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SHORT COMMUNICATION

Hepatic Metabolomic Investigation of the North American Black Bear (*Ursus americanus*) Using ^{1}H-NMR Spectroscopy

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Abstract

The growing field of metabolomics examines the end products of metabolism, metabolites, to determine physiological processes at a cellular level. Nuclear magnetic resonance (NMR) spectroscopy affords advantages such as noninvasive sample collection, minimal or no sample preparation, and conservation of samples. The objectives of this study were to determine the feasibility of NMR-based metabolomics as a screening tool for evaluating changes in North American black bear (*Ursus americanus*) metabolism utilizing samples from hunter-killed specimens and to evaluate baseline metabolic profiles for free-ranging black bear. Hepatic samples were collected from 14 legally, hunter-killed black bears. The samples were frozen, homogenized, and extracted. ^{1}H-NMR spectra were collected and analyzed. Over 30 metabolites were identified, including those involved with protein, lipid, and carbohydrate metabolism. Principal components analysis demonstrated a separation among the bears by general age groups, based on differences in several energy and amino acid biomarkers, as well as lactate. This difference may be due to variation in growth and body composition with age. Samples from hunter-killed bears were suitable for NMR-based metabolomics suggesting the use of these techniques is a practical approach for identifying components of black bear metabolism.

Introduction

The seasonal variability of metabolism in ursids holds great interest and has been studied extensively [1, 2]. The physiology of hibernation is relevant not only to wildlife biologists, but to scientists studying osteoporosis, renal disease, eating disorders, organ transplant medicine, and a wide array of other fields [2].

The growing field of metabolomics examines the end products of metabolism, metabolites, to provide insight into physiological processes. Nuclear magnetic resonance (NMR) spectroscopy techniques used in metabolomic investigations afford advantages to investigators that include the potential for minimal or noninvasive sample collection, minimal sample preparation, and conservation of samples for

other studies [3]. Proton NMR spectroscopy is particularly robust and can be used to quantitatively detect unknown metabolites without prior knowledge of the analytes.

The aim of our study was to determine the feasibility of NMR-based metabolomics as a screening tool for evaluating changes in metabolism in large free-ranging carnivores through the sampling of hunter-killed animals. We used the black bear (*Ursus americanus*) as our model and evaluated variation between individual metabolic profiles of bears of various size during legal hunts.

Methods

Hepatic samples of approximately 1-2 g were collected from 14 legally, hunter-killed black bears and immediately frozen on dry ice. Gender, weight, harvest location by county, hunter-estimated approximate time of death, and hunter identification were recorded. The first maxillary premolar was collected and submitted for age estimation (Matson's Laboratory, LLC, Missoula, Montana, USA) by cementum analysis [4, 5]. Hepatic samples were transported on dry ice and maintained at -80°C until further processing. All samples were processed, extracted, and lyophilized within 24 h of collection. All NMR spectra were obtained approximately 3 weeks after sample collection.

In the laboratory, individual hepatic samples were homogenized using a mortar and pestle chilled with liquid nitrogen. Each tissue sample was weighed, 1M perchloric acid (Sigma-Aldrich, St. Louis, Missouri, USA) was added 2-to-1 volume:weight [6], and then was stored at 4 °C overnight. The extracted samples were centrifuged at 2500 x g for 25 minutes, each supernatant was collected, pH balanced to 7.0-7.2 with potassium hydroxide (Sigma-Aldrich, St. Louis, Missouri, USA), and centrifuged again at 2500 x g for 25 minutes. The supernatant was collected, frozen at -80 C, and lyophilized. The resulting pellet was suspended with 0.1 mM trimethylsilyl propionate (TSP) in 10% deuterium oxide (Sigma-Aldrich, St. Louis, Missouri, USA), centrifuged at 3000 x g for 20 minutes, and the supernatant transferred to a 5mm NMR tube (Wilmad-LabGlass, Vineland, New Jersey, USA).

^{1}H spectra were acquired on a Bruker AVANCE 500 MHz spectrometer with Oxford Narrow Bore Magnet, HP XW 4200 Host Workstation, using Topspin 1.3 software version. We used cavprst pulse sequence with 128 transients and collected data at a temperature between 21.0 and 25.57 °C. Total correlation spectroscopy (TOCSY) and correlation spectroscopy (COSY) spectra from a single representative sample were also obtained with the same spectrometer. Data were pre-processed using ACD Labs 12.0 1D NMR Processor (Advanced Chemistry Development, Toronto, Ontario, Canada), prior to PCA. This included zero-filling to 16,000 points, baseline correction, and alignment of the reference TSP signal. For principal components analysis (PCA), regions containing TSP, residual water, and downfield of 9.0 ppm were removed. Intelligent bucket integration was used with a bin width of 0.04 ppm. The common integrals were normalized by calculating the sum of the integrals for all bins and then dividing each bin by the sum, so that the integrals for each single sample summed to 1 and were then Pareto-scaled [7]. PCA was done with JMP 10 (SAS Institute, Inc., Cary, North Carolina, USA). Peak identification was performed using Chenomx NMR Suite 7.63 (Chenomx, Edmonton, Alberta, Canada) and the Human Metabolome database [8].

Proton NMR spectra of liver homogenates from 2 of the 14 bears originally

sampled in the field were excluded from further analysis because of insufficient water suppression in their 1-D spectra leaving 12 bears in the study. These 12 bears (9 male and 3 female) had a median age was 9.25 years (Interquartile range (IQR) 4.5-10 years) and their median body weight was 233 kg (IQR 126-252 kg). Due to an error in field data collection, the approximate amount of time between death and sample collection was obtained for only 6 bears and these had a median death to sampling interval of 3 hours (IQR 2-4 hours).

Results

Signalment data and approximate time to sampling for the 12 bears included in our analysis are listed in Table 1. Figure 1 shows a representative ^{1}H-NMR spectrum for the black bear hepatic metabolome. TOCSY and COSY spectra for the same individual are depicted in Figure 2. This bear was a 3.75-year-old female, weighing 91.6 kg and was sampled approximately 3 h postmortem. This spectrum is considered representative as it shows the majority of the metabolites identified and also has excellent water suppression and shimming; however, all of the spectra obtained were subjectively very similar (Fig. 3). Table 2 lists the metabolites identified among all samples. Chemical shifts and peak shapes were assigned on the basis of data obtained from the Human Metabolome database [8].

Table 1: Signalment and approximate time to sampling for liver samples from 12 legally, hunter-killed black bears in North Carolina, USA.

Bear ID	Gender	Age (yrs)	Weight (kg)	Approximate Time to Sampling (h:min)
1	M	12,75	263,1	03:20
2	F	3,75	91,6	02:55
4	F	4,75	NA	01:55
5	M	10,75	307,1	02:50
6	M	9,75	233,1	04:30
7	M	15,75	244,5	03:50
8	M	1,75	36,3	02:00
10	M	8,75	260,4	NA
11	M	14,75	202,3	NA
12	M	9,75	307,5	NA
13	F	6,75	117,9	NA
14	M	3,75	134,7	NA

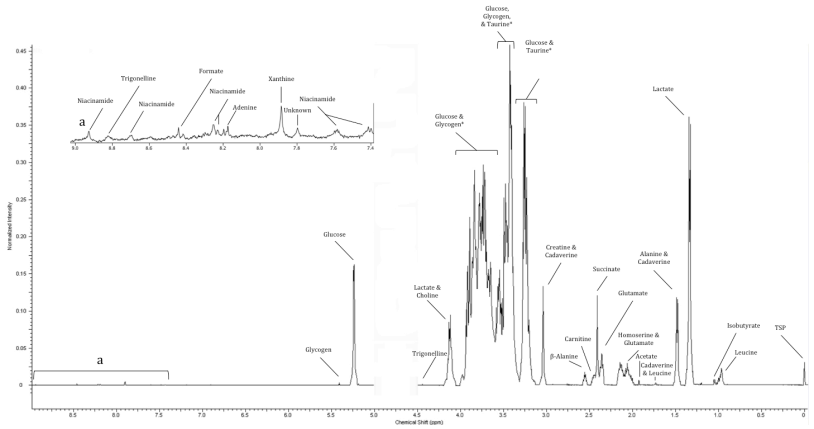

Figure 1: Example of a representative ^{1}H NMR spectrum of the black bear hepatic metabolome. The peak at 0 ppm is the external standard, TSP. This bear was a 3.75-year-old female, weighing 91.6 kg and sampled approximately 3 h postmortem. Metabolites associated with lipid (e.g. glycerol), protein (e.g. glutamate), and carbohydrate (e.g. glucose) metabolism were identified. (* = major metabolites)

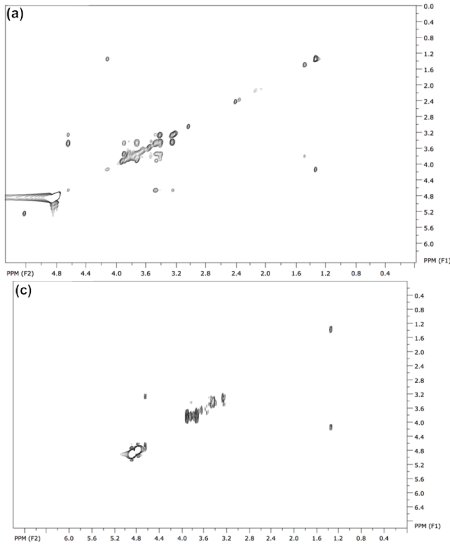

Figure 2: TOCSY (a: 0-6.0ppm; b: 7.0-9.0ppm) and COSY (c) spectra of the same representative black bear hepatic sample represented in Fig. 1. This 3.75-year-old female bear, weighing 91.6 kg was sampled approximately 3 h postmortem.

Table 2: List of compounds identified in the ^{1}H-NMR spectra of the 12 black bear hepatic metabolomes examined, and their respective chemical shifts and peak multiplicity (br s: broad singlet, d: doublet, dd: doublet of doublets, ddd: doublet of doublet of doublets, m: multiplet, q: quartet, s: singlet, t: triplet).

Metabolites	Chemical shift and peak shape, ppm
Alkaloids	
Cadaverine	1.35 (m), 1.49 (q), 2.68 (t)
Trigonelline	4.43 (s), 8.08 (t), 8.84 (t), 9.12 (s)
Amino acids	
Alanine	1.47 (d), 3.77 (q)
β-Alanine	2.54 (t), 3.17 (t)
Creatine	3.02 (s), 3.92 (s)
Glutamate	2.12 (m), 2.34 (m), 3.74 (t)
Homoserine	2.01 (m), 2.16 (m), 3.77 (m), 3.85 (dd)
Leucine	0.94 (d), 0.96 (d), 1.66 (m), 3.71 (t)
Phenylalanine	3.19 (m), 3.98 (dd), 7.32 (d), 7.36 (m), 7.42 (m)
Sarcosine	2.74 (s), 3.60 (s)
Tyrosine	3.024 (dd), 3.170 (dd), 3.921 (dd), 6.877 (m), 7.170 (m)
Valine	0.976 (d), 1.029 (d), 2.261 (m), 3.601 (d)
Cyclic amines	
Niacinamide	7.58 (dd), 8.24 (dd), 8.70 (dd), 8.92 (s)
Energy related	
Glucose	3.233 (dd), 3.398 (m), 3.458 (m), 3.524 (dd), 3.728 (m), 3.824 (m), 3.889 (dd), 4.634 (d), 5.223 (d)
Glycogen	3.40 (m), 3.60 (m), 3.80 (m), 3.96 (br s), 5.40 (br s)
Fatty acid metabolism	
Acetate	1.91 (s)
Carnitine	2.43 (dd), 3.21 (s), 3.42 (m), 4.56 (s)
Choline	3.189 (s), 3.507 (dd), 4.056 (ddd)
Glycerol	3.551 (m), 3.644 (m), 3.775 (tt)
Krebs cycle intermediates	
Fumarate	6.51 (s)
Succinate	2.41 (s)
Organic acids	
Formate	8.44 (s)
Isobutyrate	1.21 (d), 2.59 (m)
Lactate	1.40 (d)
Taurine	3.25 (t), 3.42 (t)
Purine Bases	
Adenine	8.11 (s), 8.12 (s)
Xanthine	7.89 (s)
Pyrimidine Bases	
Uracil	5.79 (d), 7.52 (d)
Unknown resonances	
Unknown	7.05 (s)
Unknown	7.80 (s)
Urea cycle	
Urea	5.78 (br s)

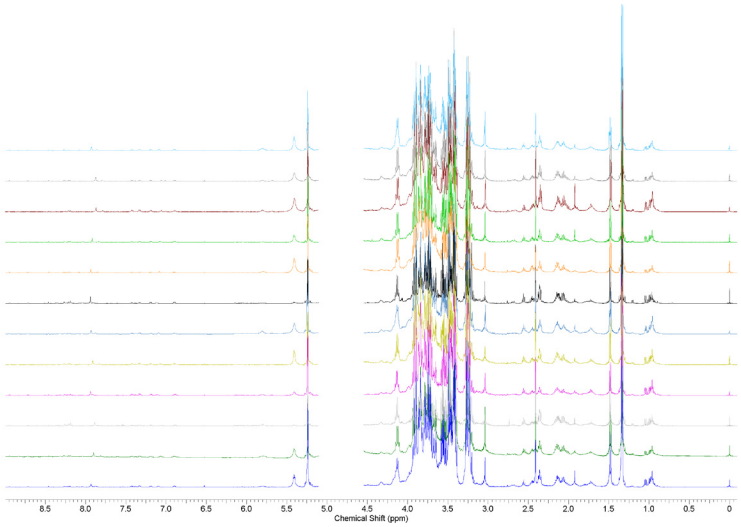

Figure 3: Collected [1]H-NMR spectra for the 12 black bears included in our analysis. The water regions have been deleted to allow better visualization of key peaks. Subjectively, the spectra appear very similar.

The PCA biplot of components 1 and 2 shows separation of bears by general age class and accounts for 56.3% of the observed variation (Fig. 4). This differentiation was traced back to energy biomarkers in the older bears and lactate and amino acid biomarkers, such as homoserine, glutamate, and leucine, in the younger bears. A single older bear was the exception to this separation. Based on this PCA, the deviation in this single bear was likely the result of differences in concentration of leucine, isobutyrate, acetate, and glutamate.

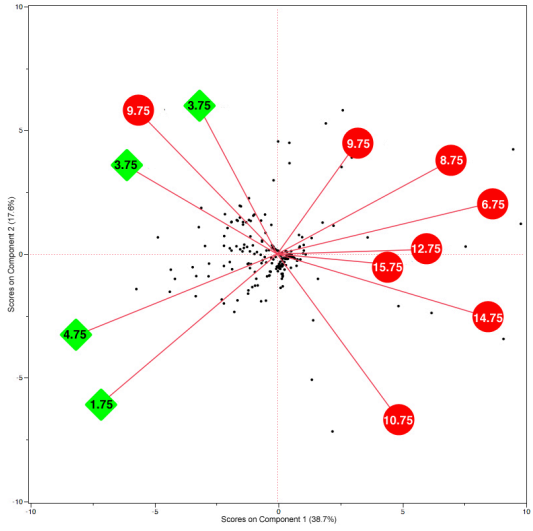

Figure 4: PCA biplot of components 1 and 2 (percent variation in parentheses) of [1]H-NMR spectra of liver from North American black bears. Younger bears are symbolized by green diamonds and older bears by red circles. Age, in years, is given within the symbol. A single older bear was the exception to this separation. Each of the small, black dots represents an integrated bin from the NMR spectra overlaid on the PCA plot.

Discussion

Metabolomics using NMR spectroscopy is becoming more prevalent as a valuable research and diagnostic modality [9-12]. A single, small sample results in vast information about an individual's metabolism, including data on metabolites not examined by routine diagnostic panels [13, 14]. The results of our study, designed to examine the feasibility of using this technology to assess free-ranging animals, show that interpretable spectra can be obtained from hunter-killed bears and supports the use of NMR-based spectroscopy to assess metabolic questions about North American black bear physiology. The application of the methodology to nonlethal sampling of other tissues or biofluids would be expected to open the potential for longitudinal studies to detect shifts in metabolism of black bear associated with a variety of environmental, dietary, and activity factors.

The sampling approach to this preliminary study did not control several potential sources of variability and error that we feel would need to be better controlled for an in-depth assessment of particular metabolic questions. In the processing of samples, we depended on the repeatability of our extraction method to provide us with equivalent samples. Use of dry weights related to total protein content obtained from subsamples partitioned from the liver tissue extracted would refine the comparability of our samples. Of particular concern for metabolomics assessment, the opportunistic nature of the sampling protocol resulted in a wide range in the time interval between death and freezing the hepatic samples. This would be expected to impact the quantitative assessment of individual metabolite concentrations in our samples. This concern, as well as concerns about our cohort size, limited the conclusions we could reliably draw from our results and we elected not to pursue statistical analyses beyond PCA. Individual bears were sampled haphazardly based on access and availability and varied in age, weight, and gender. There were other obvious weaknesses in the sampling design. The bears were assumed to be healthy, but no postmortem pathology evaluations could be performed to confirm this assumption. Nor was any attempt made to evaluate genetic relationships between individual bears. Even though the bears were collected from multiple locations within two counties the possibility of sampling closely related bears in our study cannot be ruled out. The bears sampled may have been hunted with or without the assistance of dogs, which would be expected to affect exertion and thus energetics prior to death. Stomach contents were not available for examination in our study. We expect bears were consuming a natural diet, though running bears off of bait for hunting is legal in North Carolina. Regardless, the diets of these bears undoubtedly varied, as would have the time from last meal to sample collection. An assessment of stomach contents should be considered in future studies of this nature.

Black bear nutrition is known to vary by season, life stage, and geographic locale [15]. Bears have been known to increase their body fat reserves in late autumn on a diet that is rich in fats and carbohydrates [15]. This body fat is crucial to supporting metabolism during hibernation [2, 16, 17]. Glycerol is derived from the lipid and is involved in protein, carbohydrate, and lipid metabolism in hibernating bears [17]. It acts as an active substrate for gluconeogenesis and lipogenesis and is a carbon source for the formation of amino acids [17]. Our ^{1}H-NMR hepatic metabolomic assessment identified several metabolites related to fatty acid metabolism, despite the decision

to only examine the aqueous fraction of our extraction. These compounds included glycerol, alanine, and homoserine. Assessment of these compounds and potentially the hydrophobic fraction of the extraction could provide a detailed, yet rapid, method of monitoring nutritional status or habitat quality.

The separation pattern by age class observed in the PCA was caused by differences in energy biomarkers (e.g. glucose) in the older bears and lactate and specific amino acids in the younger bears. Previously published black bear metabolism studies have noted differences between seasons, food availability, and reproductive status [16-21], but other than reports of different nutrient requirements for growing bears [22], studies on age differences in metabolism are sparse in the literature. Growth and body composition of the younger bears would contribute to this metabolic separation. Published data suggests young male bears may be underrepresented as breeders and suffer from more frequent bear-inflicted injuries due to social structure [23]. If this is the case, young male bear metabolism would differ from that of dominant, older bears. Alternatively, older males may become exhausted and cease breeding earlier in the season, and therefore increase their post-breeding food intake ahead of younger males [24]. The grouping of older individuals in our study included 6 males, but only 1 female, a sex ratio that does not support detailed assessment of metabolomic differences between the sexes. The amino acid biomarkers we observed related to protein metabolism, which may also differ between the younger and older bears. The two females included in the grouping of young animals may have been metabolically similar to the 3 males, if they had not yet become reproductively mature [25]

The separation of animals by age we observed in our data could be related to hunting method (not recorded) and/or individual bear behavior. One of the metabolites found to contribute to the separation based on age that we report was lactate. Lactate is produced during normal metabolism and increases dramatically during strenuous and anaerobic exercise. Younger bears may be more likely to run or climb compared to older animals. Another possibility, inadequately documented by our data, is that these animals may have been hunted using dogs in the chase. Even without this difference, it is possible that a hunter's first shot may have not been immediately successful, resulting in a prolonged chase and kill, contributing to lactate accumulation.

Some metabolites identified within this study were intriguing and offer the potential for expansion of our studies of bear physiology. We found the unconfirmed identification of trigonelline, an alkaloid associated with niacin metabolism in the livers of several species particularly interesting. This compound was once thought to be simply an end product of nicotinamide adenine dinucleotide synthesis pathways, but it has now been shown to function as a stored form of nicotinic acid [26]. It is of particular interest in human diabetes research and has been shown to have protective effects for the liver and kidney and improved glucose and lipid metabolism [27]. Further metabolomic study of this metabolite, and related compounds, could provide new insights into black bear hibernation.

Our study shows that samples from hunter-killed black bears can be used effectively to obtain NMR-based metabolomic data suitable for evaluating North American black bear metabolism. The methods used allowed identification of metabolites associated with protein, lipid, and carbohydrate metabolism, not found on typical clinical pathologic biochemical assays. Application of this approach to tissues and biofluids

obtainable through both lethal and nonlethal sampling in combination with careful attention to cohort construction, offers valuable opportunities to better understand the interactions between bears and their environment.

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