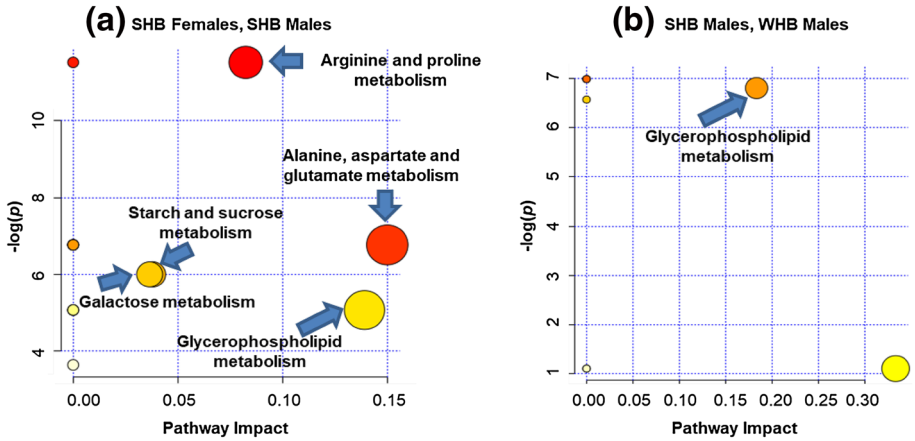

Fig. 4 Pathway analysis plot in liver of polar bears (*Ursus maritimus*) showing pathways identified as significantly impacted between groups based on the identified VIP metabolites for **a** comparisons of SHB female and SHB male bears, and **b** comparisons of male bears from the WHB and SHB. Statistical results are given in Table S5. Only pathways with significant Holm–Bonferroni adjusted *p*-values (<0.05) from pathway enrichment analysis and impact scores greater than zero from pathway topology analysis are labeled (see Table S5 for statistical results). Colors of the nodes correspond to *p* values (*red* highly significant, *white* non-significant) while their diameter is related to their impact score

metabolism as well as alanine, aspartate, and glutamate metabolism as indicated by pathway analysis (Fig. 4); however, given the multitude of physiological reactions that Gln participates in, this is a tenuous assumption (Stryer 1995; Bertolo and Burrin 2008). Correlations between Hex and Gln could be due to activation of AA-based gluconeogenic pathways, or other indirect associations, as they do intersect through several physiological processes (Newsholme et al. 2003). Polar bears do exhibit enhanced genetic signatures that suggest that their gluconeogenic and general energetic capacities are elevated compared to grizzly bears (*Ursus arctos*) (Welch et al. 2014). However, few data are available to describe how prevalent this process is, and how specific metabolite trends are related to these genetic differences.

The inverse correlations between PC aa C38:5, Hex, and the AAs, as well as the fact that lipid metabolites identified by the VIP were greater in females, while Hex and the AAs were greater in males (Fig. S3), could indicate different metabolic pathways or nutrient routing and storage between genders as has been observed in human plasma and urine (Kochhar et al. 2006). An unrelated study on adolescent humans found that the sexes could be discriminated by profiles of metabolites in the plasma; specifically, the glucose and AA concentrations were greater in males, and the unsaturated lipids and high-density lipoproteins were greater in females (Bertram et al. 2009). These patterns were attributed to greater lipid synthesis in (young) females and greater protein turnover in (young) males (Bertram et al. 2009)—and relationships in wildlife support these results (Størseth et al. 2009; Niemuth and Stoskopf 2014). Only one additional diacyl PC (PC aa C36:5) concentration was significantly different between the sexes of SHB bears and was again greater in the SHB females (Table S4). Theoretically, this may indicate a specific need for these PCs in females (for cellular signaling, membrane composition, or due to the aforementioned metabolic purposes), or greater utilization of the FAs composing the diacyl chains of these PCs in males, among other possibilities.

The structure suggested for each of these PCs by the conversion tool in MetaboAnalyst 3.0 indicates that the PCs can have FA 16:0 bound to the sn-1 position of glycerol, and an n-3 FA bound to the sn-2 position (FA 20:5 for PC C36:5 and FA 22:5 for PC aa C38:5) (Table S7); however, this is only one of many possible structural configurations for these PCs (see www.hmdb.ca for catalogues of PC structures). The concentrations of this suite of compounds are related in the SHB male–female dataset regardless; PC aa C38:5 and PC aa C36:5 had a strong, positive relationship (Spearman $r_s^2 = 0.78$, $p = 2.0 \times 10^{-7}$, $n = 24$), and both PCs were positively correlated to varying degrees with FA 20:5 and both n-3 isomers of FA 22:5 ($r_s^2 = 0.22$ to 0.50, $p = 6.1 \times 10^{-5}$ to 0.021, $n = 24$). One possible explanation for the decreased hepatic concentrations of n-3 FAs in males could be related to the need for the precursors for the synthesis of DHA (FA 20:5 and FA 22:5 among them), an important component of the plasma membranes of sperm and testicular cells (Stillwell and Wassall 2003).

Some metabolites did have relationships with other biological factors of importance in the SHB bears. Hex was the only metabolite to have any relationship with age (positive, $r_s^2 = 0.30$, $p < 0.01$, $n = 24$) (Table S6a). The age of polar bears has been found to be positively correlated with several metrics of size (Hensel and Sorensen 1980), therefore the relationship between Hex and age could simply be due to an effect of size and/or competition for resources (male bears are up to two times larger than females) (Derocher et al. 2005; Thiemann et al. 2006). As with Hex, elevated concentrations of Gln and Arg in males (Fig. S3) could also simply be due to differences in basic physiology and utilization of lipid-, protein-, and carbohydrate-related metabolic pathways between genders as is observed in humans (Kochhar et al. 2006). None of Hex, Gln, or Arg were significantly correlated with dietary tracers ($\delta^{13}C$ or $\delta^{15}N$), which themselves varied slightly between the sexes of SHB bears (Table 1), demonstrating a minimal dietary influence on these discriminatory metabolites. In fact, the only metabolite correlated with $\delta^{13}C$ (and hence diet selection) was an inverse relationship with PC aa C38:5 ($r_s^2 = 0.19$, $p = 0.04$, $n = 24$). Since ^{13}C enrichment is associated with marine food sources (i.e., a less negative $\delta^{13}C$) (Rounick and Winterbourn 1986; Kelly 2000), this could be a dietary effect of differential foraging between male and female bears. The females have an elevated concentration of this PC relative to males, and a slightly more ^{13}C-depleted $\delta^{13}C$ signature (Table 1); however, the difference in $\delta^{13}C$ values was small and not significant at 0.30‰ (KW ANOVA on ranks, $H = 14.9$, $p < 0.001$; Dunn's test = Q 1.15, $p > 0.05$). These results are thus inconclusive at this point, largely because the exact composition of the acyl or alkyl chains of the PCs and the metabolite profiles of dietary items for polar bears are not known.

Overall, the effects of diet on the VIP-identified metabolites in the sex-specific comparison were limited, as indicated by a lack of correlation between the metabolite concentrations with $\delta^{13}C$ and $\delta^{15}N$, and that the differences between male and female stable isotope values were not significant (Table 1). The $\delta^{13}C$ was significantly correlated with the only VIP-identified PC, which indicates that the limited dietary influence was most closely related to lipids, which is logical given the blubber-rich diet of polar bears for the majority of their life-cycle (Thiemann et al. 2008; Gormezano and Rockwell 2013b).

Subpopulation-influenced differences in hepatic metabolite profiles

Location-specific comparisons of SHB and WHB males demonstrated strong lipid dependence in their discrimination in the PLS-DA, particularly in relation to the diacyl PCs which comprised more than 50% of the VIP-identified metabolites (Fig. S3). In contrast to the SHB male–female model, only one non-lipid metabolite was identified, leucine (Leu). Leu is an essential AA (not synthesized de novo and taken up strictly from the diet) and is a major component of many proteins (Stryer 1995). Leu can also be a significant source of acetyl coenzyme A, which is crucial for energy production through the metabolism of proteins, carbohydrates, and lipids (Stryer 1995). Leucine was the only metabolite identified by the VIP that was significantly greater in SHB males relative to WHB males (Mann–Whitney U test, $U = 58.00$, $p = 0.042$, $n = 29$), which were themselves enriched in PCs, lysoPC a C20:3, and the unsaturated SM C24:0 (Fig. S3).

Despite the identification of Leu by the VIP, only glycerophospholipid metabolism had a significant p value and a measurable impact in the pathway model (Fig. 4; Table S5). As the exact composition of the PCs is unknown (chain length and degree of saturation indicated in the compound names are applied across the diacyl or acyl-alkyl chains), and a number of isomers can have the same formula (Ekroos et al. 2003), interpretation is somewhat limited. Investigations applying the same metabolomics platform to teleost fishes undergoing intensive migration (Benskin et al. 2014) or to fish exposed to contaminants in laboratory studies (Huang et al. 2016, 2017) have noted large-scale changes in PCs and lysoPCs, with contaminants also affecting a number of SMs. Given the crucial role of PCs and related lipids in plasma membrane structure, cellular signaling, and transport of lipoproteins, potential effects regarding these metabolites can be extensive (Cole et al. 2012).

Although the specific composition of the PCs is unknown, many were correlated with other PCs to some degree varying from very strong to very weak relationships (Table S6b). The 34- and 36-carbon diacyl PCs (PC aa C34:2, PC aa C36:2, PC aa 36:3) were particularly strongly related ($r_s^2 = 0.71$–0.83, $p = 2.0 \times 10^{-7}$, $n = 29$), as was PC aa C38:2 with those three compounds and PC aa C38:0 ($r_s^2 = 0.62$–0.78, $p = 2.0 \times 10^{-7}$, $n = 29$), which is likely to be related to the composition of the acyl chains (Table S7). LysoPCs are deacylated products of PCs formed via phospholipase enzymes (Paapstel et al. 2016). They continually alter the composition of the plasma membrane by changing the composition of the fatty acyl or alkyl chains on the PCs (Christie 2016), so relationships between lysoPCs and PCs were anticipated (Christie 2016). LysoPC C20:3 was significantly correlated to varying degrees with several unsaturated PCs (PC aa C36:2, PC aa C36:3, and PC aa C38:3; $r_s^2 = 0.30$–0.67, $p = 2.0 \times 10^{-7}$–0.0022, $n = 29$), which can utilize FA 20:3 in their structures or could be related through precursors (Table S7). PCs are also precursors to SMs and associate with them in some lipoprotein complexes for transport of lipids in blood, as well as in plasma membranes (Christie 2016). Accordingly, several of the PCs were significantly correlated with SM C24:0, particularly the saturated, longer chain diacyl and acyl-alkyl PCs ($r_s^2 = 0.54$–0.61, $p = 2.0 \times 10^{-7}$–7.3×10^{-6}, $n = 29$), though weaker relationships with unsaturated PCs were also apparent (Table S6b). This particular SM was identified among a suite of SMs associated with obesity issues in humans (Hanamatsu et al. 2014), which suggests a significant role of this SM in lipid metabolism among other potential pathways (Fig. 4).

In contrast to the gender-related comparisons, $\delta^{13}C$ was correlated with a number of metabolites in this subset of data, including negative relationships with Leu ($r_s^2 = 0.29$, $p = 0.0029$, $n = 29$), and moderate, positive relationships with four diacyl PCs (range of $r_s^2 = 0.25$–0.30, $p = 0.002$–0.0057, $n = 29$) and an acyl-alkyl PC (PC ae C36:2), though that relationship was weak ($r_s^2 = 0.21$, $p = 0.013$, $n = 29$) (Table S6b). Only weak relationships ($r_s^2 < 0.25$) were observed between metabolites and $\delta^{15}N$, though they were relatively consistent with $\delta^{13}C$ trends (Leu was negatively related with $\delta^{15}N$, and diacyl PCs were positively related). The more ^{13}C- and ^{15}N-depleted signals were related to increasing amounts of leucine (as in the SHB males), while enrichment of ^{13}C and ^{15}N (to a lesser degree) was associated with increasing concentrations of PCs (as in the WHB males), suggesting that the dietary sources of these metabolites are different. Leucine is rich in a number of foods, including proteins such as beef, chicken and fish, nuts and seeds, as well as soy and other beans, but no estimates of concentrations in seal blubber exist—though it is the second most abundant essential AA in the muscle of some seal species (Brunborg et al. 2006). However, its association with more ^{13}C-depleted $\delta^{13}C$ signatures may again indicate more terrestrial or freshwater influences in the diet of SHB bears (Rounick and Winterbourn 1986; Kelly 2000). The greater $\delta^{13}C$ and $\delta^{15}N$ signatures in the WHB were associated with greater concentrations of PCs, which could indicate continued exploitation of marine resources, particularly blubber of ringed seals (Rounick and Winterbourn 1986; Kelly 2000). However, without samples of ringed seals from this region sampled within the same year and season, this cannot be confirmed.

Subpopulation-influenced differences in metabolomic profiles of the muscle

Even though the profiles were discriminated well based on subpopulation in both the muscle and liver, very few metabolites identified by the VIP analysis were common between tissues (compare Fig. S3 and Table S9). The discriminatory metabolites in the muscle between the SHB and WHB males included Hex, Ac-Orn, FA 18:2 (n-3), FA 20:5 (n-6), FA 22:5n3c2, 2 short-chain ACs (AC C5, AC C5:1-DC), 12 diacyl PCs, and 14 acyl-alkyl PCs, and lysoPC a C20:4. In contrast to the liver, where all metabolites identified by the VIP analyses were greater in the WHB over SHB males, the concentrations of the PCs tended to be the greatest in the muscle of SHB males along with the n-3 LC-PUFAs, while Hex, FA 18:2 (n-6), and the ACs were the greatest in WHB males along with one acyl-alkyl PC (PC ae 34:3) (Table S9). The VIP-identified metabolites in the muscle are related to carbohydrate and lipid metabolism and energetics as well as cellular signalling (Hex, PCs, ACs, FAs, lysoPC C20:4) or with arginine/AA biosynthesis and catabolism (Ac-Orn) (Stryer 1995). The major pathways affected in the comparison between the male bears from each location were (1) arginine and proline metabolism (Ac-Orn) and (2) glycerophospholipid metabolism (PCs), and minor effects were observed for (3) starch and sucrose metabolism (Hex) and (4) galactose metabolism (Hex) (Fig. 5; Table S10).

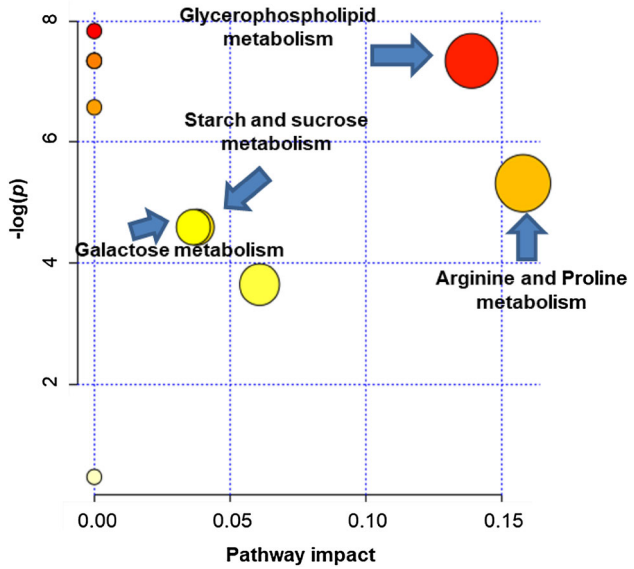

Fig. 5 Pathway analysis plot in muscle of polar bears (*Ursus maritimus*) showing pathways identified as significantly impacted in the comparison of VIP-metabolite concentrations between male bears from SHB and WHB. Only pathways with significant Holm–Bonferroni adjusted *p* values (<0.05) from pathway enrichment analysis and impact scores greater than zero from pathway topology analysis are labeled (see Table S10 for statistical results). Colors of the nodes correspond to *p*-values (*red* highly significant, *white* non-significant) while their diameter is related to their impact score

Relationships between metabolites and between metabolites, age, and stable isotopes were abundant among the VIP-identified compounds in the muscle. As in the liver, the PCs were related to trends in $\delta^{13}C$ and $\delta^{15}N$ (Table S6c). However, in contrast to the hepatic results, some diacyl and acyl-alkyl PCs identified by VIP were negatively correlated with the stable isotopes of carbon ($\delta^{13}C$: $r_s^2 = 0.14–0.30$, $p = 0.0024–0.042$, $n = 29$) and nitrogen ($\delta^{15}N$: $r_s^2 = 0.15–0.28$, $p = 0.0030–0.038$, $n = 29$), though most of the relationships were considered weak ($r_s^2 < 0.25$) (Asuero et al. 2006) (Table S6c). PC ae C34:3 behaved differently, as it had a positive relationship with $\delta^{13}C$ ($r_s^2 = 0.34$, p = 9.2×10^{-4}, $n = 29$) and a very weak positive relationship with $\delta^{15}N$ ($r_s^2 = 0.15$, $p = 0.038$, $n = 29$). PC ae C34:3 was also the only PC that was negatively correlated with other PCs to varying degrees ($r_s^2 = 0.15–0.39$, $p = 0.0003–0.037$, $n = 29$); the remaining diacyl and acyl-alkyl PCs identified by VIP tended to be significantly, positively correlated with each other. The only structure of PC ae C34:3 in the HMDB contains linoleic acid (FA 18:2, Table S7) bound in the acyl (sn-2) position, and these two compounds were positively correlated through a relatively weak relationship ($r_s^2 = 0.23$, $p = 0.0094$, $n = 29$). Linoleic acid is a dietarily essential n-6 FA and an important precursor to other n-6 FAs, including arachidonic acid (FA 20:4) which is used to produce eicosanoids during inflammatory response among other functions (though arachidonic acid was not different between bears, Table S8) (Schmitz and Ecker 2008). It is possible that PC ae C34:3 is preferentially catabolized to synthesize new FAs and PCs or is synthesized itself depending on the abundance of linoleic acid given its dietary limitations; however, this hypothesis is speculative at this point due to lack of comparative data.

Interestingly, acylcarnitines did not have any significant relationships with FAs (the substrate for β-oxidation pathways); however, AC C5:1-DC was positively correlated with PC ae C34:3 ($r_s^2 = 0.40$, $p = 1.9 \times 10^{-4}$, $n = 29$) while being negatively correlated with several other longer chained acyl-alkyl PCs (Table S6c). It is likely that these differential relationships with the PCs are related to the chain structure of the FAs bound to the membrane lipid, and their potential for use as substrates for β-oxidation in the muscle or in other energetic pathways. The n-3 PUFAs also had moderate to strong, positive correlations with the shorter chained diacyl PCs (36 carbons or less, $r_s^2 = 0.36–0.64$, $p = 2.0 \times 10^{-7}–5.1 \times 10^{-4}$, $n = 29$), but very few with acyl-alkyl PCs (Table S6c), which is indicative of the fact that FA binding to PCs is a selective and dynamic process (Cole et al. 2012; Christie 2016). The non-PC compounds (Hex,

Ac-Orn, n-3 FAs) had relatively weak, negative relationships among them ($r_s^2 = 0.14$–0.30, $p = 0.0024$–0.049, $n = 29$). These inverse relationships that could be due to the differential activation of metabolic pathways that use FAs, proteins, and Hex for energy or for storage of energy (Ac-Orn is a substrate or product in a number of reactions involving glutamate and other TCA cycle intermediates) (Stryer 1995).

These data taken together again signify a strong influence of diet on the discrimination of the metabolite profiles in the muscle between the subpopulations, which is supported by the differences between SHB females and WHB males (see Online Resources). Although sex had a clear and separate effect in the liver, this was not apparent in the muscle, as the SHB females and males could not be discriminated. The functional differences of the liver (processing and cycling of nutrients and enzymes) and muscle (utilization of nutrients for energy) are the most likely reason for these differences (Stryer 1995). Although there are differences in the size, range, and movement of male and female polar bears (Derocher et al. 2005; Laidre et al. 2013), the mechanics of locomotion and hence the metabolites consumed or produced in the muscle tissue would logically be comparable. In contrast, the metabolites in the liver vary by sex due to differences in the physiological needs of males and females, and sexual dimorphism in the liver of mammals has been a well-known fact for many years (Yates et al. 1958; Colby 1980; Waxman and Celenza 2003). The contrary relationships of $\delta^{13}C$ with PCs in the liver (positively correlated) and muscle (inversely correlated) are likely a result of nutrient routing and functions of the specific tissues in cycling lipid-related compounds and utilizing them for energy, respectively. Comparisons between compartments are further complicated by turnover times of nutrients, which can vary considerably between tissues, with the muscle turning over more slowly than the liver (though specific turnover times vary by species) (Tieszen et al. 1983; Sponheimer et al. 2006).

Applications of the metabolomics platform and future directions

The targeted metabolomics platform applied here included 156 metabolites in the final statistical models (219 analytes screened) and was successful in the discrimination of groups of polar bears based on sex (liver only) and location/subpopulation (muscle and liver), producing a unique profile of metabolites for each comparison. The majority of metabolites identified as varying significantly between subpopulations were lipid related, and many are likely due to differences in diet or differences in the composition of the food web. This assertion was supported here by differences in $\delta^{13}C$ signatures between SHB and WHB, and by correlations of VIP-identified metabolites that differentiated subpopulation but not the sexes with $\delta^{13}C$. It is worth noting that the variables that discriminated the SHB males and females found in the liver here compare well with those measured in plasma of Norwegian polar bears (Størseth et al. 2009). If the results can be confirmed to be comparable by using the targeted platform to analyze plasma samples with the liver samples from the same animals, the collection of plasma may be preferable as it does not involve traditional hunting of the bears and thus can result in larger sample sizes.

Although beyond the scope of the present study, being able to account for the dietary influence on the metabolome by using paired samples of prey species and polar bear tissues could allow for broader applications of the metabolomics platform to identify effects related to other stressors that could be exhibiting measurable effects. Additional samples from more distant subpopulations such as those from high Arctic animals would also be useful for elucidation of the sources of variation in the metabolome of polar bears. Accounting for subpopulation differences at the least is also important for future studies, though finer detailed locations (global positioning system coordinates) could also be used for more detailed modeling of both polar bears and potential prey items. Finally, controlled feeding studies with captive bears could provide unique and useful data regarding diet changes and effects on this suite of metabolites.

Acknowledgements We thank our indigenous coworkers in the North who carried out the traditional polar bear hunts and allowed samples to be collected from these animals. We also acknowledge our coworkers and technicians at Environment and Climate Change Canada (National Wildlife Research Centre, Letcher Laboratory), SGS AXYS, and with the Government of Nunavut. Susie Huang and Heather Butler (SGS AXYS) provided valuable feedback on the manuscript and statistical treatment of the data. We also thank the Northern Contaminants Programme [Indigenous and Northern Affairs Canada (INAC), Grant No. INAC-NCP-M05] for funding and supporting this project.

References

Aliferis KA, Chrysayi-Tokousbalides M (2011) Metabolomics in pesticide research and development: review and future perspectives. Metabolomics 7:35–53. doi:10.1007/s11306-010-0231-x

Alonso A, Marsal S, Julia A (2015) Analytical methods in untargeted metabolomics: state of the art in 2015. Front Bioeng Biotechnol 3:1–20. doi:10.3389/fbioe.2015.00023

Asuero A, Sayago A, González AG (2006) The correlation coefficient: an overview. Crit Rev Anal Chem 36:41–59

Benskin JP, Ikonomou MG, Liu J, Veldhoen N, Dubetz C, Helbing CC, Cosgrove JR (2014) Distinctive metabolite profiles in in-migrating sockeye salmon suggest sex-linked endocrine perturbation. Environ Sci Technol 48:11670–11678. doi:10.1021/es503266x

Bertolo RF, Burrin DG (2008) Comparative aspects of tissue glutamine and proline metabolism. J Nutr 138:2032S–2039S

Bertram HC et al (2009) Nuclear magnetic resonance-based metabonomics reveals strong sex effect on plasma metabolism in 17-year-old Scandinavians and correlation to retrospective infant plasma parameters. Metab Clin Exp 58:1039–1045. doi:10.1016/j.metabol.2009.03.011

Boertje RD, Ellis MM, Kellie KA (2015) Accuracy of moose age determinations from canine and incisor cementum annuli. Wildl Soc B 39:383–389. doi:10.1002/wsb.537

Brunborg LA, Julshamn K, Nortvedt R, Frøyland L (2006) Nutritional composition of blubber and meat of hooded seal (*Cystophora cristata*) and harp seal (*Phagophilus groenlandicus*) from Greenland. Food Chem 96:524–531. doi:10.1016/j.foodchem.2005.03.005

Buang Y, Wang YM, Cha JY, Nagao K, Yanagita T (2005) Dietary phosphatidylcholine alleviates fatty liver induced by orotic acid. Nutrition 21:867–873. doi:10.1016/j.nut.2004.11.019

Bundy JG, Davey MP, Viant MR (2009) Environmental metabolomics: a critical review and future perspectives. Metabolomics 5:3–21. doi:10.1007/s11306-008-0152-0

Cai X, Li R (2016) Concurrent profiling of polar metabolites and lipids in human plasma using HILIC-FTMS. Sci Rep 6:36490. doi:10.1038/srep36490

Christie W (2016) Phosphatidylcholine. The American Oil Chemists Society. http://lipidlibrary.aocs.org/Primer/content.cfm?ItemNumber=39351. Accessed 14 Dec 2016

Clarke CJ, Haselden JN (2008) Metabolic profiling as a tool for understanding mechanisms of toxicity. Toxicol Pathol 36:140–147. doi:10.1177/0192623307310947

Colby HD (1980) Regulation of hepatic drug and steroid metabolism by androgens and estrogens. Adv Sex Horm Res 4:27–71

Cole LK, Vance JE, Vance DE (2012) Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochim Biophys Acta 1821:754–761. doi:10.1016/j.bbalip.2011.09.009

Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (2008) COSEWIC assessment and update status report on the polar bear (*Ursus maritimus*) in Canada. COSEWIC, Ottawa

Derocher AE, Lunn NJ, Stirling I (2004) Polar bears in a warming climate. Integr Comp Biol 44:163–176. doi:10.1093/icb/44.2.163

Derocher AE, Andersen M, Wiig Ø (2005) Sexual dimorphism of polar bears. J Mammal 86:895–901. doi:10.1644/1545-1542(2005)86[895:SDOPB]2.0.CO;2

Dunn WB, Ellis DI (2005) Metabolomics: current analytical platforms and methodologies. Trends Anal Chem 24:285–294. doi:10.1016/j.trac.2004.11.021

Ekroos K, Ejsing CS, Bahr U, Karas M, Simons K, Shevchenko A (2003) Charting molecular composition of phosphatidylcholines by fatty acid scanning and ion trap MS3 fragmentation. J Lipid Res 44:2181–2192. doi:10.1194/jlr.D300020-JLR200

Ellis RP, Spicer JI, Byrne JJ, Sommer U, Viant MR, White DA, Widdicombe S (2014) ^{1}H NMR metabolomics reveals contrasting response by male and female mussels exposed to reduced seawater pH, increased temperature, and a pathogen. Environ Sci Technol 48:7044–7052. doi:10.1021/es501601w

Environment and Climate Change Canada (ECCC) (2014) Maps of global and Canadian sub-populations of polar bears and protected areas. ECCC. https://www.ec.gc.ca/nature/default.asp?lang=En&n=F77294A3-1. Accessed 18 Jul 2016

Gormezano LJ, Rockwell RF (2013a) Dietary composition and spatial patterns of polar bear foraging on land in western Hudson Bay. BMC Ecol 13:51. doi:10.1186/1472-6785-13-51

Gormezano LJ, Rockwell RF (2013b) What to eat now? Shifts in polar bear diet during the ice-free season in western Hudson Bay. Ecol Evol 3:3509–3523. doi:10.1002/ece3.740

Hanamatsu H et al (2014) Altered levels of serum sphingomyelin and ceramide containing distinct acyl chains in young obese adults. Nutr Diabetes 4:e141. doi:10.1038/nutd.2014.38

Hansen BH, Degnes K, Øverjordet IB, Altin D, Størseth TR (2013) Metabolic fingerprinting of arctic copepods *Calanus finmarchicus*, *Calanus glacialis* and *Calanus hyperboreus*. Polar Biol 36:1577–1586

Hensel RJ, Sorensen FE (1980) Age determination of live polar bears. Bears 4:93–100

Hines A, Oladiran GS, Bignell JP, Stentiford GD, Viant MR (2007) Direct sampling of organisms from the field and knowledge of their phenotype: key recommendations for environmental metabolomics. Environ Sci Technol 41:3375–3381

Hobson KA, Welch HE (1992) Determination of trophic relationships within a high Arctic food web using δ13C and δ15 N anaylsis. Mar Ecol Prog Ser 84:9–18

Huang SS, Benskin JP, Chandramouli B, Butler H, Helbing CC, Cosgrove JR (2016) Xenobiotics produce distinct metabolomic responses in zebrafish larvae (*Danio rerio*). Environ Sci Technol 50:6526–6535. doi:10.1021/acs.est.6b01128

Huang SSY, Benskin JP, Veldhoen N, Chandramouli B, Butler H, Helbing CC, Cosgrove JR (2017) A multi-omic approach to elucidate low-dose effects of xenobiotics in zebrafish (*Danio rerio*) larvae. Aquat Toxicol 182:102–112. doi:10.1016/j.aquatox.2016.11.016

Idle JR, Gonzalez FJ (2007) Metabolomics. Cell Metab 6:348–351. doi:10.1016/j.cmet.2007.10.005

Kelly JF (2000) Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. Can J Zool 78:1–27

Kochhar S, Jacobs DM, Ramadan Z, Berruex F, Fuerholz A, Fay LB (2006) Probing gender-specific metabolism differences in humans by nuclear magnetic resonance-based metabonomics. Anal Biochem 352:274–281. doi:10.1016/j.ab.2006.02.033

Laidre KL, Born EW, Gurarie E, Wiig O, Dietz R, Stern H (2013) Females roam while males patrol: divergence in breeding season movements of pack-ice polar bears (*Ursus maritimus*). Proc Biol Sci 280:20122371. doi:10.1098/rspb.2012.2371

Letcher RJ, Gebbink WA, Sonne C, Born EW, McKinney MA, Dietz R (2009) Bioaccumulation and biotransformation of brominated and chlorinated contaminants and their metabolites in ringed seals (*Pusa hispida*) and polar bears (*Ursus maritimus*) from East Greenland. Environ Int 35:1118–1124. doi:10.1016/j.envint.2009.07.006

Letcher RJ et al (2010) Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. Sci Total Environ 408:2995–3043. doi:10.1016/j.scitotenv.2009.10.038

Lin CY, Viant MR, Tjeerdema RS (2006) Metabolomics: methodologies and applications in the environmental sciences. J Pestic Sci 31:245–251

Matson G, Van Daele L, Goodwin E, Aumiller L, Reynolds H, Hristienko H (1993) A laboratory manual for cementum age determination of Alaska brown bear first premolar teeth. Alaska Department of Fish and Game, Anchorage

McKinney MA et al (2011a) Regional contamination versus regional dietary differences: understanding geographic variation in brominated and chlorinated contaminant levels in polar bears. Environ Sci Technol 45:896–902. doi:10.1021/es102781b

McKinney MA et al (2011b) Flame retardants and legacy contaminants in polar bears from Alaska, Canada, East Greenland and Svalbard, 2005–2008. Environ Int 37:365–374. doi:10.1016/j.envint.2010.10.008

McKinney MA et al (2012) Trophic transfer of contaminants in a changing arctic marine food web: Cumberland Sound, Nunavut, Canada. Environ Sci Technol 46:9914–9922. doi:10.1021/es302761p

McMeans BC, Arts MT, Olin JA, Benz GW (2009) Stable-isotope comparisons between embryos and mothers of a placentatrophic shark species. J Fish Biol 75:2464–2474

Menni C, Zierer J, Valdes AM, Spector TD (2017) Mixing omics: combining genetics and metabolomics to study rheumatic diseases. Nat Rev Rheumatol 13:174–181. doi:10.1038/nrrheum.2017.5

Morris AD, Letcher RJ, Dyck MG, Chandramouli B, Cosgrove JR (2016) Investigating relationships between contaminant and metabolomics profiles in polar bears (*Ursus maritimus*) from the Hudson Bay Region of Canada. Society of Environmental Toxicology and Chemistry, 7th World Congress, Orlando, FL, USA

Muir DC et al (2006) Brominated flame retardants in polar bears (*Ursus maritimus*) from Alaska, the Canadian Arctic, East Greenland, and Svalbard. Environ Sci Technol 40:449–455

Muir DCG et al (2013) Occurrence and trends in the biological environment. In: Muir DCG, Kurt-Karakus P, Stow J (eds) Canadian arctic contaminants assessment report (CACAR) on persistent organic pollutants—2013. Aboriginal Affairs and Northern Development Canada, Ottawa, pp 273–422

Newsholme P, Lima MMR, Procopio J, Pithon-Curi TC, Doi SQ, Bazotte RB, Curi R (2003) Glutamine and glutamate as vital metabolites. Braz J Med Biol Res 36:153–163

Niemuth JN, Stoskopf MK (2014) Hepatic metabolomic investigation of the North American black bear (*Ursus americanus*) using ^{1}H-NMR spectroscopy. Wildl Biol Pract 10:14–23. doi:10.2461/wbp.2014.10.3

O'Kane AA, Chevallier OP, Graham SF, Elliot CT, Mooney MH (2013) Metabolomic profiling of *in vivo* plasma responses to dioxin-associated dietary contaminant exposure in rats: implications for identification of sources of animal and human exposure. Environ Sci Technol 47:5409–5418. doi:10.1021/es305345u

Paapstel K, Kals J, Eha J, Tootsi K, Ottas A, Piir A, Zilmer M (2016) Metabolomic profiles of lipid metabolism, arterial stiffness and hemodynamics in male coronary artery disease patients. IJC Metab Endocr 11:13–18. doi:10.1016/j.ijcme.2016.05.001

Rounick JS, Winterbourn MJ (1986) Stable carbon isotopes and carbon flow in ecosystems. Bioscience 36:171–177. doi:10.2307/1310304

Sadler T, Kuster C, von Elert E (2014) Seasonal dynamics of chemotypes in a freshwater phytoplankton community—a metabolomic approach. Harmful Algae 39:102–111

Samuelsson LM, Bjorlenius B, Forlin L, Larsson DGJ (2011) Reproducible ^{1}H NMR-based metabolomic responses in fish exposed to different sewage effluents in two separate studies. Environ Sci Technol 45:1703–1710. doi:10.1021/es104111x

Schmitz G, Ecker J (2008) The opposing effects of n-3 and n-6 fatty acids. Prog Lipid Res 47:147–155. doi:10.1016/j.plipres.2007.12.004

Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA (2016) Untargeted metabolomics strategies-challenges and emerging directions. J Am Soc Mass Spectrom 27:1897–1905. doi:10.1007/s13361-016-1469-y

Skelton DM, Ekman DR, Martinovic-Weigelt D, Ankley GT, Villeneuve DL, Teng Q, Collette TW (2013) Metabolomics for in situ environmental monitoring of surface waters impacted by contaminants from both point and nonpoint sources. Environ Sci Technol 48:2395–2403. doi:10.1021/es404021f

Sponheimer M et al (2006) Turnover of stable carbon isotopes in the muscle, liver, and breath CO_2 of alpacas (*Lama pacos*). Rapid Commun Mass Spectrom 20:1395–1399

Stillwell W, Wassall SR (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid. Chem Phys Lipids 126:1–27

Størseth TR, Bytingsvik J, Standal IB, Hansen BH, Jenssen BM (2009) Gender differences in the metabolic profiles of blood plasma from polar bear (*Ursus maritimus*) analyzed by ^{1}H-NMR metabolomics. In: 5th international conference of the metabolomics society, Edmonton

Stryer L (1995) Biochemistry, 4th edn. Freeman & Company, W.H

Thiemann GW, Iverson SJ, Stirling I (2006) Seasonal, sexual and anatomical variability in the adipose tissue of polar bears (*Ursus maritimus*). J Zool 269:65–76

Thiemann GW, Iverson SJ, Stirling I (2008) Polar bear diets and arctic marine food webs: insights from fatty acid analysis. Ecol Monogr 78:591–613. doi:10.1890/07-1050.1

Tieszen LL, Boutton TW, Tesdahl KG, Slade NA (1983) Fractionation and turnover of stable carbon isotopes in animal tissues: implications for δ13C analysis of diet. Oecologia 57:32–37

van Ginneken V et al (2007) Metabolomics (liver and blood profiling) in a mouse model in response to fasting: a study of hepatic steatosis. Biochim Biophys Acta 1771:1263–1270. doi:10.1016/j.bbalip.2007.07.007

Waxman DJ, Celenza JL (2003) Sexual dimorphism of hepatic gene expression: novel biological role of KRAB zinc finger repressors revealed. Genes Dev 17:2607–2613. doi:10.1101/gad.1154603

Welch AJ, Bedoya-Reina OC, Carretero-Paulet L, Miller W, Rode KD, Lindqvist C (2014) Polar bears exhibit genome-wide signatures of bioenergetic adaptation to life in the arctic environment. Genome Biol Evol 6:433–450. doi:10.1093/gbe/evu025

Xia J, Wishart DS (2011) Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. Nat Protoc 6:743–760. doi:10.1038/nprot.2011.319

Xie J, Sinelnikov I, Han B, Wishart DS (2015) MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Res 43:W251–W257

Yates FE, Herbst AL, Urquhart J (1958) Sex difference in rate of ring A reduction of delta 4-3-keto-steroids *in vitro* by rat liver. Endocrinology 63:887–902. doi:10.1210/endo-63-6-887

Zhang D et al (2015) Changes in the milk metabolome of the giant panda (*Ailuropoda melanoleuca*) with time after birth—three phases in early lactation and progressive individual differences. PLoS ONE 10:e0143417. doi:10.1371/journal.pone.0143417