Serum metabolomics of Alaskan sled dogs during endurance racing

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Abstract

Long distance racing sled dogs are fed diets high in fat since lipid metabolism has long been thought to be the major substrate utilised during endurance racing. However, recent studies suggest that sled dogs are equally, if not more, dependent on carbohydrate metabolism. Considering the metabolic disparity regarding the energetics of endurance exercise, our study aimed to explore the serum metabolomic profiles of sled dogs running a 1,609 km (1000 mile) race. We hypothesised that there would be amino acid depletion due to gluconeogenesis and alteration in the citric acid cycle (CAC) based on the limited carbohydrate diet they consume. Serum was obtained from 6 Alaskan sled dogs approximately 24 h prior to the race (Whitehorse), at the midrace checkpoint (Dawson City), and again at the finish (Fairbanks). Serum was analysed using liquid chromatography–mass spectrometry for over 200 metabolites involved in amino acid, lipid, and carbohydrate metabolism with MetaboAnalyst Software 3.0. Major metabolic changes observed were decreased free fatty acids and enhanced acyl-carnitine derivatives during the race compared to baseline. Serum depletion of nearly all amino acids except for branched chain amino acids and phenylalanine was observed suggesting extensive protein catabolism. Many of the CAC intermediates were variable with increases in abnormal end glycation products. These results highlight that sled dogs display general amino acid depletion for pyruvate, acetyl CoA and CAC pathway intermediates with increased carnitine bound lipid metabolites, suggesting rate limiting beta-oxidation during endurance exercise, particularly at mid race. Further metabolomic studies to assess the influence of exercise and nutritional regimens are warranted to better understand substrate utilisation in working dogs.

Keywords: plasma metabolite fingerprinting, metabolism, canine athletes, nutrition, energetics

1. Introduction

During long distance endurance racing, Alaskan sled dogs undergo intense physiological and metabolic demands. Energetic studies have found that racing sled dogs have similar energy requirements to other breeds at rest in a thermoneutral environment, but may require as much as 40,000 to 50,000 kJ/day during a race (Hinchcliff et al., 1997a; Loftus et al., 2014). The physiological stresses (ambient temperature, rough terrain, distance travelled, weight pulled) encountered dictate extensive increases in daily energy expenditure and potential upregulation of metabolic pathways in order to meet the energy demand. Canine metabolism demonstrates a greater capacity for lipid utilisation than people with an enhanced ability to mobilise and oxidise fats (Hammel et al., 1977; Paul and Issekutz, 1967; Weber et al., 1996). Foundational studies have shown that fat is a major substrate metabolised during endurance exercise rather than carbohydrate; therefore consuming a high fat (>50% of metabolisable energy (ME)), high protein (>30% of ME), and low in carbohydrate (0-22% of ME) diet may be advantageous for increasing stamina if fed for 12 or more weeks before competition (Hill, 1998; Hinchcliff et al., 1997a; McKenzie et al., 2008; Reynolds et al., 1994, 1995; Roberts et al., 1996). Higher fat diets appear to enhance overall caloric consumption in the face of energetic extremes and decreases coprophagy and hypoglycemia.
associated with attempts to feed higher carbohydrate diets (Kronfeld, 1973; Kronfeld et al., 1977). Previous studies have established that canine athletes have the ability to sustain exercise without reliance on dietary carbohydrates and that after an adequate period of adaptation, prolonged feeding of a high fat diet safely enhances the availability of lipids for oxidation and preserves muscle glycogen for more rigorous exercises. (Hammel et al., 1977; Reynolds et al., 1994). Studies in long distance racing sled dogs have also shown that their skeletal muscle has remarkable glyconeogenic capability: glycogen depletion in the first days of exercises was progressively replenished to greater than 50% of resting concentrations after 4 consecutive days of prolonged submaximal exercises even when fed a modest-carbohydrate, high-fat diet (McKenzie et al., 2005, 2008).

These canine feeding recommendations differ drastically from reported human ultra-endurance athletes’ who need 50-65% of ME in carbohydrates, 20-35% of ME in lipids and 15-25% of ME in protein to maintain weight and energy balance (American College of Sports Medicine, 2000, 2009). Recent data in competitive sled dogs shows a dependency on carbohydrate oxidation during endurance exercise, even when consuming less than 17% carbohydrates (Miller et al., 2015a). The study by Miller and colleagues suggests that carbohydrate substrates were sustained by gluconeogenesis from protein, glycerol and increased lactate oxidation. Gluconeogenesis uses protein as a substrate when glycogen is not available and most competitive sled dog diets are over 30% ME from protein (Loftus et al., 2014). Increases in blood urea nitrogen, often observed in competitive sled dog racing studies, might reflect this route of protein metabolism (Burr et al., 1997; Ermon et al., 2014; Hinchcliff et al., 1997b; McKenzie et al., 2007; Wakshlag et al., 2010).

It has been widely demonstrated in human and animals, such as horses and rodents, that the metabolome is highly modified by physical exercise (Huang et al., 2010; Le Moyec et al., 2012, 2014; Lewis et al., 2010; Luck et al., 2015; Monleon et al., 2014; Mukherjee et al., 2014; Pechlivanis et al., 2013, 2014; Xiang et al., 2015; Yan et al., 2009). Compared with more conventional techniques (such as routine biochemistry, muscle biopsies, arteriovenous differences, pulmonary gas exchange, or isotopic tracers) metabolomics performs a global, non-dedicated analysis of a whole organism’s metabolic status as reflected in serum or other bodily fluids at one point in time (Nicholson and Wilson, 2003). Our objective was to examine the serum metabolome of endurance sled dogs to further characterise alterations in pathways involved in protein, fat and carbohydrate metabolism. We hypothesised that endurance sled dogs will show a global depletion of amino acids due to increased gluconeogenesis over the course of an ultramarathon race. The aim of this study was evaluate a broad metabolite profile including fatty acid, citric acid cycle (CAC) intermediates, and amino acids and their metabolites before racing, mid-race, and immediately after racing 1,582 km (983 miles).

2. Material and methods

Animals

In accordance with Cornell University Institutional Animal Use Committee and the Yukon Quest Board of directors, six well-conditioned Alaskan sled dogs that participated in the 2015 Yukon Quest were recruited for this prospective study. Enrolled dogs were from a single team (winning team of the 2015 Yukon Quest) and were consuming the same generalised meal as published previously (Loftus et al., 2014) under similar feedings regiments. All dogs underwent a physical examination by a veterinarian before the race and were deemed healthy for racing. The race consisted of approximately 1,600 km over varied terrain and under variable winter weather conditions. At the midpoint, a mandatory rest period of 36 h was taken.

Sample and data collection

All dogs had a brief physical examination and had their body condition scores evaluated according to the one to nine grading system (Mawby et al., 2004) at the time of all blood draws. Venepuncture was conducted approximately 24 h prior to the race (Whitehorse, Yukon Territory), at midrace (Dawson City, Yukon Territory, 775 km (480 miles)), and again at the finish (Fairbanks, Alaska, 1,582 km (983 miles)). All blood samples were collected within 2 h of the dogs’ arrival at the midway check-point and at the finish before feeding. Ten ml of whole blood was collected from all six dogs via jugular (22-gauge needle and 12 ml syringe), transferred to a coagulation tube and centrifuged at 4,000×g for 10 min. Serum was dispensed into one ml aliquots and frozen on dry ice for transportation to the principal investigator’s laboratory. Samples were stored at -80 °C until thawed three months later for untargeted liquid chromatography-mass spectrometry (LC-MS).

Liquid chromatography-mass spectrometry

Serum samples were prepared by thawing samples and 20 μl of serum was added to 80 μl ice-cold water in a 1.5 ml microcentrifuge tube on ice, followed by the addition of 400 μl ice cold methanol and the tube was vortexed rigorously for one min before centrifuged at 20,000×g for 10 min at 4 °C. The supernatant was transferred to a new polypropylene micro-centrifuge tube and dried in a vacuum concentrator at room temperature. The dry pellets were reconstituted into 30 μl sample solvent (water:methanol:acetonitrile, 2:1:1, v/v) and further analysed by LS-MS at the Duke University Molecular and Physiology Institute. Ultimate 3000 UHPLC (Dionex, Thermo Fisher Scientific, Waltham, MA, USA) was coupled to Q Exactive Plus-Mass spectrometer (QE-
Metabolomic analysis

242 distinct metabolites were identified by LC-MS of which 210 could be identified with valid Kyoto Encyclopedia of Genes and Genomes (KEGG) identification and 32 metabolites were unnamed based on KEGG identification. 87 of these 210 identified metabolites were found to be significantly \( P \leq 0.05 \) different from baseline at midpoint and/or the end of the race (Supplementary Table S1). Upon manual analysis of the 32 metabolites not identified by KEGG identification, eight metabolites (creatine, creatinine, lactate, acetamidopropanal, L-leucine/L-isoleucine, L-proline, oleic acid and palmitate) were found to be significantly changed from baseline and were therefore included (Supplementary Table S1). Consequently, a total of 95 metabolites were different over time during racing.

Lipid metabolites

Nearly all by-products of fatty acids metabolism were significantly upregulated mid-race due to increase in carnitine bound intermediates (Figure 1). Significant decreases in free fatty acids (arachidonic acid, docosahexaenoate, 8-11-14-eicosatrienoic acid, phytic acid/arachidic acid) and fatty acids metabolism (L-palmitoylcarnitine) were observed. These fatty acid changes were most pronounced at mid-race. Cholesterol biosynthetic intermediate R-mevalonate was significantly decreased throughout the race. Metabolites that had significant increases in mid-race with a return to baseline by the end of the race included some of the fatty acid intermediates (L-palmitoylcarnitine, L-octanoylcarnitine, perillic acid), and intermediates of the linoleic acid metabolism (9-10-hydroxyoctadec-12-Z-enoate/12-13-hydroxyoctadec-9-Z-enoate).

Protein and metabolites

Amino acids aspartate, arginine, glutamate, glutamine, cystathione, serine, threonine, and glycine were significantly lower at mid-race and remained decreased, whereas intermediates were increasing throughout the race (Figure 2). One enzyme (beta-carboline), one cysteine and methionine metabolism pathway intermediate (2-keto-4-
methylthiobutyrate), two tryptophan derivatives (anthranilate and 5-methoxyindoleacetate), one intermediate of the histidine metabolism (methylimidazole acetic acid), one metabolite of alanine, aspartate and glutamate (5-phospho-beta-D-ribosylamine) were significantly elevated by race end. Although there was a general depletion and production of metabolic intermediates of most amino acids, there was a significant increase in branched chain amino acid (BCAA) leucine/isoleucine at mid-race and race finish, as well as mild significant increases in L-phenylalanine and L-methionine.

**Carbohydrate metabolites**

Glycerophospholipid and glycerolipid metabolism (G3P, choline phosphate, galactosylglycerol), as well as CAC intermediates (succinate, 2-cis-aconitate, pyruvate) were found to be significantly elevated (Figure 3). Glycolysis as represented by increases in lactate at mid-race and the finish suggesting continued utilisation of glucose by race finish. In addition, atypical gluconeogenic intermediates D-glucitol, galactitol and L-iditol showed large multi-fold increases which were significant.

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**Figure 1. Relative fold changes in fatty acid (lipid) metabolites.** Heatmaps show changes in metabolites compared to baseline time 1 (green line), mid-race time 2 (magenta line) and at the end of the race time 3 (pink line). Shades of blue and red represent respectively fold-decrease and fold-increase of a metabolite in time (see colour scale).
Nucleic acids, xenobiotic and other metabolites

Trans-xenobiotic compound derivate from oral joints supplements or by-products of collagen consumption (increases of D-glucosamine 6-phosphate) as well as pyrimidines metabolites (cytosine and 2-doxycytidine) were significantly increased by the end of this endurance exercise (Figure 4). Purine metabolites (guanosine, xanthine) were significantly diminished by race finish. Some neurotransmitter and vitamin metabolic by-products were significantly increased by the end of the race (kynurenes, pyridoxal, ecgonine and phenyl acetate). Potential xenobiotic contaminants were also significantly decreased over the course of the race (benzoate, coumarin).

4. Discussion and conclusions

Metabolomics

Metabolomic approaches help define patterns and identify pathways of altered substrate metabolism through a non-targeted approach that fails to provide specific concentrations of metabolites; however it provides a starting point to identify specific pathways and metabolites for more targeted investigations. As expected, we identified...
changes in metabolites reflecting heightened utilisation of energy substrates in several metabolic pathways, including increased glycolysis, lipolysis, and amino acid catabolism. Simultaneously, we unexpectedly identified many increased byproducts of fatty acid metabolism and abnormal glycation end products as increased metabolic products of exercise. It has been established that during the first stage of exercise, muscle glycogen is the primary fuel for muscle contraction, whereas circulating glucose and nonesterified fatty acids become essential with increasing length of exercise at submaximal intensities. The mechanisms behind the ability to sustain prolonged submaximal work in exercising dogs has been thought to be due to mitochondrial volume and lipid substrate utilisation (Hill, 1998; McKenzie et al., 2008; Reynolds et al., 1994; Wasserman and Cherrington, 1991; Weber et al., 1996) suggesting that glycogenolysis is significantly reduced during prolonged endurance exercises in favour of fatty acid metabolism. The liver plays an important regulatory role during exercise; the sum of changes in hepatic glycogenolysis and gluconeogenesis are closely correlated to the increased glucose uptake by the working muscle (Wasserman and Cherrington, 1991). The liver must conserve and extract certain products (such as ammonia, alanine, and glutamine as well as lactate, glycerol, and certain amino acids) to establish increases in glucogenic pathways potentially converting nitrogen into
urea or utilising them for protein synthesis (Wasserman and Cherrington, 1991). Gluconeogenesis during endurance exercise has not been examined extensively; however, recent studies in trained and untrained sled dogs during endurance activity show significant glucose utilisation attributed to either glycogenolysis or gluconeogenesis of amino acids (Miller et al., 2015a). Our results suggest there is significant amino acid depletion and loss of body condition (speculated to be muscle catabolism) which may be contributing as significant substrates for energy during endurance racing. Hence, endurance sled dogs appears to be reliant on a protein catabolism for gluconeogenesis similarly to lipid beta-oxidation.

**Lipid metabolism**

Prior serum assessment has shown marked depletion of non-esterified fatty acids during endurance racing (Ermon et al., 2014); yet our metabolomic profiles demonstrate accumulation of carnitine bound fatty acids of various lengths. Since carnitine is required to transport fatty acids across the mitochondrial membrane for oxidation, this finding lends credence to the idea of a potential bottle neck or rate limiting effect in the beta oxidation of fatty acids during exercise. Prior non-esterified fatty acid measurements have not examined carnitine bound derivatives which appear to be potential contributors, and

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**Figure 4. Relative changes nucleic acid, xenobiotic, contaminants and other metabolites. Heatmaps show changes in metabolites compared to baseline time 1 (green line), mid-race time 2 (magenta line) and at the end of the race time 3 (pink line). Shades of blue and red represent respectively fold-decrease and fold-increase of a metabolite in time (see colour scale).**
suggests that future work to understand the partitioning of fatty acid pools may be necessary. These derivatives along with the increase presence of N6N6N6-trimethyl-L-lysine, a (precursor of carnitine) suggests decreased mitochondrial translocation of fats as the reason for this accumulation and not carnitine deficiency; although increased production and release cannot be excluded. This altered fatty acid metabolism was strongest at mid-race and returned close to baseline by the end of the race. Interestingly, a prior study demonstrated increases in esterified carnitine levels between days three and five of starvation in dogs (Rodriguez et al., 1986), and carnitine concentrations in cats with hepatic lipodisosis show higher free, total and esterified carnitine concentrations in serum and tissue during enhanced beta-oxidation (Jacobs et al., 1990). Plasma L-carnitine increase has also been associated with positive adaptation to exercise due to improved muscle oxidative capacity as previously shown in humans (Kuehnbaum et al., 2015). Our carnitine bound fatty acid increases suggest that this may be an adaptation to exercise; however, free plasma carnitine did not rise, which may be due to increased conjugation to fatty acids or potential competition for amino acids for gluconeogenesis.

**Protein catabolism**

Simultaneously to increased carnitine bound fatty acids, nearly all amino acids were depleted despite high protein and caloric intake (Loftus et al., 2014), suggesting increased amino acid metabolism which is most likely due to the need for gluconeogenesis. Nearly all prior studies examining the serum biochemistry of endurance racing sled dogs exhibit significant increases in serum urea nitrogen which is presumed to be due to protein catabolism, gastrointestinal bleeding or decreased renal blood flow (Burr et al., 1997; Ermon et al., 2014; McKenzie et al., 2007). This overall depletion of amino acids, and decreased body condition scores (a reflection of both fat and lean mass depletion), throughout the race suggest that protein substrates are involved in meeting energy needs during endurance racing and are certainly not only spared for protein synthesis and anabolic purposes.

Interestingly, the amino acids leucine and isoleucine were elevated in the serum, which is contrary to the other amino acids in this study. Leucine is a ketogenic BCAA that is metabolised to acetyl-CoA before its subsequent oxidation in the CAC, whereas isoleucine is both a glucogenic and ketogenic BCAA. The rate of leucine oxidation varies depending on the availability of other carbon sources within a given tissue. Other studies have noted parallelism between leucine oxidation and the catabolism of carbohydrate in muscle (De Godoy et al., 2014; Hood and Terjung, 1987; Pechlivanis et al., 2013); however, species differences as well as sprinting vs endurance exercise make these direct comparisons difficult. BCAA elevations also have been reported in people and horses after exercise and are involved in muscle and liver energy supply (De Lorenzo et al., 2003; Le Moyec et al., 2014). Others have found similar increases in serum leucine after exercise which were attributed to alterations in gastrointestinal metabolism (Halseth et al., 1997; Hamada et al., 1999; Williams et al., 1996) and/or metabolic hepatic bypass due to their unique processing and signalling capability within skeletal muscle during consecutive days of exercise (Henriksson, 1991; Zanghi et al., 2015). This BCAA mobilisation has been concomitant with increased concentrations of aromatic amino acids, tyrosine and phenylalanine (markers of protein and muscle breakdown which may be seen during abnormal hepatic metabolism or starvation) (Monleon et al., 2014). Our results show that L-phenylalanine, L-methionine and tyrosine metabolites, were increased in the plasma of sled dogs at mid race and/or the end of racing. Serum phenylalanine and tyrosine significantly increase from baseline immediately after a moderate exercise bout in dogs; although it rapidly returns to baseline concentrations by 15 min after exercise (Zanghi et al., 2015). Overall, these increases in BCAA and some aromatics contrasting dramatic decreases in primarily gluconeogenic amino acids appears unique to endurance sled dogs. These findings suggest that there could be potential to spare amino acid catabolism with the proper carbohydrate, or protein and carbohydrate supplementation during endurance exercise; contrary to the present supplemental feeding during ultramarathon racing with high protein and fat intake.

**Carbohydrates and citric acid cycle**

CAC intermediates are generally derived from carbohydrate or amino acid intermediates and appear to be amplified due to increases in gluconeogenesis as previously demonstrated by Miller et al. (2015a). Increases in anaerobic by-products (lactate, pyruvate) and glycerol 3-phosphate (which is a glycolysis intermediate) suggest increased glycolysis for anaerobic metabolism. The modest rate limiting entry of pyruvate to the CAC leads to lactate increases. Although we believe that the increased pyruvate in the bloodstream is secondary to increased production, it could also be secondary to decreased clearance. Nevertheless, completion of ultra-endurance exercise with evidence of increased pyruvate is a novel finding refuting the previous conception that dogs exercising at submaximal effort for hours rely on primarily oxidation of fat. It is well established that lipid oxidation sustains low-intensity or submaximal exercise; however, higher intensity activities may rely more heavily on carbohydrate oxidation (Roberts et al., 1996). These conceptual principles were built in different breed undergoing short bursts of exercise and time to fatigue on different levels of dietary fat and carbohydrates (Brooks and Mercier, 1994; Downey et al., 1980). During the Yukon Quest, dogs regularly sustain speeds of 13–16 km/h for up to 6–8 h per day proving to be
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metabolite generation of long distance endurance sled dogs are relying on the rest time in Dawson and may push dogs harder as they approach this mandatory layover or the mandatory 36 h rest coming into Dawson (coming to a mandatory 36 h rest) as dogs are likely to be in a deeper negative calorie balance before serum collection. To the authors' knowledge, the sled dogs were studied and does not represent the full spectrum of canine ultramarathon athletes. The study is potentially under-powered to identify minor metabolic alterations, however our goal was to examine the more extreme changes in metabolites and to introduce a novel method for others to build upon. The global information gained regarding end glycation product formation, carnitine bound fatty acid increases, and protein catabolism provide that basis for future more targeted investigations in metabolites of interest.

It must also be recognised that these results are only a three specific ‘snap-shots’ in time without a full history of recent feed consumption on the trail or intensity of exercise before serum collection. To the authors’ knowledge, the sled dogs are likely to be in a deeper negative calorie balance coming into Dawson (coming to a mandatory 36 h rest) as compared to finishing the race in Fairbanks, since mushers are relying on the rest time in Dawson and may push dogs harder as they approach this mandatory layover or the competitiveness of the race as mushers make a final push to finish in Fairbanks Alaska.

5. Conclusions

These metabolomics profiles provide a comprehensive single point examination into the substrate utilisation and metabolite generation of long distance endurance sled dog racing. The alteration in this study demonstrate that adaptation to endurance exercise involves simultaneous protein catabolism and glycolytic alterations as well as generation of glycation end product. Depletion of nearly all amino acids, except for the noted elevations in hepatic BCAA and phenylalanine, suggest that sled dogs are at least partially reliant on protein and carbohydrate metabolism during exercise. The increases in carnitine bound fatty acid derivatives suggest a potential rate limiting event during beta-oxidation of fatty acids during prolonged aerobic exercise. This data is unique and important as it better defines metabolic pathways in endurance sled dogs, provides novel insight into protein and carbohydrate use as a substrate, and lays a foundation for further classification and targeted research for better management programs for the endurance canine athlete to optimise performance as well as improve safety and recovery.

Limitations

There are a number of limitations in studies of this nature. Firstly, mass spectrometry-based strategies for metabolomics provide only a pseudo-quantitative and qualitative information allowing for the monitoring of changes that occur within a system over time, but not true concentrations of metabolites. In addition, this screen provides only a limited number of metabolites which is not a complete pathway examination. Furthermore, analyses rendered from the software system may not be a true representation or differences or nuances in canine metabolism since the software is designed for human and murine use and may not reflect ideal models for canine metabolism (McKenzie et al., 2008).

Our study only evaluated six of the healthiest dogs from the winning team; therefore only a few of the most elite dogs were studied and does not represent the full spectrum of canine ultramarathon athletes. The study is potentially under-powered to identify minor metabolic alterations, however our goal was to examine the more extreme changes in metabolites and to introduce a novel method for others to build upon. The global information gained regarding end glycation product formation, carnitine bound fatty acid increases, and protein catabolism provide that basis for future more targeted investigations in metabolites of interest.

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The ultimate test for endurance activity with evidence that glycolytic metabolism is occurring well into a multi-day endurance event. Surprisingly, sugar alcohol by-products of metabolism (iditol, arabitol, myo-inositol) were also found to be elevated suggesting alteration in carbohydrate or fatty acid metabolism. Since these substances are not expected as normal end products of metabolism, we speculate that they reflect an abnormal cellular process producing glycation end products.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/CEP180010.

Table S1. Metabolic changes in plasma during exercise.

References


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