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Discovering the Impact of Diet, Genetic, and Sex Effects on Adipose, Skeletal Muscle, and Liver Metabolomes in Mice Using Untargeted Liquid-Chromatography Mass-Spectrometry

Ann Elizabeth Wells
University of Tennessee, awells13@vols.utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Ann Elizabeth Wells entitled "Discovering the Impact of Diet, Genetic, and Sex Effects on Adipose, Skeletal Muscle, and Liver Metabolomes in Mice Using Untargeted Liquid-Chromatography Mass-Spectrometry." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Brynn H. Voy, Major Professor

We have read this dissertation and recommend its acceptance:

Vitaly Ganusov, Robert W. Mee, Jonathan Wall

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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"Discipline is remembering what you want"
-David Campbell

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ABSTRACT

The metabolome is the end product of all biochemical reactions in the body and is sensitive to external perturbations, such as diet, genetics, sex, disease, and environment. Understanding the dynamic relationship between metabolites and external inputs is key to delineating the metabolic underpinnings of disease. Mass-spectrometry based metabolomics provides a means through which the metabolome can be systemically and systematically profiled to discern alterations across thousands of metabolites at a time. The research in this dissertation characterized the effects of diet, genetics, and sex on the tissue metabolome. Adipose, skeletal muscle, and liver tissue were chosen due to their role in energy metabolism. In the first study, we characterized the effects of five diets (Japanese, ketogenic, Mediterranean, American, and standard chow) on the tissue metabolome, across both sexes. This study described the metabolic response to diets that altered in macronutrient ratio and composition, independent of genetic differences. Our results revealed that liver tissue was most sensitive to metabolic changes but responded similarly for diet, sex, and sex-by-diet interaction while adipose and muscle remained largely stable. In the second study, we characterized the effects of four strains (A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ) on the tissue metabolome, across both sexes. This study assessed the metabolic response to differing genetic backgrounds represented by various predispositions to metabolic disease, independent of diet. Our results showed that strain exerted the largest effect on metabolites across all three tissues. In contrast, sex and sex-by-strain interaction had little effect on adipose, muscle, and liver. The third study characterized the effects

of diet, genetics, and sex on the tissue metabolome. We assessed the metabolic response to diet, genetics, sex, and their interactions to determine which factor/s were potentially driving metabolic differences. Strain exerted the largest effect across all tissues. Liver was most sensitive to metabolite changes when all factors and interactions were collectively assessed. Overall, the results in this dissertation aid in the understanding of complex relationships between diet, genetics, and sex at the systems-level. Furthermore, it was shown that tissues responded differentially to the same stimuli highlighting the need to consider sample type when performing metabolomics.

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CHAPTER ONE

GENERAL EXPERIMENTAL DESIGN

The goal of this dissertation was to identify "metabotypes", phenotypes defined by distinct strain- and diet-dependent metabolite profiles, which could be linked to systems' phenotypes relevant to metabolic disease.

Little is known about the metabolic response to whole diets at the tissue level and even less is known about how genetic background intersects with diet to alter these responses. Metabolomics is an analytical tool that allows us to obtain relative abundances of individual metabolites within whole tissue or cells. It allows us to identify how metabolism is affected on a systems level.

This project is part of a larger project designed to identify how gene by diet interactions affect colon tumor initiation. The first phase of the project assessed how gene by diet interaction affected physiology in the absence of tumors. This study focused on the identification of metabolites during the first phase of the project.

Males and females across four strains of mice (A/J, C57Bl/6J, NOD, and FVB) were fed one of five diets (Japanese, Mediterranean, ketogenic, American, and standard chow) for 7 months. These mice were chosen due to their varying susceptibility to azoxymethane, a chemical carcinogen; however, these mice also have altering susceptibilities to metabolic disease. Mice were fed diets as libitum for 12 weeks, to acclimate to the diets, prior to metabolic testing. During metabolic testing mice were placed in metabolic chambers to measure respiratory exchange rate, oxygen consumption, activity levels, and heat output.

The research in this dissertation measured thousands of metabolites within adipose, muscle, and liver tissue across multiple diets and genetic backgrounds for both sexes with the aim of understanding how all three factors intersect to modify the tissue

metabolome. The objectives of this study were: 1. Characterize the relative effects of genetics, sex, diet, and their interactions on tissue metabolomes, 2. Identify tissue-level metabolic effects of diets that are commonly recommended for health, and 3. Link tissue metabolite profiles to systems phenotypes relevant to metabolic syndrome.

Chapter 3 will provide the bioinformatics approach designed to deal with tissue metabolomics, including experimental procedure, sample preparation, and statistical procedures. Chapter 4 will characterize the effects of diet and sex on a single genetic background in adipose, skeletal muscle, and liver tissue. Chapter 5 will characterize the effect of strain and sex on the tissue metabolome when mice were fed a single diet. Chapter 6 will characterize the effects diet, genetics, and sex on the tissue metabolome. Chapter 7 will conclude the research presented in this dissertation and provide future directions.

CHAPTER TWO

METABOLOMICS AND ITS ROLE IN DISSECTING SYSTEMS-LEVEL

EFFECTS OF DIET, GENETICS, AND SEX

2.1 Energy Balance and Diet

More than two-thirds of the United States (U.S.) population is classified as overweight or obese [1-3]. Obesity is associated with the comorbidities type 2 diabetes, hypertension, heart disease, and certain cancers [4]. In addition, obesity is the 5th leading cause of death worldwide [3]. Obesity and its comorbidities are an economic burden. As of 2008, the estimated annual cost of obesity in the U.S. was $147 billion [5].

Obesity is traditionally accepted as the physiological response to excess caloric intake and low physical activity. Despite a large body of research investigating the relationship between obesity and diet and lifestyle interventions, no clear relationship has been elucidated. Current recommendations encourage individuals to increase their physical activity [6] and to consume a healthy eating pattern [7]. According to the Dietary Guidelines for Americans a healthy eating pattern consists of a variety of vegetables, fruits, grains, fat-free or low-fat dairy, a range of proteins consisting of lean meats, eggs, legumes, and seafood, and oils with limited consumption of saturated fats, sugar, and sodium [7]. Caloric intake is also considered important to maintaining a healthy lifestyle, in addition to the types of food consumed. Caloric intake for an individual is dependent on a number of factors; activity level, age, weight, sex, and height [8]. The aim of a healthy eating pattern and limiting caloric intake is to modulate energy metabolism. Energy metabolism at the cellular level is the production of ATP from nutrients. Nutrients from food come in the form of three macronutrients; protein, carbohydrates, and fat.

2.1.1 Protein

Protein synthesis occurs through the transcription of DNA to RNA and the translation of RNA. This process is known as the central dogma in biology. Transcription of DNA generates messenger RNA (mRNA) and transfer RNA (tRNA), which are necessary to build proteins. The nucleotide sequences of mRNA are translated into amino acids, which bind together to create proteins. Proteins are generated from twenty amino acids. Nine of these amino acids are essential for humans and mice, which means they must be provided from dietary sources (Table 2.1). In addition to creating proteins from individual amino acids, individual amino acids can be incorporated into various metabolic reactions to produce glucose through gluconeogenesis and ATP through the TCA cycle.

Although dietary intake of protein makes up approximately 1% of proteins stored in the body [9] various levels of protein in the diet have been investigated. Consumption of a high-protein diet can increase satiety; however, there is some concern that the liver's capacity to convert nitrogen to urea may be exceeded inducing a disease state with long-term adherence [10]. Furthermore, a high-protein diet can prevent muscle catabolism during weight loss [11].

Table 2.1: Twenty Amino Acids

Non-essential Amino Acids	Essential Amino Acids
Alanine	Histidine
Arginine	Isoleucine
Asparagine	Leucine
Aspartate	Lysine
Cystine	Methionine
Glutamic acid	Phenylalanine
Glycine	Threonine
Ornithine	Tryptophan
Proline	Valine
Serine	
Tyrosine	

2.1.2 Carbohydrates

The simplest form of carbohydrates is monosaccharides. All monosaccharides are made up of carbons, hydrogens, and oxygens with the empirical formula $C_m(H_2O)_n$. Monosaccharides consist of a single glucose, fructose, galactose, or xylose. Other forms of carbohydrates can be built from monosaccharides. Disaccharides (sucrose, lactose, maltose, and trehalose) and polyols (sorbitol and mannitol) are made up of two monosaccharides. Oligosaccharides (raffinose, oligofructoses, and maltodextrins) are made up of three to nine monosaccharides. Polysaccharides are made up of more than nine monosaccharides. Polysaccharides consist of starches (amylose, amylopectin, and glycogen) and non-starches (cellulose and pectins). Carbohydrates are broken down into monosaccharides through digestive enzymes, such as α-amylase in order to utilize for energy metabolism. Monosaccharrides, disaccharides, and polyols are generally known as simple carbohydrates while oligosaccharides and polysaccharides are known as complex carbohydrates.

Fructose and galactose can be converted into glucose for energy metabolism. Adipose, muscle, and liver through glucose transporters take up glucose in an insulin-dependent manner while excess glucose is stored as fat through *de novo* lipogenesis. Carbohydrates through dietary intake make up 50-100% of carbohydrate stores in the body, which is stored as glycogen [9].

The relationship between carbohydrate intake and health remains controversial. Like protein, intake of carbohydrates has been shown to induce satiety for at least 90 minutes postprandial [12]. Furthermore, current recommendations have suggested limiting fat intake to improve cardiovascular disease; however, a recent study has shown that high-carbohydrate diets have been associated with higher total mortality across the world [13, 14]. Conversely, high-carbohydrate diets have improved insulin sensitivity, lowered LDL cholesterol and reduced weight [15, 16].

2.1.3 Lipids

Lipids can act as receptors, serve as the plasma membrane of a cell, or be broken down into non-esterified fatty acids for energy. Lipids consist of triacylglycerols, phospholipids, sphingolipids, as well as others. Triacylglycerols consist of three fatty acids attached to a glycerol backbone and serve as the primary component of adipose tissue. Triacylglycerols can be broken down via lipoprotein lipase into glycerol and non-esterified fatty acids. In order for lipids to be accessible for metabolism breakdown of triacylglycerols is necessary. Non-esterified fatty acids, also known as free fatty acids, can then be shuttled to the mitochondria for ATP production. Free fatty acids are classified a short-chain, medium-chain, and long-chain fatty acids. Short-chain fatty

acids have six or fewer carbons, medium-chain fatty acids contain between eight and fourteen carbons, and long-chain fatty acids contain more than fourteen carbons. All fatty acids, regardless of length, consist of a carbon backbone and a carboxylic acid. Furthermore, fatty acids can be classified as either saturated of unsaturated. Saturated fatty acids contain hydrogens at all carbon atoms while unsaturated fatty acids contain at least one double bond.

Two fatty acids, linoleic acid and α -linoleic acid, are essential for humans and mice and can only be provided by diet. These fatty acids are necessary to build omega-3 and omega-6 fatty acids. Omega-3 and 6 fatty acids are named omega-3 and 6 because their first double bond is at the third and sixth carbon from the methyl end. Omega-3 fatty acids convert to eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) while omega-6 fatty acids convert to arachadonic acid (AA). DHA and EPA are considered anti-inflammatory and AA is considered pro-inflammatory [17]. High omega-6 to omega-3 ratios have been linked to obesity and diabetes and create a pro-inflammatory response [17].

Fat intake makes up less than 1% of fat stores in the body [9]. While consumption of a high protein diet induces satiety, high intakes of fat have not resulted in the same levels of satiety for any length of time [12], which may lead to overconsumption of food. For this reason high-fat diets and fats, in general, have been touted as the primary contributor to obesity; however, the ketogenic diet, which is primarily made up of fat has not induced an obesogenic state in individuals suggesting another mechanism [18, 19].

2.2 Health and Diet

Diet modification has treated a host of diseases including type 2 diabetes, epilepsy, Celiac's disease, irritable bowel disease (IBS), and cardiovascular disease [20-26]. Diet, however, can be used for more than disease reversal. Individuals may use diet to maintain a healthy weight or improve athletic performance [27-29]. The following four diets were chosen for this dissertation due to their known affects on human health. These diets were formulated to mimic the macronutrient and dietary composition of a human diet.

2.2.1 American (Western) Diet

With the industrial revolution refined grains, oils, sugars, and high fructose corn syrup were developed. These foods became the staples of a western diet, particularly after WWII. The diet that stemmed from the industrial revolution and the term western became synonymous due to the association of industrialization with western countries, such as the U.S. and England. From 2007-2008 the average caloric intake was 2,504 and 1,771 for men and women, respectively [30, 31]. When calories were broken down by macronutrient approximately 50% were attributable to carbohydrates, nearly 16% to protein, and roughly 33% to fat with 11% of fat being saturated fat [30-32]. The Dietary Guidelines for Americans were developed in 1980 and have been updated and revised every five years to promote a healthy diet based on published research. In addition to dietary recommendations the guidelines assess the current state of nutrition in the United States. According to the 2015-2020 Dietary Guidelines the average U.S. adult under-consumed vegetables (<1-2 cups per day) but exceeded the dietary

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recommendations for total grains, protein, sugars, saturated fats, and sodium [7].

Current dietary guidelines recommend 2.5 or more cups of vegetables, three cups or

less of dairy, 7 ounces of protein, and less than 10% of calories from sugar or saturated

fats. Despite these recommendations current research has shown an inverse

relationship between vegetable consumption and weight gain, heart disease, and

diabetes [33, 34]. This suggests that more than 2.5 cups of vegetables should be

consumed to receive maximal health benefits. In agreement with the recommendations

over-consumption of sugar showed an increased risk of cardiovascular disease and

metabolic syndrome, according to the NHANES [35, 36]. As individuals pursue food that

is pre-made, quick, and hyper palatable, diseases such as obesity, type 2 diabetes,

certain cancers, and cardiovascular disease have also increased [37, 38]. Furthermore,

as countries become westernized and consume more foods from an American diet the

aforementioned diseases follow [39]. Since the wide adoption of the American diet both

locally and globally, deaths due to preventable diseases, mentioned above, have

become some of the top causes of death [40].

2.2.2 Japanese Diet

The Japanese diet is low in fat and high in carbohydrate [41]. Rice, soybeans,

fish, some meats, oils, fermented foods, and vegetables typically make up the Japanese

diet [42]. After WWII the Japanese population began eating what is now considered a

traditional Japanese diet, mentioned above. Preceding WWII the Japanese population

was impoverished and primarily ate a diet of rice. The association between the

Japanese diet and longevity developed as a result of this alteration in dietary pattern.

Prior to WWII the average life expectancy of Japanese men and women was 42.8 and 51.1 years, respectively [43]. From 1946 to 1951 life expectancy rose by 18 years for men and 13.7 years for women. The life expectancy of the Japanese population continued to increase until Japan had the highest life expectancy in the world in 1979 [43]. As of today the average Japanese individual lives to 85, which is ranked third worldwide [44]. The high life expectancy of the Japanese population drew considerable attention to their eating habits. Okinawa, Japan, in particular, is known for having a large number of centenarians [45]. Okinawans tend to be resistant to acculturation and therefore maintain a traditional diet [45]. In 2002, the same scientists that research the Okinawan centenarian population published The Okinawa program [46]. This book provided dietary guidelines based on the Japanese diet in Okinawa popularizing the Japanese diet in the United States.

Rates of coronary heart disease (CHD) and cancer, in Japan, are some of the lowest in the world [47-49]. Investigating CHD in Japanese-Americans revealed the same rates of CHD when consuming a traditional Japanese diet; however, when Japanese-Americans consumed a western diet they had a 3-5-fold increase in CHD prevalence [47]. Furthermore, Japanese individuals that moved to the U.S. and adopted a western diet had increased rates of certain cancers [48]. Individual markers of health are improved through the consumption of a Japanese diet. In the Japanese population, consumption of fish and soy were associated with higher HDL cholesterol and folate levels [50].

Studies investigating the Japanese diet in mice also show significant health improvements. Mice fed a Japanese diet had lower liver weights, adiposity levels,

serum glucose, insulin levels, and triglyceride levels compared to their counterparts fed a Mediterranean diet [51]. Furthermore, modification of the Japanese diet to contain more fish, soy, seaweed, vegetables, fruit, and dashi, a fermented seasoning reduced serum LDL cholesterol, liver total cholesterol, and IL-6 levels compared to mice fed a traditional Japanese diet [41]. Individual components of a Japanese diet were also shown to have protective effects. Consuming green tea in conjunction with a high-fat diet diminished the negative effects of the high-fat diet in mice [52].

2.2.3 Mediterranean Diet

The Mediterranean diet consists of a moderate level of carbohydrates and fats. Individuals eating a Mediterranean diet consume a low intake of saturated fats, meats, and dairy; high intake of vegetables, cereals, nuts, fruits, and legumes; and a moderate intake of fish and wine [53-55]. Ancel Keys was the first to promote a Mediterranean diet in the U.S.. Investigating the diets of 12,000 men across seven countries in a study, known as The Seven Countries study, he revealed that the Mediterranean diet had protective effects of CHD [56, 57]. The Mediterranean diet, however, didn't become popular in the U.S. until the 1990's. Despite the promotion of the Mediterranean diet by Ancel Keys decades earlier, Walter Willet is often accredited with creation of the Mediterranean diet in the U.S. [58, 59].

In addition to the Japanese diet, consumption of a Mediterranean diet contributes to some of the longest life expectancies in the world [51]. Besides long life expectancies, the Mediterranean diet can improve specific markers of health. When assessing the health of an individual triglyceride levels, HDL, LDL, and blood glucose

levels are measured. These markers have been linked to CVD, NAFLD, and type 2 diabetes [26, 60-62]. A healthy individual will have triglyceride levels that are less than 150 mg/dL, HDL levels that are greater than 60mg/dL, LDL levels that are less than 100mg/dL, and fasting glucose levels that are less than 100mg/dL [63, 64]. Furthermore, menopausal women are assessed for osteoporosis. The Mediterranean diet has been shown to increase bone mineral density and reduce triglyceride levels [55, 65]. The Mediterranean diet has also been shown to improve energy metabolism in obese individuals. Sirt 4 is a mitochondrial ADP-ribosyltransferase that aids in the downregulation of insulin secretion via inhibition of glutamate dehydrogenase 1 [66]. Obese individuals adhering to a Mediterranean diet showed improved sirt 4 levels [54]. Additionally, adherence to a Mediterranean diet lowered their body mass index, fasting blood glucose, LDL, and increased HDL levels [54].

2.2.4 Ketogenic Diet

The ketogenic diet was formally developed in the 1920's to treat epilepsy [20, 21, 67]. Prior to the ketogenic diet starvation was the primary method to treat epilepsy [67]. The term "ketogenic" was coined due to the production of ketone bodies as a result of the diet [67]. The Ketogenic diet is predominantly comprised of fat. The original diet was developed by M.G. Peterman and consisted of 1g of protein per kilogram of body weight in children, 10-15g of carbohydrates per day, and the rest of the calories in fat [67, 68]. Ketosis is the basis of a ketogenic diet and occurs when fatty acid oxidation becomes the primary source of energy for the body in response to glucose depletion. As a result three ketone bodies—β-hydroxybutyrate, acetoacetate, and acetone are produced.

The ketogenic diet has also been used to treat symptoms of autism [69] and as an adjuvant treatment for cancer [70, 71]. In addition to the therapeutic effects of the diet, interest in the ketogenic diet has continued to gain popularity despite few studies showing the efficacy of the ketogenic diet as a weight loss tool [18, 72, 73]. Moreover, endurance athletes use a ketogenic to increase their endurance by reducing the need for glucose supplementation during an event and improving their maximum volume of oxygen (VO_2 max). Off-road cyclists fed a ketogenic diet utilized significantly more free fatty acids during exercise than cyclists on a mixed diet [29]. VO_2 max was also significantly higher and RER was significantly lower in cyclists fed a ketogenic diet [29]. The ketogenic diet can also improve these markers for weeks after cessation of the diet. Individuals fed a ketogenic diet showed a reduced RER after 20 days and remained low at 40 days even after cessation of the ketogenic diet [72].

2.3 The Role of Genetics in Diet and Disease

Diets are used to lose weight, improve health, or treat disease; however the efficacy of a diet can be influenced by genetic background. Studying dietary responses in humans can be challenging for many reasons, including but not limited to the inability for biological replicates, compliance, high-heterozygosity, and issues with self-reporting methods. Regardless, several human studies have been able to determine the genetic effects on diet and disease [45, 74, 75]. Postprandial plasma triglyceride levels are a risk factor for cardiovascular disease. Individuals fed a high-fat diet were assessed for genetic variants associated with plasma triglyceride levels. Two SNPs were associated with triglyceride response revealing genetic variants that may increase plasma

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triglyceride levels [76].

Mice are a good model to represent humans due to their genetic similarity, the ability for biological replications, and their inherent compliance. Inbred strains of mice are developed through the breeding between siblings or parent and progeny. In order for a mouse line to become homozygous must be bred for at least 20 sequential generations (F20). After 20 generations mice become 98.7% homozygous at all loci [77], which allows for biological replication. In addition to biological replication inbred mice make it possible to compare multiple scientific studies potentially produced at different times and in different locations. This allows researchers to comprehensively study the biology of a particular mouse strain. Due to the inbred nature of a mouse strain, however, it can be difficult to extrapolate results beyond the genetic breed. For this reason, multiple strains can be utilized to represent genetic diversity and allow us to better understand how individual variation can influence physiological responses to dietary inputs. For example, diet induced obesity has been shown to be induced in C57BL/6J mice, while A/J and NOD/ShiLtJ mice are obesity resistant [78-81]. Furthermore, genetic background has been shown to influence the severity of disease. Ob/ob mice on a FVB/NJ background developed severe diabetes while ob/ob mice on a C57BL/6J mice developed hyperglycemia [82]. Treatment of disease through diet has also been shown to have differing levels of success due to genetic background. Seizures have been successfully controlled through a ketogenic diet; however, between 10-40% of patients do not experience a reduction in seizures, which is thought to be due to inherent genetic differences [83]. Investigating the effects of genetic background on mice consuming a ketogenic diet revealed that C57BL/6J and DBA/2J mice

remained seizure-free while FVB/NJ and A/J mice both continued to experience seizures [83]. In order to assess the role of genetics in our study four strains (A/J, NOD/ShiLtJ, FVB/NJ, and C57BL/6J) were chosen in this dissertation for their altering susceptibilities to common metabolic diseases.

2.3.1 A/J

A/J mice were developed by LC Strong in 1921 [84]. They are a cross between a Bagg albino and a Cold Spring Harbor albino. A/J mice are high susceptible to cancer and are used as a carcinogen induced cancer model [85, 86]. Conversely A/J mice are resistant to diabetes, obesity, atherosclerosis, insulin resistance, and glucose intolerance [78, 84, 87]. In addition to metabolic markers A/J mice were shown to have low levels of activity [88].

2.3.2 C57BL/6J

The C57BL/6J mouse was developed using mice provided by Abbie Lanthrop, a high school teacher [77]. Clarence Little, along with Leonell Strong, and E.C. MacDowell created the C57BL/6J line around 1918 [77]. In contrast to A/J mice, C57BL/6J mice are susceptible to diet-induced obesity, type 2 diabetes, and atherosclerosis [78, 82, 89-91]. C57BL/6J also show high levels of activity [88]

2.3.3 FVB/NJ

FVB/NJ mice were developed in 1966. Outbred Swiss mice were bred for a resistance or sensitivity to histamine challenge after receiving a pertussis vaccination [92]. It was discovered that the sensitive line was also sensitive to the Friend leukemia

virus B strain (FVB) so this generation was then inbred specifically to develop the FVB/NJ strain [92]. FVB/NJ mice are susceptible to certain cancers, have higher basal body temperature, activity, and anxiety levels [93, 94]. Additionally, FVB/NJ mice are homozygous for the Pde6b^{rd1} allele resulting in retinal degeneration [92].

2.3.4 NOD/ShiLtJ

NOD/ShiLtJ mice developed from the inbreeding of the Cataract Shionogi strain [95]. NOD/Shi mice developed at the F6 generation, marked by elevated fasting blood glucose. The F6 generation was then inbred and at the F20 generation, female mice spontaneously developed insulin-dependent diabetes [95]. These mice were then inbred in 1974 to develop the NOD/ShiLtJ strain. NOD/ShiLtJ mice are a model for type 1 diabetes [96]. They are characterized by the leukocyte infiltration of pancreatic islets. In addition NOD/ShiLtJ females are the first to show decreased in pancreatic insulin, typically around 12 weeks, while males express this phenotype weeks later [81].

2.4 The Role of Sex in Diet and Disease

Research investigating diet and disease over the last century has predominantly used males as their subjects; however, it has been shown that females differentially respond to diet and disease in some cases. The largest difference between males and females relates to their sex hormones. Females primarily produce estrogen and progesterone while males produce testosterone. Physiologically females tend to have a higher proportion of body fat relative to men, regardless of ethnic group [97]. There are also sex differences when it comes to utilization of fats and carbohydrate at rest and

during exercise. Women store more fatty acids as triglycerides while men oxidize free fatty acids at the resting state [98], which may be due to estrogen levels in women [99]. Conversely, during exercise, women preferentially use fatty acids over carbohydrates while men preferentially use carbohydrates [98]. Free fatty acids have also been shown to have differential effects on insulin sensitivity. Free fatty acids in men played a role inhibiting tissue insulin sensitivity but had no effect on insulin sensitivity in women [100]. Furthermore, post-menopausal women or ovariectomized mice had increased insulin resistance [101].

Disease prevalence can also be due to sex dimorphisms. Seventy-eight percent of autoimmune disease diagnoses are women [102]. In contrast, men are more likely to develop type 1 diabetes [103]. Additionally, both men and women are nearly equally at risk for CVD; however, women have a higher rate of death [104].

2.5 Metabolomics

Chromatographs are used to separate analytes (i.e. compounds in a sample). The first chromatograph was developed by Mikhail Semenovich Tsvett in the early 1900's. This chromatograph was able to separate plant pigment. Paper chromatography was developed several decades later in 1941 by Richard Synge and Archer Martin. This allowed for the separation of colorless compounds as the solvent spread across the paper. Compounds would spread across the paper based on their solvency in the chosen solution. Paper chromatography was the primary method used until NMR was developed in the early 1950's. NMR spectroscopy determines the physical and chemical

properties using the magnetic properties of the atomic nuclei [105]. The intramolecular magnetic field that surrounds an atom in a whole molecule alters the resonance frequency, which provides structural information. A major advantage to NMR is sample recovery, but the inability to resolve compounds due to low sensitivity must be considered. Less than a decade after the development of NMR, in 1959, the first gas chromatographer was coupled to a mass spectrometer occurred [106]. Analytes measured using gas chromatography-mass spectrometry (GC-MS) must be volatile limiting the types of analytes that can be analyzed, however, the resolving power—the ability to provide a specific mass resolution (chapter 3.6.2)—is higher than NMR due to higher sensitivity. The first paper published using metabolomics as it is currently thought of analyzed urine and breath using GC to discern nutritional differences in 1971. Robinson and Pauling measured approximately 250 metabolites in breath and 280 metabolites in urine.

Electrospray ionization (ESI) coupled with liquid chromatography and mass spectrometry was developed in 1989 [107] (see section 3.6.1 for details). The addition of ESI was an important technological advancement in metabolomics because it allowed for the ionization and measurement of larger molecules, such as metabolites, with very little fragmentation. This paved the way to be able to measure metabolites, such as peptides, nucleic acids, and other polar hydrophilic compounds.

The term metabolomics was not coined until 1998 by S.G. Oliver, et. al. [108]. Metabolomics is the quantification and identification of metabolites that are left by biochemical reactions that occur throughout the body. These measurements are usually taken at a single point in time. Metabolomics is usually thought of as the phenotypic

response to the completion of the central dogma (Fig. 2.2). Metabolites in the body are influenced by diet, disease, environment, genetics, and many other factors. To understand the number and types of metabolites that could be identified the human metabolome project was formed. The first draft of the human metabolome was completed in January 23, 2007. This metabolome consisted of approximately 2,500 metabolites, 1,200 drugs, and 3,500 food components.

There are two major types of metabolomics: untargeted and targeted. Untargeted metabolomics can detect thousands of metabolites while targeted metabolomics measures a set of pre-defined metabolites or pathways. Untargeted metabolomics can investigate metabolic disruptions due to an external perturbation without prior biological information. This allows for the potential to identify novel metabolites, which may be a biomarker of disease. The sensitivity of the measurements, however, can be lower compared to targeted metabolomics due to the large range of metabolites being measured and the inability to perform absolute quantification [109]. In contrast, targeted metabolomics can allow for absolute quantification of metabolites of interest by comparing metabolites of interest to known quantities of their respective ^{13}C-labeled standard. Targeted metabolomics, however, does not measure unknown metabolites. Conversely, an untargeted approach allows for the identification of unknown metabolites (metabolites that are not defined by a retention time and mass-to-charge ratio). Although these metabolites cannot be identified they can potentially be linked to diseases as the field of metabolomics progresses.

With the potential to identify thousands of metabolites in a single sample bioinformatics tools have been employed to hand the complex data. Furthermore, due

to the potential limitations in detection, data must be handled carefully to deal with missing values, background noise, and alterations in retention time due to column drift (see chapter 3).

Most metabolomics studies use non-invasive samples (i.e. serum, urine, etc.) to identify metabolites rather than tissue. This provides a global metabolite profile but does not provide any tissue specific information; however, understanding how tissue metabolites are influenced has become of interest recently. Using untargeted metabolomics, adipose, muscle, and liver tissue were assessed to identify alterations in the metabolome due to diet, genetic, and/or sex effects.

2.5.1 The Role of Metabolomics in Understanding Diet

Diet and disease are inextricably linked. Understanding how diet alters underlying metabolic pathways can provide insight into how whole diets and/or supplements influence health and ultimately disease. Many studies use low-fat and high-fat diets to assess diet differences. Metabolomics studies have investigated the differences between high- and low-fat diets in serum, liver, feces, and urine. Metabolites involved in energy metabolism and amino acid metabolism were all increased with the consumption of a high-fat diet [110, 111]. Furthermore, individuals consuming a Nordic diet, which consists of whole-grains, berries, root vegetables, and fatty fish showed altered amino acid metabolism and fatty acid profiles [112].
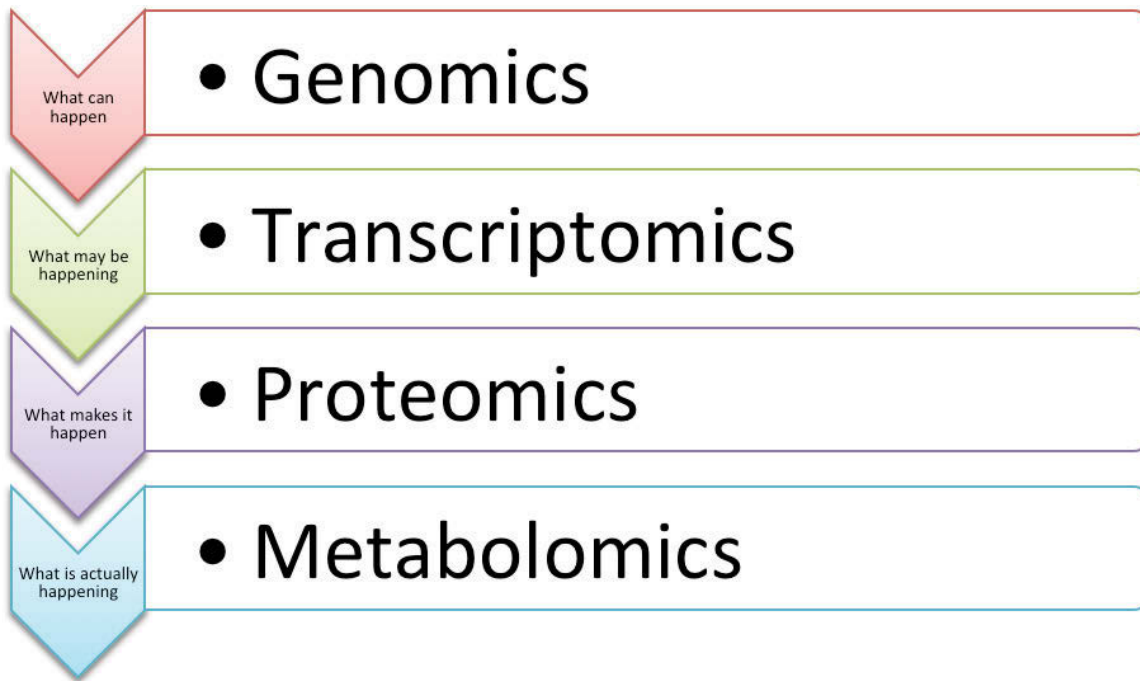
Figure 2.1: Depiction of the Central Dogma through –omics studies.

Individual dietary components can also influence the metabolome. Consumption of meat provides an exogenous influx of amino acids into the body. Investigating humans consuming a vegan diet, which consists of zero animal products, had lower amino acid levels compared to omnivores [113]. Additionally, specific metabolites can be associated with dietary components. Vegetable intake was positively correlated with phenylacetylglutamine while red-meat intake was associated with o-acetylcarnitine, which aids in the transport of acetyl-CoA to the mitochondria during fatty acid oxidation [114]. Moreover, increased pipecolic acid and s-methylcysteine levels in serum for both humans and mice are associated with consumption of dry beans [115].

It has been shown that individual dietary components can be attributable to changes in the metabolome; however, dietary components can interact to modulate the effects of one another with the potential to cancel out deleterious effects. Metabolites influenced by a high-fat diet in mice were mediated by consumption of green tea [52]. In addition to diet-driven alterations in the metabolome, caloric differences can also affect the metabolite levels. Metabolites in the TCA cycle and glycolysis were altered by energy-restriction [116].

2.5.2 The Role of Metabolomics in Understanding Disease

Metabolomics can be used to identify biomarkers that can predict a disease state or as a means to identify potential treatment targets. Leucine, isoleucine, and valine are branched-chain amino acids (BCAA), which are necessary for protein synthesis and metabolism of glucose. Levels of BCAA were increased in obese individuals, type 2 diabetics, and individuals displaying insulin resistance [117-119]. Furthermore,

increases in body mass index—a tool used to determine overweight and obesity—in addition to obesity were associated with increased levels of amino acids [117, 119]. Moreover, alterations in amino acid metabolism and tryptophan metabolism were also altered in obese individuals resulting in increased levels of kynurenine, kynurenic acid, and quinolinate [119, 120].

The ability to predict the development of disease is important for prevention. As of 2015, it is estimated that approximately 30 million individuals have diabetes in the U.S. [121]. The Framingham Heart Study profiled 2,383 individuals to identify relationships between body mass index and cardiometabolic traits. Plasma metabolites from the Framingham Heart study identified 2-aminoadipic acid as a predictor of developing diabetes. This longitudinal study showed that increased levels of 2-aminoadipic acid were associated with a >4-fold risk of developing diabetes up to 12 years prior to developing type 2 diabetes [122].

Non-alcoholic fatty liver disease (NAFLD) is marked by inflammation and excess storage of fat in the liver. Chronic NAFLD can lead to cirrhosis. Dysregulation of amino acids has been linked to NAFLD [123]. Additionally, obesity-associated NAFLD in humans revealed increased BCAA in serum in individuals with steatosis [124].

Although metabolic diseases alter amino acid metabolism, obesity treatments can reverse these alterations. Both dietary intervention and gastric by-pass surgery decreased branched chain amino acids and other amino acids [125]. In addition to alterations in metabolites, glucose homeostasis was improved, which may be due to lowering BCAA levels [125].

The development or treatment of metabolic diseases has been shown to alter the metabolome; however, bodily injury can also alter the metabolome. Injury to the anterior cruciate ligament (ACL) resulted in changes in the following pathways in synovial fluid: glycine, serine, and threonine; arginine and proline; alanine, aspartate, and glutamate [126]. Additionally, joint injury resulted in changes in several amino acids, including hydroxyproline and proline [126]. Amino acids have been shown to play a role in joint health [127]. Furthermore, hydroxyproline and proline are critical for collagen formation [128].

2.5.3 The Role of Metabolomics in Understanding Genetics

Genetics may influence an individual's response to diet or disease. Metabolomics can assess how different genetic backgrounds influence the underlying metabolome. Leptin is important in the regulation of energy balance and reduces appetite. Dysregulation of hunger signals can occur if an individual becomes leptin resistant. Furthermore, absence of leptin or the leptin receptor can also alter hunger signals. Two genetically obese mouse models, db/db (leptin receptor deficient) and ob/ob (leptin deficient), showed an increase in BCAA and a decrease in other amino acids [129]. Db/db mice, however, are also a model for type 2 diabetes. Glycoxylate, a biomarker for diabetes [130] was elevated in db/db mice while lysine and arginine were decreased [129].

Type 1 diabetes (T1D) is a polygenic autoimmune disorder that results in the destruction of pancreatic β -cells. NOD/ShiLtJ, a T1D mouse model, were compared to C57BL/6J mice to assess metabolic alterations due to type 1 diabetes. Metabolites in

the TCA cycle were higher in C57BL/6J mice with the exception of glutamic acid, succinate, and asparate [96]. Glutamic acid is converted to gamma-aminobutyric acid (GABA) via glutamic acid decarboxylase (GAD). NOD/ShiLtJ mice express anti-GAD enzymes, which prevent the conversion of glutamic acid to GABA.

Humans are inherently genetically diverse making it difficult to separate genetic influences from environmental influences; however, differences in metabolites can still be assessed as potential biomarkers of disease. Genome wide associations studies can be combined with metabolomics to identify genetic alterations linked to metabolic differences. Genetic variants that correspond to metabolic phenotypes have been matched to metabolic pathways. For example, FADS1 is an enzyme that aids in the metabolism of omega-3 and omega-6 fatty acids. SNPs containing the FADS1 gene have been shown to explain up to 10% of the variance in glycerophospholipd concentrations [131].

2.6 Summary

Metabolic diseases in the U.S. and worldwide continue to rise despite current treatments. Metabolomics studies have revealed a complex relationship between metabolic diseases, diet, and genetics. Branched chain amino acids as well as other amino acids were often altered in response to diet or disease, although it is not clear why. Some metabolic differences are also due to sex. The complex relationships between diet, genetics, and the potential role of sex have yet to be studied in a

comprehensive way. Understanding the influence of each and their interactions may

provide insight into how the metabolome is altered to affect physiology.

CHAPTER THREE

EXPERIMENTAL AND COMPUTATIONAL METHODS TO PERFORM

NUTRITIONAL METABOLOMICS

3.1 General Workflow to Perform Tissue Level Metabolomics

Untargeted metabolomics was performed for all experiments discussed using liquid chromatography coupled with electrospray ionization quadropole mass spectrometry (Fig. 3.1). To understand and characterize how diet, genetics, and sex interact to influence the metabolome adipose, skeletal muscle, and liver tissue were flash frozen from four strains of mice after being fed five diets for 6-7 months. Extracting metabolites from tissue was not inconsequential due to potential metabolic shifts if the tissue samples degrade. As a result, tissue samples are kept frozen throughout the extraction process as well as all equipment and solutions. High-performance liquid chromatography separated metabolites based on size and polarity. Metabolites are then ionized through electrospray ionization and transferred to the mass analyzer and detector. This is where mass-to-charge (m/z) and relative abundance are measured in a full mass spectrum (MS1). This dataset is then compared against known retention times and m/z to identify metabolites. Using retention time and m/z to identify metabolites is moderately specific; however, some metabolites such glucose-1-phosphate and glucose-6-phosphate cannot be discerned with using tandem mass spectrometry (MS2). The steps mentioned above allow us to identify and quantify thousands of metabolites in complex tissue samples across multiple interactions. Each step is discussed in detail below.

HCD Cell   C Trap   HyperQuad Mass Filter with Advanced
Quadrupole Technology (AQT)

Advanced Active Beam
Guide (AABG)

RF Lens

Orbitrap
Mass Analyzer
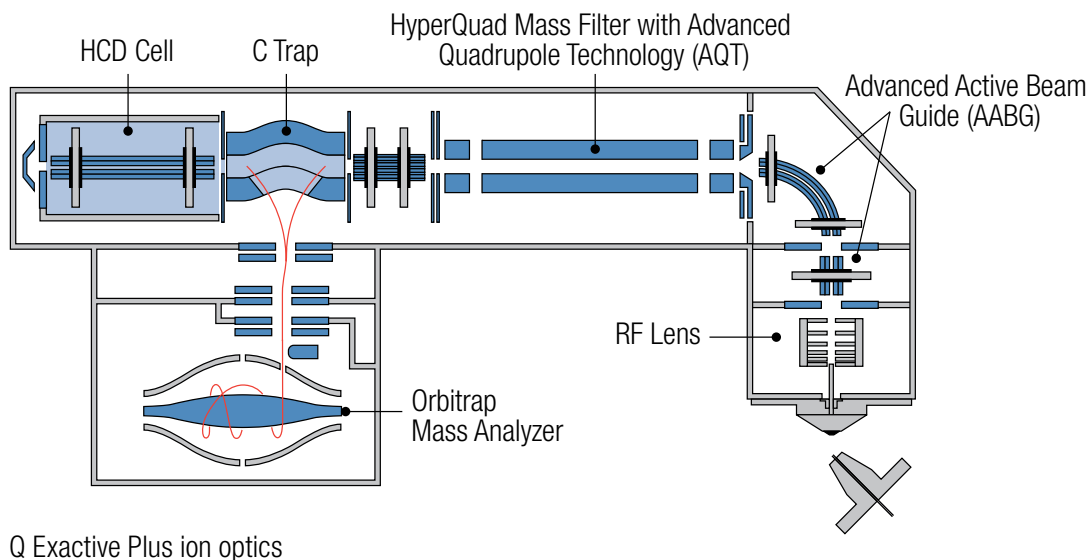
Q Exactive Plus ion optics

Figure 3.1: Schematic of Q-Exactive Plus Hybrid Quadropole-Orbitrap Mass Spectrometer (Image source: Thermo Scientific Q Exactive Plus Orbitrap LC-MS/MS System product specification sheet: https://assets.thermofisher.com/TFS-Assets/CMD/Specification-Sheets/PS-63912-LC-MS-Q-Exactive-Plus-Orbitrap-PS63912-EN.pdf)

3.2 Sample Preparation

Metabolite measurements typically come from cells, due to their ability to easily perform flux analysis and quantitative labeling. Metabolite measurements, however, from tissue can provide insight into systemic changes despite the additional challenges; tissue cannot be easily labeled, quantifying turnover rates is difficult at best, and preventing degradation of the sample during the extraction process requires special care. Measurements from a single time point can provide a lot of information regarding long-term changes and which metabolites are sensitive to perturbations and which are not. Tissue metabolites also provide a much more comprehensive picture since tissues interact within the body.

Adipose, skeletal muscle, and liver tissue was collected from male and female mice from A/J, NOD/ShiLtJ, C57BL/6J, and FVB/NJ strains. Mice were fed one of five diets (Japanese, ketogenic, Mediterranean, Western, and standard chow) for a total of 6-7 months. Dissections were performed by Dr. David Threadgill's laboratory and samples were sent on dry ice to the University of Tennessee-Knoxville. Mice were staggered throughout the experiment due to volume. Although I was largely uninvolved with mouse work, I did participate in dissections and assisted with some laboratory work while visiting Dr. Threadgill's laboratory for a total of two weeks. Samples were stored at -80°C and remained frozen until methanol extraction.

3.3 Metabolite Extraction

Different methods can be utilized to extract metabolites from biological samples to analyze hydrophilic (e.g. methanol, freeze thaw methanol) or hydrophobic

metabolites (e.g. Hydrochloric acid, chloroform, and methanol:chloroform) . Currently, there are no methods that extract all of the metabolites present in a sample; therefore, the extraction method is crucial to identifying metabolites of interest. Two solvents; methanol and methanol:water were chosen for metabolite extraction in the following studies. One hundred percent methanol and 80:20 methanol:water were chosen for their low decomposition rates of high energy molecules [132].

3.4 Internal Standard

Internal standards in mass spectrometry are used to quantify metabolite abundances or to control for run-to-run variation when running a high volume of samples that exceed the capacity of the mass spectrometer. To quantify metabolites a purified version of the metabolite of interest is used. Quantification requires that the metabolite of interest is labeled with ^{13}C. A known quantity is injected into the mass spectrometer and is subsequently compared across all samples of interest to quantify the abundance of the metabolite in each sample. A single purified metabolite with known m/z and retention time can also be used to measure run-to-run variation ensuring that metabolites are being properly identified.

Another method that is less common is to uniformly label bacteria, such as *Escherichia coli* (*E.Coli*) and spike samples of interest. This allows for multiple metabolites to be assessed at the same time. To uniformly label bacteria, bacteria are grown on ^{13}C-glucose agar plates and then transferred to ^{13}C-glucose liquid cultures. The bacteria are then tested for uniform labeling on the mass spectrometer before being

used as an internal sample for any experiment. ^{13}C-labeled *E. Coli* NCM3722 were spiked into all samples for each experiment discussed.

3.5 Liquid Chromatography

Extraction of metabolites from tissue results in a complex mixture of compounds. A reverse phase liquid chromatography column was used for experiments discussed. Liquid chromatography is used to separate mixtures of compounds in a high throughput manner. There are two separation methods that can be performed to accomplish this; normal phase liquid chromatography (NPLC) and reverse phase liquid chromatography (RPLC). Normal phase liquid chromatography refers to a situation when the stationary phase is more polar than the mobile phase, while reverse phase liquid chromatography refers to a situation where the stationary phase is less polar than the mobile phase. RPLC retains hydrophobic compounds on the column while allowing hydrophilic compounds to be released quickly. NPLC results in the opposite response. Choice of column is important depending on the type of molecules of interest.

3.6 Mass Spectrometry Instrumentation

All mass spectrometers consist of an ionization source, a mass analyzer, and a detector. The compounds of interest are first ionized with a negative or positive charge and converted into a gaseous substance. These gaseous ions then pass on to the mass analyzer where they are resolved according to their mass-to-charge ratio. Finally, the ions that emerge from the analyzer are detected and their relative abundance is

measured. The relative abundance value is then converted into an intensity signal, which is then graphed with the m/z ratio as the independent variable and the relative abundance as the dependent variable.

3.6.1 Electrospray Ionization

Electrospray Ionization (ESI) is a soft ionization method that is used to ionize compounds with very little fragmentation. This allows biologists to identify and analyze large, nonvolatile molecules that were previously difficult to capture [107]. ESI is performed by applying a voltage to a capillary tube that transfers the eluent to the mass spectrometer. The following experiments were performed using a spray voltage of 3kV. The eluent that moves through the capillary tube becomes a protonated eluent and develops a Taylor cone at the tip of the capillary tube [133]. When the voltage is applied, a fine spray of liquid is ejected from the Taylor cone. This liquid breaks into tiny like-charged droplets. As these like-charged droplets move towards the mass spectrometer their solvent evaporates due to the heated capillary. As the droplet size shrinks their surface charge density increases until they hit the "Rayleigh stability limit" [134]. This is when their surface tension can longer be sustained and they undergo "Coloumb fission" [135]. Smaller droplets are continuously formed until positively charged ions in the gas-phase are formed. These will then pass through the heated capillary and into the mass analyzer (Fig. 3.2). The nitrogen sheath gas was set to a flow rate of 10psi and the capillary temperature for the following experiments was set at 320°C.

3.6.2 Mass analyzer and Detector

The mass analyzer is where ions are separated by their m/z ratio. There are many different types of mass analyzers. Each one is specific to a subset of molecule types. When considering which analyzer to use figures of merit must be considered. Figures of merit consist of five items: mass resolution, mass resolving power, mass accuracy, mass precision, and dynamic range. Mass resolution is the observed mass-to-charge divided by the smallest distance that two m/z can be separated, while mass resolving power is the ability of the mass spectrometer to provide a specified value of mass resolution [136]. Mass resolution is also defined at 50% of the maximum peak height for an Orbitrap mass spectrometer. Mass accuracy is defined as the difference between the measurement result and the exact mass of a measured value. Mass precision (or sensitivity) is the difference between independent mass measurements [137]. Dynamic range is the range between the smallest m/z and the largest m/z that the analyzer can detect.

The Orbitrap is used in the following experiments (Fig. 3.1). It has a mass range of 50-6,000 m/z, a dynamic range of >5,000:1, a mass accuracy of <1PPM RMS internally and <3PPM RMA externally, and a sensitivity of 500fg buspirone on column S/N 100:1 [138]. Samples in the following experiments were analyzed with a resolution of 140,000. A scan window of 85 to 800 m/z was used from 0 to 9 minutes, and a window of 110 to 1000 m/z from 9 to 25 minutes in the following studies.
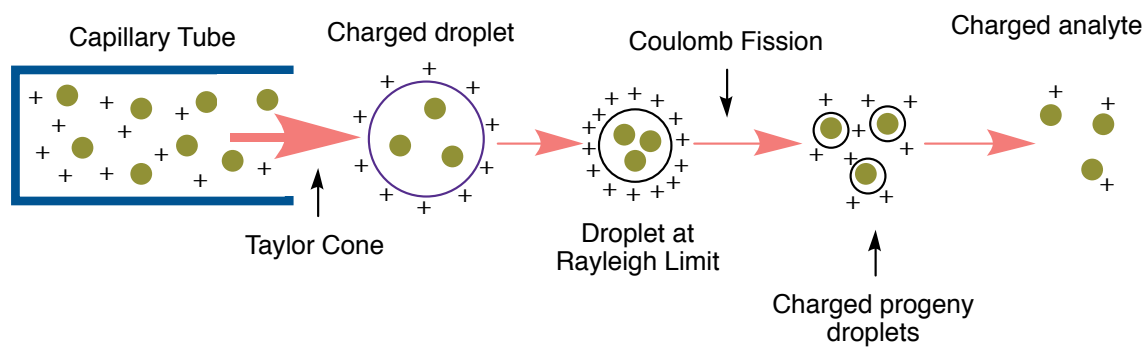
Figure 3.2: Schematic Depicting Formation of Charged Analyte from Electrospray Ionization

Before ions are analyzed they must go through the RF-lens, quadropole, and C-trap. The RF-lens and quadropole store and isolate ions, which are scanned by m/z before being transported into the C-trap to measure their mass.

3.7 Analysis of Metabolomics Data

Full MS1 allows for thousands of molecules with different m/z to be scanned and measured. To properly analyze any biological data as many sources of variation must be removed as possible. High-throughput data, such as metabolomics, creates a unique set of issues that must be addressed due to computational and instrumentation limits. To limit external variation the experimental design must be considered. Samples should be randomized according to the experimental design to reduce run-to-run variation on the mass spectrometry. If samples cannot be computationally processed together then they should be randomized again before picking peaks, otherwise systematic variation may be introduced.

Metabolites measured from the mass spectrometer are defined by their retention time on the column and their m/z ratio. The first step in defining a metabolite involves correcting for column drift. As the column in the mass spectrometer is used the metabolites tend to remain attached to column longer. To obtain the correct retention time an algorithm to correct for column drift must be used. Many programs automatically correct for column drift, including MAVEN, the program used to identify metabolites for the following studies.

3.7.1 MAVEN

Raw files are created by Xcalibur, which is the software associated with a Thermofisher Scientific mass spectrometer. These files must be converted to an mzML file, which can be read by MAVEN. Conversion can occur via msconvert [139]. MAVEN is an open source data analyzer for metabolomics data [140, 141]. When samples are loaded into MAVEN total ion chromatograms are corrected based on retention time automatically, for each sample (Fig. 3.3). A list of known metabolites; metabolites that have a retention time and m/z, can be uploaded to identify metabolites. Metabolites can then be manually chosen or automatically chosen. All of the following experiments used both manual and automatic peak picking techniques. Identified metabolites were manually chosen and peak abundance was integrated by mass (±5 ppm) and retention time. Unidentified metabolites were chosen using an algorithm with the following settings: minimum peak width, 5; minimum signal/blank ratio, 3 or greater; minimum peak intensity, 10,000; and minimum peak/baseline, 3. Unidentified peaks were filtered manually to remove those that did not meet the above criteria.

3.8 Normalization of Data

3.8.1 Imputation

Often there are missing values in metabolomics data, which are generally thought to be below the sensitivity of the mass spectrometer. As a result, researchers replace the missing values with one of several techniques. Our study used k-Nearest Numbers (kNN) imputation to replace missing values. kNN imputation averages k-
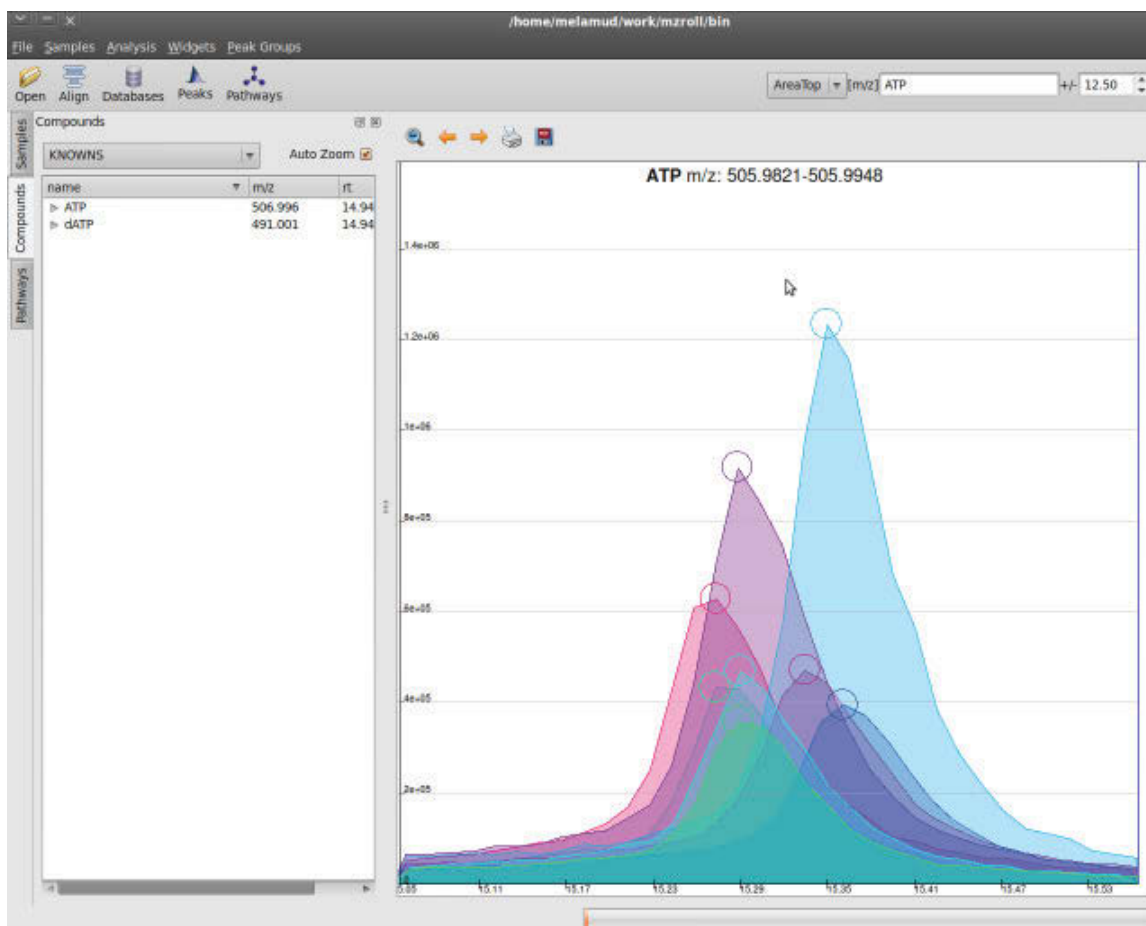
Figure 3.3: Example of Peak Analysis in MAVEN software (Image source: http://genomics-pubs.princeton.edu/mzroll/index.php?show=screenshots)

neighbors to determine a value for the missing number. Using a small k allows for noise to be more influential while a large k will reduce the local effects of the data allowing the data to become more generalizable. To find the k-nearest neighbors a Euclidean metric is used (eq. 3.1) [142].

Equation 3.1

$$dist(A,B) = \sqrt{\frac{\sum_{i=1}^{m}\left(x_i - y_i\right)^2}{m}}$$

where m is the dimensionality of the data matrix and A and B are data vectors. Replacing missing values in metabolomics is important to the matrix structure for downstream analysis. Missing values are not well tolerated in certain multivariate analysis techniques, such as partial least squares discriminant analysis (PLS-DA) or principal component analysis (PCA). Metabolites that were missing 70% or more sample measurements were removed from analysis prior to kNN imputation.

3.8.2 Covariates

Metabolomics data may have confounding factors so covariates are sometimes needed. Metabolite abundance is sensitive to the quantity of sample measured, usually in a linear fashion. For this reason it is important to adjust metabolite abundance for sample weight to correct the metabolite abundance. Another important covariate to consider including is an internal standard. Run-to-run variation may occur on a mass spectrometer. One way to correct for this is to run a single internal standard of known

mass and retention time. Another method is to spike samples with a ^{13}C-labeled

bacterial species, such as *E. Coli*, to standardize metabolite measurements across

runs. This allows for direct comparisons between individual metabolites within a known

sample and individual metabolites within a ^{13}C-labeled sample of known quantity. An

internal standard should correct for any sample variation from run-to-run given that the

internal standard is homogenous across all samples.

Metabolites measured from the ^{13}C *E. Coli* internal standard were matched with

their corresponding metabolite; otherwise metabolites measured were matched with a

^{13}C metabolite of the same class according to their chemical taxonomy. Classes were

identified using the Human Metabolome Database [143-145]. Tissue weight and internal

standard were treated as covariates for the statistical models in this dissertation to

control for biological and technical variation.

3.8.3 Scaling and Transformations

Due to variation across mice, even within the same strain, it is important to

consider scaling methods. Additionally, scaling across metabolites may be necessary to

interpret high and low signals. Scaling can occur across samples, metabolites, or both,

which allows for variation reduction. Pareto scaling mean centers the data and then

divides by the square root of the standard deviation. This shrinks high signal data

without losing low signal data. Pareto scaling was performed on each metabolite, across

samples, in this dissertation. Mice were then median normalized, across metabolites,

and cube-root transformed to create a normal distribution.

3.9 Statistical Techniques Used to Analyze Metabolomics Data

3.9.1 Benjamini-Hochberg to Control for Type 1 error

The Benjamini-Hochberg procedure controls the expected proportion of significant effects that are in fact type 1 errors (or false positives). This is called the False Discovery Rate (FDR). In this dissertation, we will set an upper bound for FDR of $q = 5\%$. Let $p_{(1)} \leq p_{(2)}...\leq p_{(k)}$ be the ordered p-values for m independent tests. For our given upper bound $q = 0.05$, determine k to be the maximum i for which

Equation 3.2

$$p_{(i)} \leq \left(\frac{i}{m}\right)q.$$

Then the k smallest p-values all correspond to significant effects, controlling for FDR $\leq$ q.

Another method to correct for type 1 error is the Bonferroni correction, which is a way to control the family wise error rate. The family wise error rate is the probability of having one or more false discoveries when performing multiple hypothesis tests. To perform the Bonferroni correction the significant p-value is multiplied by the total number of tests. The Benjamini-Hochberg procedure is more appropriate for metabolomics data because it is less conservative than the Bonferroni correction. Since the Bonferroni correction is a family wise error rate it reduces the number of type 1 errors at the cost of increasing the number of type 2 errors (or false negatives). In metabolomics data this

can result in a large reduction in the number of significant metabolites due to the volume of metabolites being measured. The Benjamini-Hochberg procedure, however, controls the expected proportion of false discoveries allowing for a less stringent cutoff. As a trade-off this procedure allows for a percentage of type 1 errors to remain in the analysis in order to gain power while the Bonferroni correction attempts to remove all type 1 errors at the cost of power. All of the following experiments used the Benjamini-Hochberg procedure to adjust for multiple comparisons.

CHAPTER FOUR

TISSUE LEVEL DIET AND SEX-BY-DIET INTERACTIONS REVEAL UNIQUE METABOLITE AND CLUSTERING PROFILES USING UNTARGETED LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ON ADIPOSE, SKELETAL MUSCLE, AND LIVER TISSUE IN C57BL6/J MICE

Ann Wells[1], William T. Barrington[2], Stephen Dearth[3], David W. Threadgill[2], Shawn R. Campagna[1, 3], Brynn H. Voy[1, 4]

[1]University of Tennessee-Knoxville, UT-ORNL Graduate School of Genome Science and Technology, [2] Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, [3]University of Tennessee-Knoxville, Department of Chemistry, [4]University of Tennessee-Knoxville, Department of Animal Science

Ann Wells' contribution included: tissue extractions, data analysis, and primary writing of the manuscript.

4.1 Abstract

Diet is a common tool used for weight loss or to improve health. Obesity, however, contributes to the intersection between diet and disease due its effect on risk of developing type 2 diabetes, hypertension, cardiovascular disease, stroke, osteoarthritis, and certain cancers. Various dietary profiles are associated with effects on health, yet little is known about the effects of diet at the tissue level. Using untargeted metabolomics, this study aimed to identify changes in water-soluble metabolites in C57BL/6J males and females, fed one of five diets (Japanese, ketogenic, Mediterranean, American, and standard mouse chow) for seven months. Metabolite abundance was examined in liver, skeletal muscle, and adipose tissue for sex, diet, and sex-by-diet interaction. Analysis of variance (ANOVA) suggests that liver tissue is most metabolically active under dietary changes compared to adipose and skeletal muscle.

The ketogenic diet was distinguishable from other diets for both males and females, according to partial least squares discriminant analysis. Pathway analysis revealed that the majority of pathways affected play an important role in amino acid metabolism in liver tissue. Not surprisingly, amino acid profiles were affected by dietary patterns in skeletal muscle. Few metabolites were significantly altered for adipose tissue relative to skeletal muscle and liver tissue indicating it was largely stable, regardless of diet alterations. The results of this study revealed that the ketogenic diet had the largest affect on physiology, particularly for females. Furthermore, metabolomics revealed that diet affects metabolites in a tissue-specific manner; however, liver was most sensitive to dietary changes.

4.2 Introduction

Diet is arguably the most commonly used tool for weight loss or to improve health. The physiological consequences of diet manifest in its close association with disease. Four (diabetes, cardiovascular disease (CVD), cancer, and stroke) of the top 10 causes of death in the United States are associated with diet[146]. Obesity is a major contributor to the intersection between diet and disease due its effect on risk of developing type 2 diabetes, hypertension, cardiovascular disease, stroke, osteoarthritis, and certain cancers[2, 147, 148]. In the United States (U.S.) more than two-thirds of the population is classified as overweight or obese. Globally, overweight and obesity are now related to more deaths than underweight, except in parts of Asia and sub-Saharan Africa [2]. Accordingly, a recent meta-analysis indicated that approximately 40% of the

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U.S. general population is attempting to lose weight at any given time, and primarily through changes in diet [149].

Various dietary profiles around the globe are associated with effects on health. For example, American diet is used to describe the high fat and sucrose intake that are characteristic of the United States and, increasingly, of other developed countries. Consumption of an American diet is associated with increased risk of obesity and its co-morbid disorders in both humans and experimental models [37, 38]. Conversely, dietary patterns that are enriched in fiber, certain unsaturated fats, and plant-derived bioactive compounds are associated with beneficial effects on body weight and on risks of diet-related syndromes such as cardiovascular disease and diabetes. Diets characteristic of Japan and the Mediterranean region both contain relatively high intake of polyunsaturated fatty acids and fiber- and flavonoid-rich fruits and vegetables, with modest consumption of the sugar and saturated fat that are hallmarks of the American diet. Accordingly, both the Japanese and Mediterranean eating patterns are associated with reduced risk of obesity and metabolic syndrome. Rates of coronary heart disease (CHD), hypertension, and cancer in individuals following a Japanese diet have remained low compared to individuals following an American diet [47-49, 150, 151]. Likewise, consumption of a Mediterranean diet is associated with reduced risk of cardiovascular disease, type 2 diabetes and obesity compared to Western-style eating patterns [26, 152, 153]. In stark contrast to complex carbohydrate-rich diets, like Japanese and Mediterranean diet profiles, ketogenic or Atkins-type regimens, which consist primarily of fat and protein, are also widely popular for weight loss. An inherent assumption of diet regimens is that they alter cellular metabolism, particularly in

important metabolic tissues such as adipose and liver. However, despite the widely accepted relationship between diet and health, and the prevalent use of diets for weight loss, relatively little is known about the fundamental impact of diet on metabolism at the tissue level.

Mass spectrometry-based metabolomic platforms now enable global, discovery-based profiling of tissue and circulating metabolomes. Accordingly, metabolomics has emerged as a tool with which to understand the impact of diet on metabolism and to identify metabolites and pathways that are associated with disease [90, 110, 154]. Metabolomics studies on the effects of diets in humans and mice frequently use samples that can be obtained noninvasively (e.g., plasma, erythrocytes or urine). Less is known, however, about the impact of diet at the tissue level, and the extent to which tissues may respond to diet in a similar manner. The objective of this study was to use metabolomics to determine the effects of diet on metabolomes at the tissue level and to associate tissue metabolites with systems level phenotypes of energy utilization and body composition in mice. Four common dietary profiles (American, Japanese, Mediterranean and ketogenic) were chosen because of their relationships to various aspects of metabolic health in humans, and because they vary widely in composition. Mouse diets were formulated to represent both the macronutrient profiles and sources that are characteristic of each of these four eating patterns, while maintaining similar caloric values and sufficient amounts of micronutrients. The C57BL/6J strain was used because it has been extensively characterized as a model of diet-induced obesity. Both males and females were used to evaluate the relative effects of diet, sex, and sex-by-diet interaction on tissue metabolism. Untargeted metabolomics was used to

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comprehensively profile the metabolomes of liver, skeletal muscle, and adipose tissue because of their roles in energy balance [155, 156].

4.3 Materials and Methods

4.3.1 Animals and Diets

All husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Four-week old C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were allowed to acclimate for 14 days and consumed a standard mouse chow (PicoLab Mouse Diet 20, LabDiet, St. Louis, MO) during this period. At 42 days of age, five male and five female mice were assigned to each of five diets: American, Mediterranean, Japanese, ketogenic, or chow. Diets were designed in collaboration with Research Diets, Inc. (New Brunswick, NJ) to contain calories, macronutrient ratios, sources of ingredients, fiber content, and lipid profiles that are reflective of each diet pattern. (Table 4.1, macronutrient ratios, Appendix I and J, detailed formulations). Mice were maintained on a 12-hour light/dark cycle throughout the study. At age 18 weeks, after 12 weeks on the diets, mice were housed in Phenomaster metabolic chambers (TSE Systems, Inc.) for 48 hours for measurement of metabolic rate and activity. The chambers measured respiratory exchange rate (RER), volume of oxygen (VO_2), and heat output via heat dissipation, and activity level by laser detection. Activity levels measured voluntary movement of the mouse in the x and y plane. Mice were euthanized at seven months of age by CO_2 asphyxiation. Perigonadal adipose tissue

was dissected and weighed as a measure of adiposity. Samples of adipose tissue, the left lobe of the liver and the vastus medialis, vastus lateralis, and rectus femoris muscle were snap-frozen in liquid N_2 and stored at $-80^{\circ}C$ for metabolomics analysis.

Table 4.1: Dietary Composition

	Ketogenic D12052706	American D12052705	Mediterranean D12052702	Japanese D12052703	chow D12052701
Fat	84% Butter, Lard	35% Corn oil, butter, Olive oil	42.6% Olive oil	11% Soybean oil, Olive oil	18% Soybean oil
Carbohydrate	None	50% Corn starch, Wheat starch, Sucrose, potato starch	44.69% Wheat starch, Sucrose, Fructose	76% Rice starch, Sucrose	63% Corn starch
Protein	16% Casein	15% Soy, Beef, Egg white	12.71% Soy, Fish, Beef	13% Soy, Fish	19% Casein

4.3.2 Metabolite Extraction and Liquid Chromatography Mass Spectrometry

Frozen tissue samples were pulverized under liquid N. Approximately 25 mg of pulverized tissue were weighed and extracted sequentially in methanol and then in methanol:water (4:1), as previously described [157]. Supernatants were dried under nitrogen and reconstituted in 160 µL of sterile MilliQ water. Internal standard (60 µL of a ^{13}C-labeled *E.Coli* metabolite pool) was added to each sample.

4.3.3 Preparation of ^{13}C-labelled E. Coli metabolite pool

Cultures of *Escherichia coli* NCM3722 were grown on minimal media ^{13}C-glucose agar plates and passed five times. Single colonies were then transferred to minimal media ^{13}C-glucose liquid cultures (0.4% w/v made from 99% U-^{13}C-glucose, Cambridge

Isotope Laboratories). Liquid cultures were set back five times before samples were extracted and run on mass spectrometer to ensure complete ^{13}C-labeling of all metabolites.

E. Coli cells were extracted by vacuum filtering three 10 mL aliquots, per culture, for three different cultures (grown to ~0.4 OD_{600}) through Magna nylon membrane filters (0.45 micron, 47 mm filter, Maine Manufacturing, Sanford, ME). The filters were transferred face down into petri dishes containing 1.3 mL of extraction solvent (40:40:20 HPLC grade methanol, acetonitrile, water with 0.1M formic acid) chilled to -20 ˚C. The extraction was allowed to proceed for 15 min at -20 ˚C. The following extraction was carried out in a room maintained at 4 ˚C unless otherwise specified. The filters were rotated so that the cell side was on top and rinsed by pipetting the extraction solvent over the face of the filters. The extraction fluid was then transferred to 1.5 mL centrifuge tubes and an additional 300 µL of new extraction fluid was used to wash the filters again. The remaining extraction solvent was also transferred to the 1.5 mL centrifuge tube and centrifuged for 5 minutes (16.1 rcf). The resulting supernatant was transferred to new vials and the pelleted cell was resuspended in 50 µL of extraction solvent. The extraction was allowed to proceed for 15 min at -20 ˚C at which time the samples were centrifuged for 5 min (16.1 rcf). The supernatant was transferred to the vials and another 50 µL of extraction solvent was added to the pelleted cell repeating the previous extraction once more. Vials containing all of the collected supernatant were dried under a stream of N_2 until all the extraction solvent had been evaporated. Following the resuspension of extracted E. coli residue in 300 µL of sterile water, samples were physically averaged and 10 mL were directly spiked into unlabeled samples.

4.3.4 Metabolomics Data Processing

Raw files generated by Xcalibur were converted to mzML, an open-source format, using msconvert [139]. An open source data analyzer for metabolomics, MAVEN [140, 141] (Princeton University) was used to correct total ion chromatograms based on retention time automatically, for each sample. Known metabolites were manually chosen and peak abundance was integrated by mass (±5 ppm) and retention time. Unknown metabolites were chosen using an algorithm with the following settings: minimum peak width, 5; minimum signal/blank ratio, 3 or greater; minimum peak intensity, 10,000; and minimum peak/baseline, 3. Unknown peaks were filtered manually to remove those that did not meet the above criteria.

4.3.5 Statistical Analysis

All statistical analyses were performed in the language R (3.1.0 and 3.2.2) [158]. An ANOVA model was used to identify significant effects of diet, sex, and sex-by-diet interaction on physiological traits and metabolites. Tukey's Honest Significant Difference (HSD) was used for post-hoc testing. Significance for physiological traits was based on raw p-values ($p < 0.05$).

Metabolite peak area data files were read into R using the package XLConnect [159]. Metabolites that were missing 70% or more sample measurements were removed from analysis. Missing values in the remaining metabolites were imputed using k-nearest numbers from the function impute [160] (Additional File 1 and 2). Metabolites were matched to their corresponding ^{13}C-labeled internal standard, or to a ^{13}C-labeled standard of the same compound class. Class types were identified using the Human

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Metabolome Database [143-145]. Prior to statistical analyses, a linear model was created for each metabolite using the terms sex, diet, sex*diet, tissue weight and internal standard (eq. 4.1):

Equation 4.1

$$Metabolite = sex + diet + sex * diet + tissue\ weight + int\ ernal\ s\tan dard$$

Coefficients for the terms for tissue weight and internal standard were used to adjust metabolite abundance for technical variation using eq. 4.2:

Equation 4.2

$$Adjusted\ metabolite = metabolite + tissue\ weight\ coefficient(mean\ tissue\ weight - tissue\ weight) + int\ ernal\ s\tan dard\ coefficient(mean\ int\ ernal\ s\tan dard - int\ ernal\ s\tan dard)$$

Adjusted metabolites were Pareto scaled across all mice for each metabolite using the package MetabolAnalyze [161], normalized to the median across all metabolites for each mouse, and cube root transformed to create a normal distribution. Normalized metabolite values were analyzed for effects of sex, diet and sex*diet. Data were assessed for normality using Q-Q plots, residuals, and the Shapiro-Wilks test. False discovery rate for ANOVA and correlation analyses was set to 5% using the method of Benjamin-Hochberg [162]. Venn diagrams were created using the package VennDiagram [163].

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4.3.6 Correlation Analysis

Associations between physiological measurements and metabolite measurements across tissues were assessed using Pearson correlation from the package Hmisc [164] and the package R.Utils [165]. Correlation p-values were FDR-adjusted using the Benjamini-Hochberg procedure. Correlations were visualized using the Cytoscape app Metscape [166].

4.3.7 Functional Pathway Analysis

Overrepresentation pathway analysis was performed using Metaboanalyst [167]. KEGG IDs were input and compared against the mouse KEGG reference metabolome. Statistical significance of pathway overrepresentation was evaluated using Fisher's exact test. Pathway topology was performed using relative-between centrality.

4.4 Results

4.4.1 Physiological Measurements Reveal Differences Among Diet and Sex-by-diet

Body composition and indirect calorimetry were used to define system-level effects of diet and sex on metabolism. Body weight, adiposity, oxygen consumption (VO_2), respiratory exchange ratio (RER), and heat output were all significantly influenced by diet but in a sex-dependent manner ($p_{sex \times diet} < 0.05$; Table 4.2). In contrast, activity level was significantly affected by sex ($p = 0.036$) but not by diet or sex-by-diet interaction. Females consuming the Mediterranean diet were significantly

heavier than those fed the ketogenic or standard chow diets and numerically heavier

than all four diet groups (Fig. 4.1A). Ketogenic females also weighed significantly less

than those consuming the American diet (Fig. 4.1A). The heaviest males were those

eating the American diet, which weighed significantly more than males fed the standard

or Japanese diets (Fig. 4.1A). Evidence of interaction between sex and diet were clearly

visible in adipose tissue, particularly with regard to the ketogenic diet. This diet

produced the leanest females but the fattest males, based on relative weight of the

abdominal fat pad (Fig. 4.1B). Ketogenic diet females were significantly leaner than

Mediterranean females. The Japanese diet yielded the leanest males ($2.1 \pm 0.71\%$),

which had significantly less adipose tissue than those fed the Mediterranean ($4.96 \pm$

0.24%, p=0.015), ketogenic ($6.25 \pm 1.15\%$, p=0.006), and American ($4.29 \pm 0.94\%$,

p=0.002) diets. Oxygen consumption in both sexes was lowest in mice consuming the

Japanese diet, which is consistent with its lower fat content compared to other diets

(Fig. 4.1C). Differences in fat and carbohydrate content of the diets were reflected in

RER values, which were comparable between males and females. Mice of both sexes

consuming the ketogenic diet had the lowest RER values (0.73), and differed

significantly from all other diets (Fig. 4.1D). In males, the high carbohydrate content of

the Japanese diet was reflected in a significantly higher RER value than in all other

diets. Heat output (an indicator of energy expenditure), was higher in females than

males across diets. In both sexes, heat output was lowest in mice fed the Japanese diet

and highest in those on the ketogenic diet (Fig. 4.1E). Activity levels varied but were not

significantly affected by diet or sex-by-diet (Fig. 4.1F).

Table 4.2: P-values for Effects of Sex, Diet, and Sex-by-diet Interaction on Weight and Metabolism.

	Sex	Diet	Sex-by-diet
Weight	<0.001	<0.001	0.013
Adiposity	0.004	0.005	0.001
VO_2	0.002	<0.001	0.302
RER	0.222	<0.001	0.004
Heat Output	<0.001	<0.001	0.011
Activity	0.036	0.367	0.856

$p < 0.05$ is considered significant
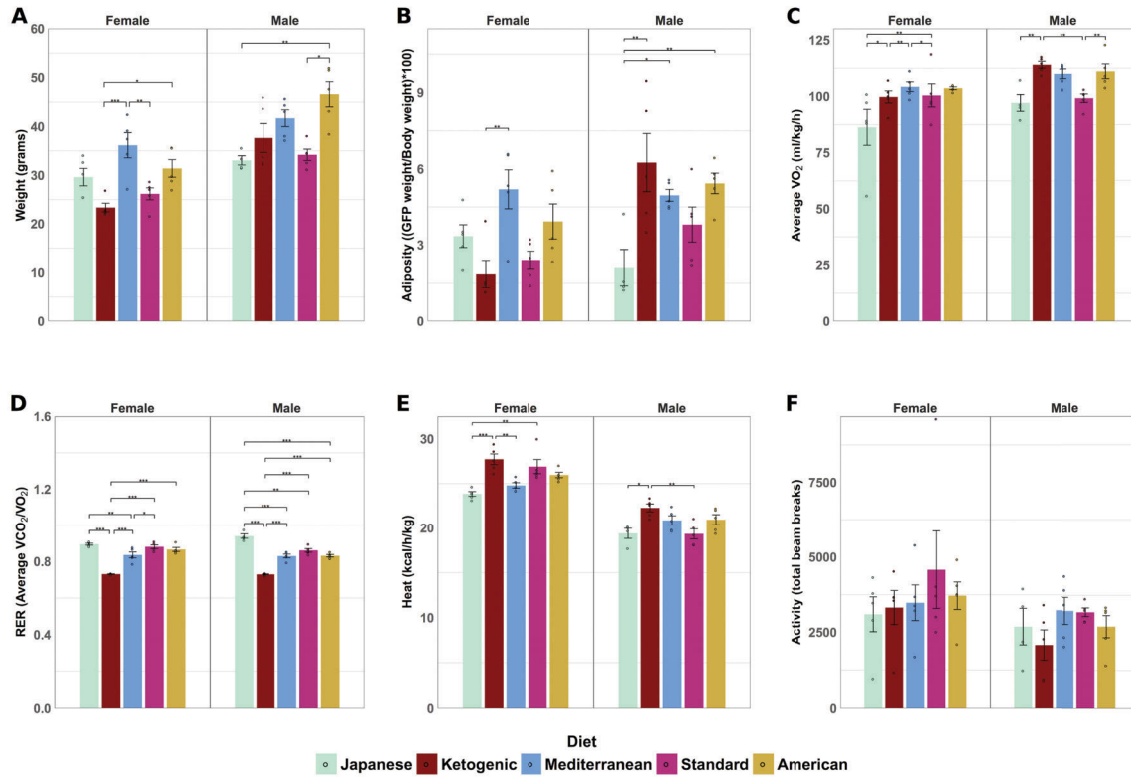
Figure 4.1: Effects of sex and diet on weight and metabolic phenotypes; N=5/ sex and diet group, avg. ± std. dev. Dots represent individual mice within sex-by-diet combination. Horizontal bars represent pairwise comparisons performed using Tukey's HSD post-hoc analysis; * p<0.05, ** p<0.01, and *** p<0.001 Body weight (A) and adiposity (B) were measured at 28 weeks of age, after 16 weeks on the experimental diets. Adiposity is expressed as the relative weight of the perigonadal adipose fat pads. Oxygen consumption (C), RER (D), heat output (E), and activity (F) were measured during a 48 hr. period when mice were housed in Phenomaster metabolic cages.

4.4.2 Significant Effects of Sex, Diet, and Sex-by-diet Interaction on Tissue Metabolomes

Liver, skeletal muscle, and adipose tissue are fundamental to systemic energy balance and play central roles in the body's response to diet. Global metabolomic profiling of these tissues was used to characterize the effects of sex and diet on tissue metabolism. A total of 184 known compounds, those for which m/z and retention time have been mapped to specific metabolites on our platform, were detected in one or more tissues. In addition, several thousand features corresponding to unknown metabolites (6,245, 4,909, and 4, 477) were detected in liver, skeletal muscle, and adipose tissue, respectively.

An ANOVA model was used to identify metabolites that were robustly affected by sex, diet, and sex-by-diet interaction (Appendix A, Additional File 3, Fig. 4.2). A total of 85 metabolites (46% of all known metabolites detected) were significantly affected (FDR < 0.05) by diet, sex, or diet interacting with sex in one or more tissues (Fig. 4.2A). Approximately one-third (28 of 85) of these metabolites were influenced by diet in a sex-dependent manner in at least one tissue. Some metabolites differed only by sex (23), with no influence of diet in any tissue. Likewise, 18 metabolites differed only between diets, with no impact of sex in any tissue. Of the 41 known metabolites that were significantly affected in one or more tissues by diet or sex-by-diet interaction, only two (proline and hydroxyproline) were altered in all three tissues (Appendix A).

The impact of both sex and diet on tissue metabolomes was most apparent in liver. Twenty-six metabolites responded to the variation in diets but in a manner that differed between males and females (Fig. 4.2B). An additional 30 metabolites were

affected by diet similarly in both sexes. Likewise, 31 metabolites differed between males

and females independently of diet effects. Nineteen metabolites were only affected by

sex and showed no response to diet, while 18 differed between diets but not between

males and females. In contrast to liver, the majority of metabolites in muscle and

adipose tissue did not show sex-dependent responses to diet (Figs. 4.2C, D). Only

three metabolites were significant for sex-by-diet interaction in adipose tissue (Fig.

4.2C), and none in muscle (Fig. 4.2D). Within adipose tissue the relative effect of sex

was greater than that of diet, with eight metabolites differing significantly between males

and females (Fig. 4.2C). Only three metabolites were affected by diet alone. Fourteen

metabolites showed effects of sex in muscle, and 11 of diet, with four influenced by both

factors independently (Fig. 4.2D). Unsupervised hierarchical clustering was performed

to visualize the relatedness of each of the 10-sex/diet groups, based on similarities

between hepatic metabolomes (Fig. 4.3). Males and females clustered separately,

indicating that the effect of diet did not override intrinsic metabolic differences between

males and females.
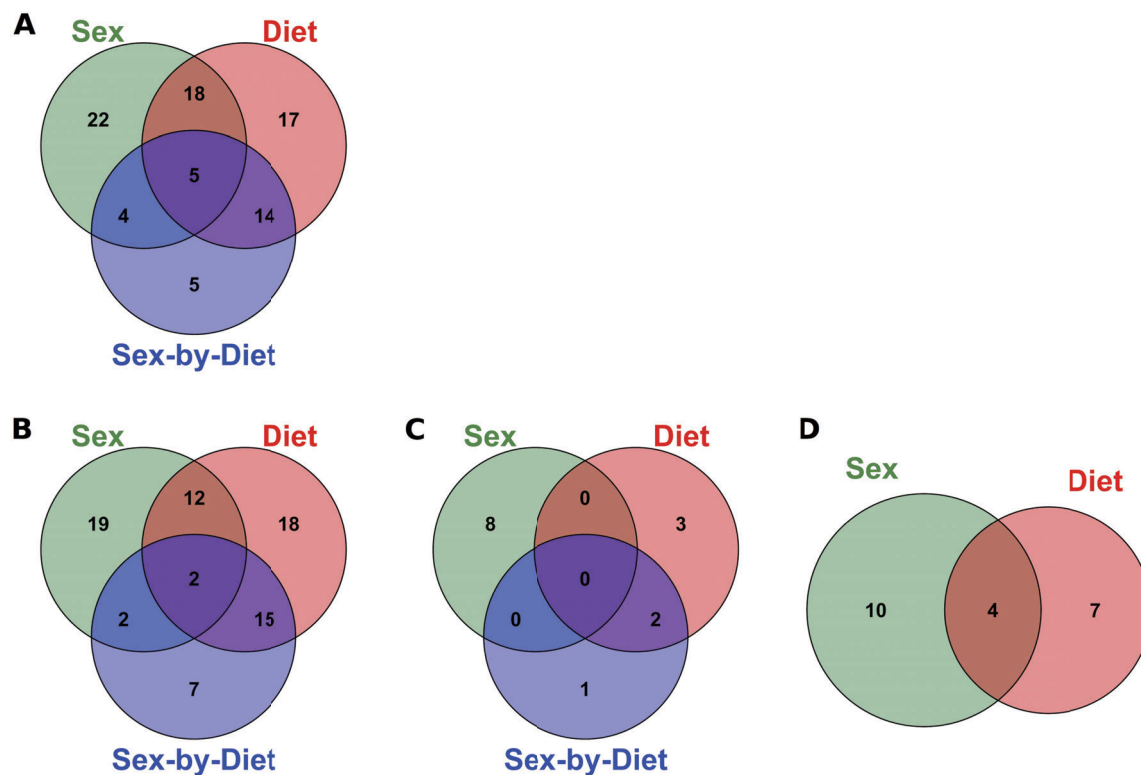
Figure 4.2: Shared and unique effects of sex, diet, and sex-by-diet interactions on tissue metabolomes. The sets of metabolites that differed significantly, based on ANOVA (FDR < 0.05) by sex, diet, or sex-by-diet interaction across tissues, (A), and in liver (B), adipose (C), and muscle (D) were visualized for shared and factor-specific effects using Venn diagrams. Only sex and diet are shown for muscle (D) because there were no metabolites significantly affected by the interaction term in the ANOVA model. Metabolites significantly affected by the interaction term were excluded from those significant for main effects of sex or diet.
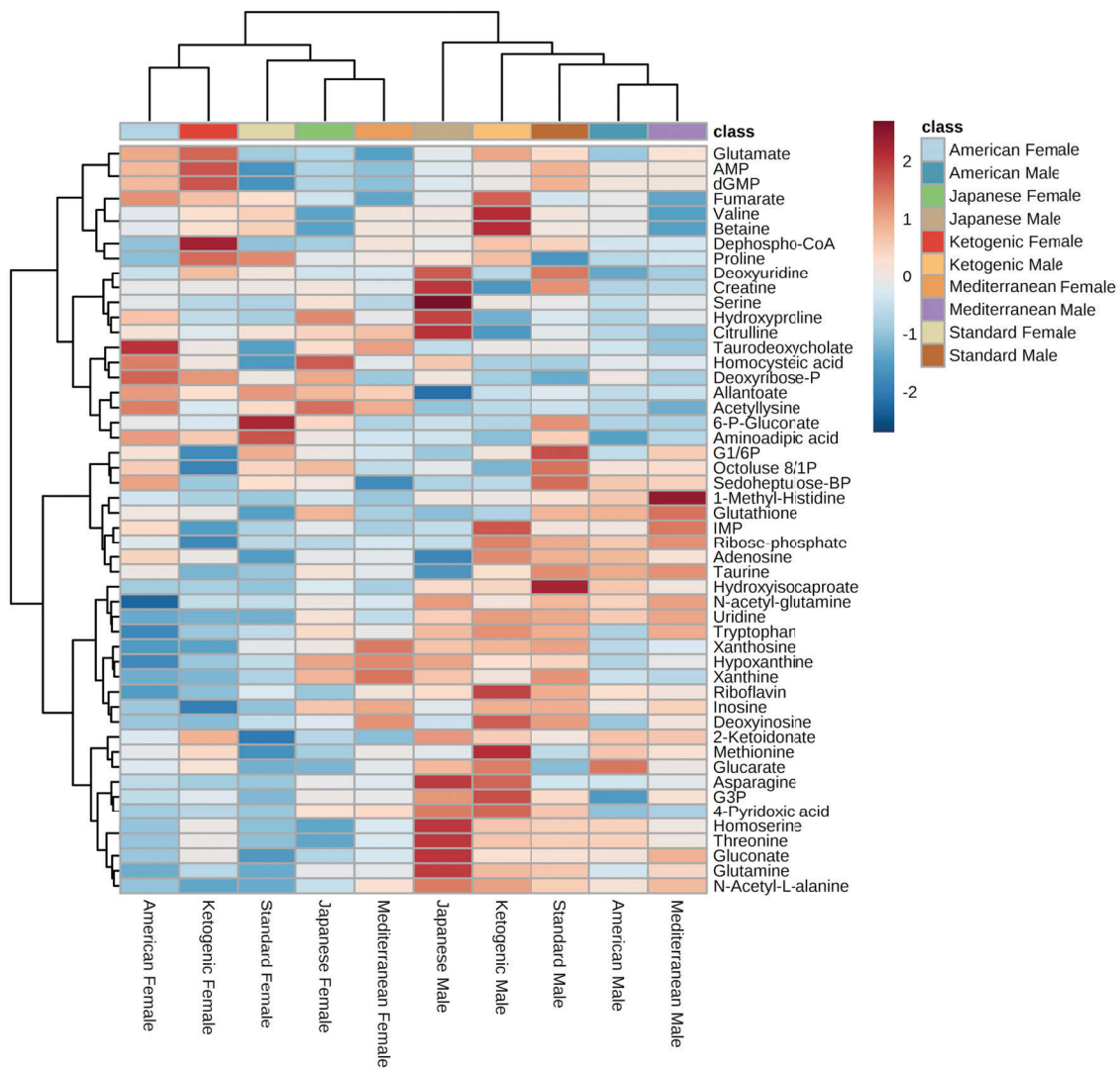
Figure 4.3: Hierarchical clustering of sex/diet groups based on metabolite abundance in liver.
Heatmaps were generated in Metaboanalyst (v3.0) using group averages of the 50 metabolites
that varied the most across sex and diet combinations. Samples were normalized to median
values and values were scaled using Pareto scaling. Z-score was used to determine scale.
Abbreviations: Deoxyribose-P: Deoxyribose-phosphate, 6-P-Gluconate: 6-phosphogluconate,
G1/6P: Glucose-1/6-phosphate, Sedoheptulose-BP: Sedoheptulose-bisphosphate, G3P: Glycerol-
3-phosphate

4.4.3 Functional Annotation of Diet and Sex Effects

The sets of metabolites affected by the interaction between diet and sex, and by sex and diet independently, were functionally annotated using KEGG pathways (Table 4.3, Fig. 4.4). Sex and diet interacted to significantly affect purine metabolism in liver ($p < 0.001$), with nine metabolites mapping onto this pathway (referred to as hits). Interactions between sex and diet also influenced amino acid metabolism. Six metabolites are included in the KEGG pathway aminoacyl-tRNA biosynthesis ($p = 0.001$), which represents incorporation of amino acids into protein. Additional pathways (pantothenate and CoA biosynthesis, arginine and proline metabolism, and valine, leucine, and isoleucine biosynthesis) contain metabolites that were affected by sex-by-diet interaction but were not significantly overrepresented after adjusting for FDR (> 0.05). Metabolites affected by diet, independently of sex, also represented components of amino acid metabolism, including an additional four metabolites in the aminoacyl-tRNA biosynthesis pathway. Other aspects of amino acid metabolism (e.g., arginine and proline metabolism) were represented by at least two metabolites. Only two metabolites affected by diet are components of the TCA cycle. Sex affected aspects of pyrimidine (hits=3), riboflavin (hits=2), and beta-alanine (hits=2) metabolism, as well as pantothenate and CoA biosynthesis (hits=2) but no pathways were significantly overrepresented (FDR > 0.05).

Diet significantly affected amino acid metabolism (aminoacyl-tRNA biosynthesis, $p = 0.002$) in muscle, but metabolites affected by sex did not map onto specific KEGG pathways (i.e., < 2 metabolites/pathway). In adipose tissue, the eight metabolites that differed between males and females included components of purine metabolism (hits=3)

Table 4.3: KEGG Pathway Enrichment of Metabolites Affected by Diet, Sex and Sex-by-diet Interaction.

Tissue	Factor	Pathway	Hits	P-value	(k/m)q
All Tissues	**Sex-by-diet**	Purine metabolism	**10**	**<0.001**	**0.002**
		Aminoacyl-tRNA biosynthesis	**7**	**<0.001**	**0.002**
	Diet	Arginine and proline metabolism	**5**	**<0.001**	**0.004**
	Sex	Purine metabolism	**6**	**0.002**	**0.002**
Liver	**Diet**	Arginine and proline metabolism	**4**	**0.001**	**0.003**
	Sex-by-diet	Purine metabolism	**9**	**<0.001**	**0.005**
		Aminoacyl-tRNA biosynthesis	**6**	**0.001**	**0.005**
Muscle	**Diet**	Aminoacyl-tRNA biosynthesis	**3**	**0.002**	**0.02**
Adipose	**Sex**	Purine metabolism	**3**	**0.003**	**0.013**

Figure 4.4: Sex-by-diet influenced purine metabolism. Purine metabolites affected by sex-by-diet were plotted. Horizontal bars represent significant pairwise comparisons performed using Tukey's HSD post-hoc analysis; * p<0.05, ** p<0.01, *** p<0.001.

AMP

IMP

Adenosine

Inosine

Hypoxanthine

Xanthine

Diet
Japanese Ketogenic Mediterranean Standard American
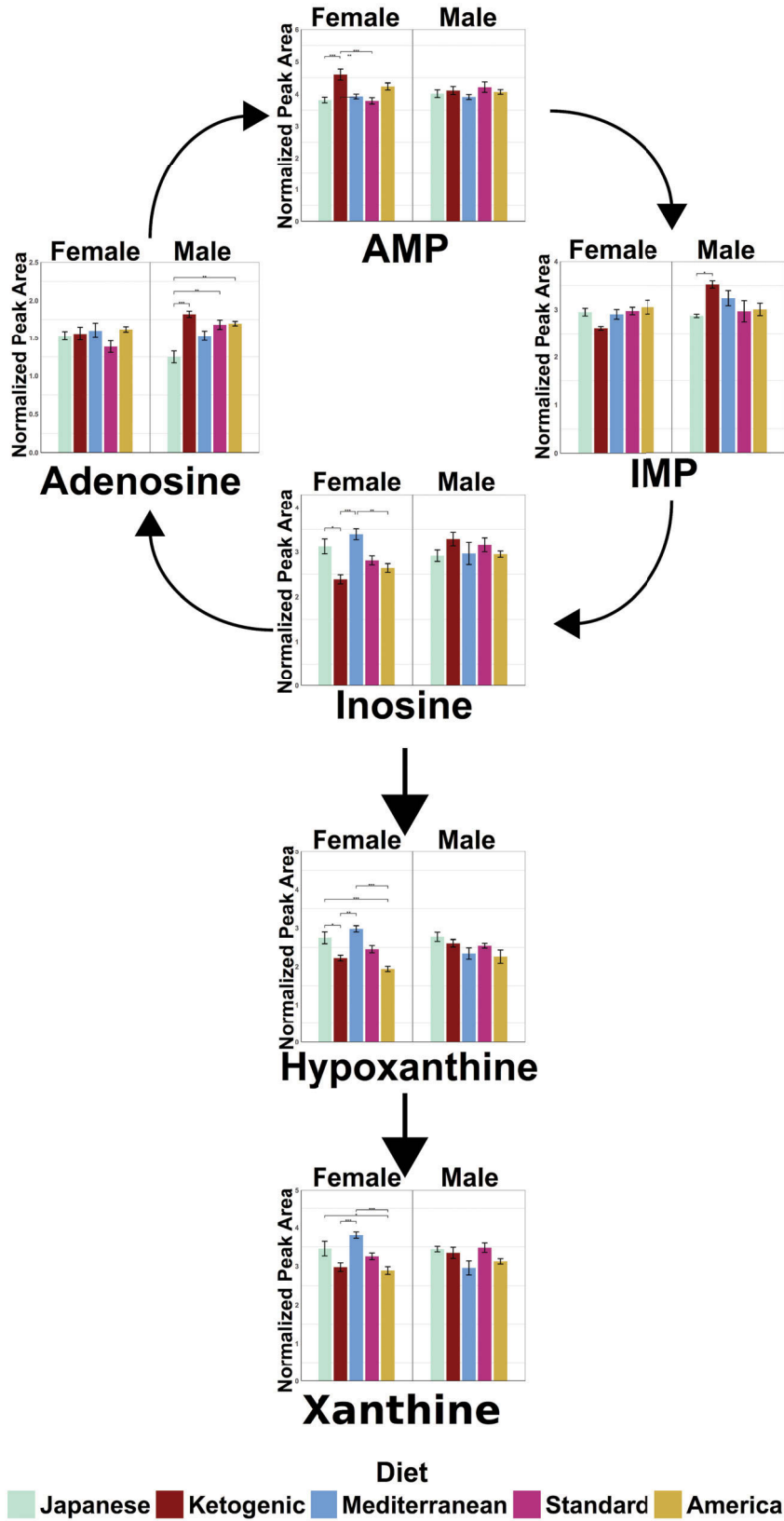
and arginine and proline metabolism (hits=2). The small numbers of metabolites affected by sex-by-diet interaction (hits=3) or diet alone (hits=2) in adipose tissue were not matched to pathways.

4.4.4 Relationships Between Known Metabolites and Phenotypes

The relationships between metabolite profiles in each tissue and systems level energy balance traits were identified using a correlation-based approach. Energy expenditure and adiposity were significantly correlated with 27 and 20 metabolites, respectively, across the three tissues, with the majority of associations due to hepatic (17 heat, 12 adiposity) and adipose (5 heat, 8 adiposity) metabolomes (Fig. 4.5). Partial correlation was used to adjust for spurious correlations to body weight, after which 15 (4 in adipose and 11 in liver) metabolites remained significantly correlated with adiposity and 19 (5 in adipose and 16 in liver) with heat output. In muscle, four metabolites (pantothenate, N-acetyl-glutamate, orotate, and kynurenine) were correlated with heat output and none with adiposity.

The relationships between tissue metabolites and systems level energy balance traits were assessed by constructing a correlation-based network. The network consists of metabolites (39, 8 and 5 from liver, adipose and muscle, respectively) that were significantly correlated with at least one energy balance trait and with one or more metabolites in any of the three tissues (Fig. 4.6, Appendix B). The majority of the metabolites that were correlated within and across all tissues were purine metabolites, amino acids, and energy molecules. Pantothenate, vitamin B5, is a metabolite that is necessary for coenzyme A synthesis. Pantothenate in skeletal muscle was positively

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correlated with pantothenate (r=0.68) in adipose tissue. Pantothenate in both tissues was positively correlated with aminoadipic acid (adipose, r=0.59; muscle, r=0.46), allantoate (r=0.58, 0.69), and butyryl-CoA (r=0.51, 0.47) and negatively correlated with n-acetyl-glutamine (r=-0.66, -0.73), n-acetyl-L-alanine (r=-0.57, -0.49), in liver. Pantothenate in both tissues was also positively correlated with s-adenosyl-L-homoCysteine (r=0.66, 0.51) in adipose tissue. Another B vitamin, riboflavin, in liver, was positively correlated with adiposity (r=0.50); however, it was not correlated with any metabolites in adipose or skeletal muscle. Methionine, in liver, was negatively correlated with arginine (r=-0.47), erythrose-4-phosphate (r=-0.48), and lysine (r=-0.48), in skeletal muscle. Riboflavin is necessary for the formation of FAD and FMN.

4.5 Discussion

Diet is a primary determinant of health, but the fundamental relationship between dietary intake and metabolism at the tissue level is poorly understood. We used an untargeted metabolomics approach to investigate the effects of diet and sex on tissue metabolomes, and to relate metabolites to body composition and energy expenditure. Experimental diets were designed to model eating patterns (American, Japanese, Mediterranean, and ketogenic) that are known to affect various aspects of metabolic health in humans. Diet composition varied widely in terms of the sources of ingredients, relative contribution of animal- and plant-based ingredients, and macronutrient and micronutrient composition. In combination with sex, these diets imposed a strong and diverse stimulus on tissue metabolism. This allowed us to query the extent to which tissue metabolomes were influenced by diet, sex, and their interactions, and to
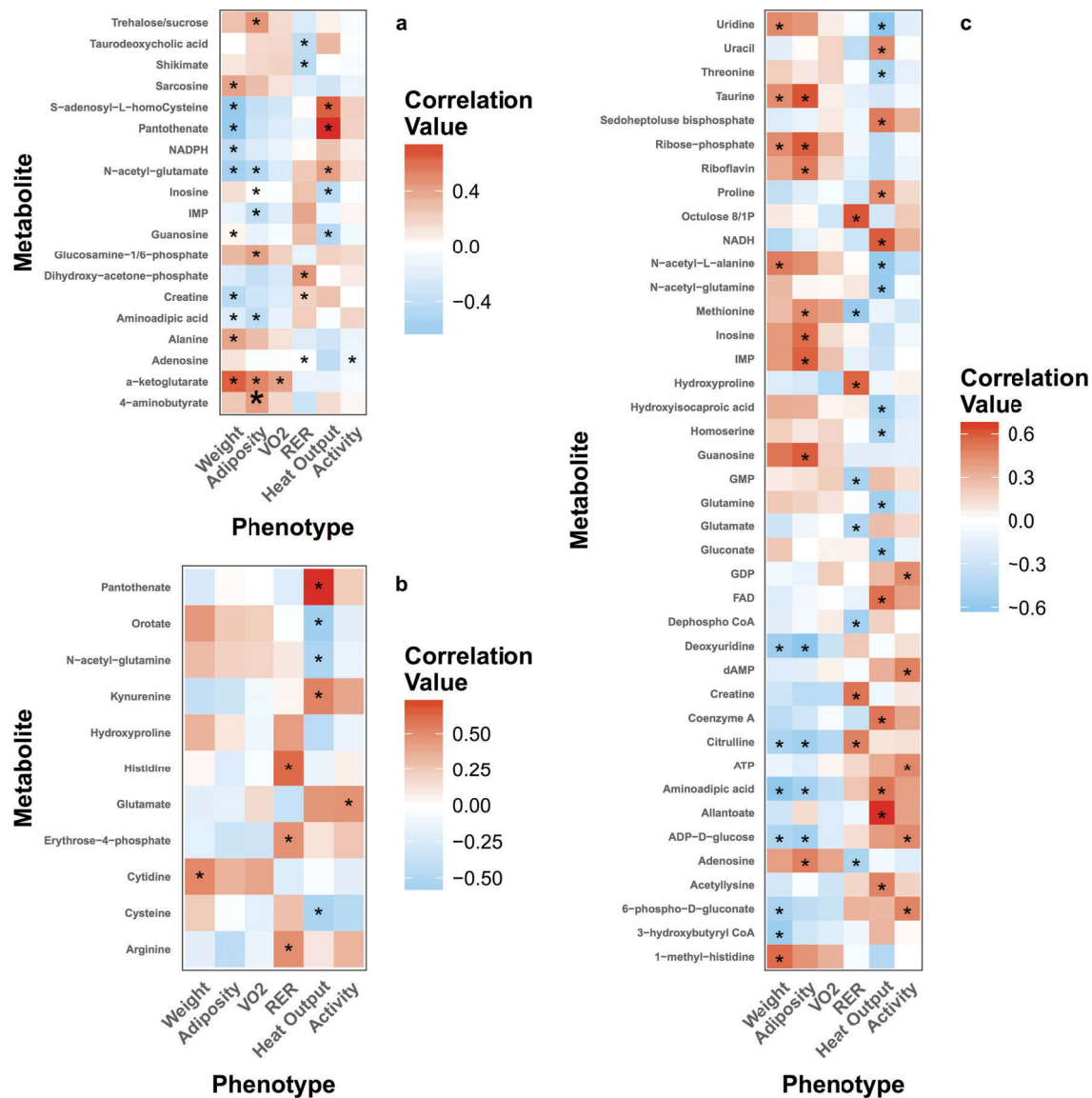
Figure 4.5: Correlations between metabolites and system phenotypes. Pearson correlation was used to associate metabolites in adipose (A), muscle (B), and liver (C) with weight, adiposity, and metabolic phenotypes. Significant correlations (FDR < 0.05) are represented by an asterisk (*). For metabolites that were significantly correlated for adiposity and/or heat output, partial correlation was used to determine if the relationship between metabolite and trait was due to spurious correlation with body weight. Correlations that remained significant are indicated by a bold asterisk (*).
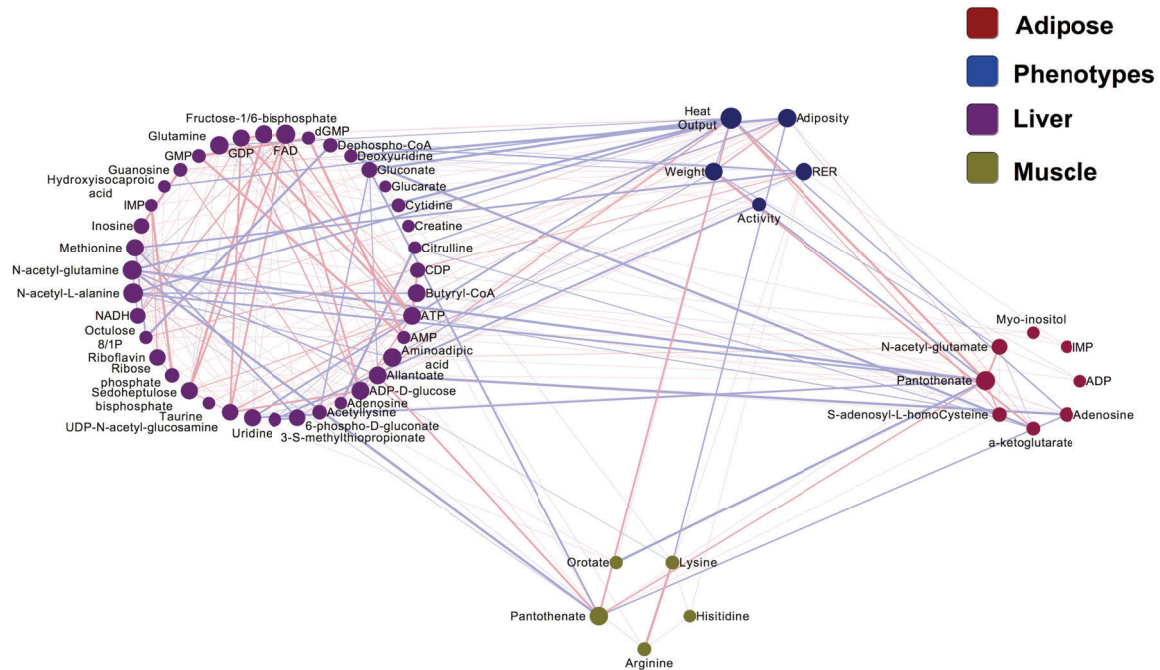
Figure 4.6: Cross-tissue metabolite and phenotype network. Pearson correlation was used to associate metabolites within and across each tissue with weight, adiposity, and metabolic phenotypes. Only significant correlations (FDR < 0.05) are shown. To assess relationships within and across tissues and phenotypes, metabolites are clustered by their respective tissue or phenotype. The size of each node represents the number of edges connected to the node. A red line represents positively correlated metabolites, while a blue line represents negatively correlated metabolites.

characterize their relative impact on liver, adipose tissue, and skeletal muscle. Our model, which included 10 unique combinations of diet and sex, also enabled us to use correlation to relate metabolites to body composition and energy expenditure.

Because of the sex-dependent effects of diet on weight and energy expenditure phenotypes, we expected that a considerable proportion of metabolites would be influenced by diet in a sex-dependent manner. This was true in liver, in which the number of metabolites affected by the interaction (n=26) was comparable to those responding to diet (n=30) and sex (n=31) independently. In contrast, the metabolomes of adipose tissue and muscle showed little evidence of sex-specific response to diet, which may be due to the fact that liver is one of the first tissue to receive and digest nutrients from the diet. Only three metabolites responded to diet in a sex-dependent way in adipose tissue, and none in muscle. Approximately one-third (9 of 27) of metabolites in liver that were influenced by sex-by-diet interactions are involved in purine metabolism. Purine metabolism includes the synthesis and breakdown of purine nucleotides, which are building blocks of nucleic acids and cofactors that are required for energy metabolism. While these functions are fundamentally important for all cells, recent metabolomic studies associate this pathway with adaptation to energetic challenges and to metabolic syndrome. Progressive exposure to cold rapidly increased purine metabolites in brown adipose tissue, in parallel with mitochondrial thermogenesis [168]. No effects of cold exposure were observed in white adipose from the same animals, suggesting a relationship to mitochondrial uncoupling and fatty acid oxidation. Metformin is a widely used anti-diabetic drug that improves glycemia and lipidemia through the energy-sensing kinase AMPK and other targets in liver. Metformin

administration to diet-induced obese increased purine metabolites in serum [169]. Circulating levels of purine metabolites have also been shown to discriminate between obese patients with and without metabolic syndrome [170]. How flux through the purine metabolism pathway may regulate cellular utilization is not yet clear. One potential mechanism is through effects on 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an intermediate in the de novo purine synthesis pathway [171]. AICAR interacts with the gamma subunit of AMPK, inducing a conformational change that enhances its phosphorylation and activation [172]. This AMP-mimicking action of AICAR has resulted in its widespread use as an AMP analog in experimental studies, although the physiological effects of endogenous AICAR in mammals are unknown [173]. We recently proposed that endogenous AICAR mediates anti-obesity actions of the polyherbal supplement Zyflamend, which both alters tissue purine metabolism and activates AMPK [174]. Whether AICAR levels were altered in the current study has not been determined because we do not consistently detect it with sufficient sensitivity for quantitation in tissues. Additional, more focused studies are necessary to determine the consequences of variation in purine metabolism and how this pathway might influence the response to diet.

Various aspects of amino acid metabolism were influenced by sex-by-diet interactions in liver, as well as by diet alone in muscle. Twelve of the 20 amino acids differed between diets in at least one tissue, along with numerous amino acid metabolites. The percentage of protein in each diet was sufficient for mice but varied across diets (from ~ 13 – 19%). In addition, different types of protein were used, from both plant and animal sources. Some of the effects of diet may therefore be attributed

to variation in amino acid composition of the diets. Other changes in amino acids and their metabolites likely resulted from metabolic adaptations to varying levels of carbohydrates and fat. Metabolomic studies have been valuable in identifying relationships between amino acid metabolism and various aspects of metabolic health that had previously gone undetected [175-177]. In particular, several analyses of the serum metabolome in humans have revealed a link between branched chain amino acid metabolism and cardiometabolic health [117, 178]. A recent, large scale study of individuals in the Framingham Offspring cohort determined that a broad collection of serum amino acids, and not just branched chain species, are associated with abdominal obesity, BMI, insulin resistance, lipidemia and other metabolic traits [119]. Our study design was not of sufficient scope to link specific tissue amino acid levels to the metabolic traits that we measured. We also did not profile the serum metabolome and thus cannot determine how tissue amino acid changes are reflected in those in circulation. Nonetheless, assuming that these tissues contribute to serum amino acid profiles, our results suggest that diet may influence the risk of metabolic disease through effects on tissue amino acid metabolism.

The Framingham Offspring cohort study described above also identified aminoadipic acid, which we found to be significantly influenced by diet in liver and muscle, as a novel metabolite that predicts future development of type 2 diabetes in normoglycemic individuals [119]. Other metabolomic studies have also associated this metabolite, which is a poorly understood intermediate in lysine catabolism, with metabolic health in humans [170] and in Zucker rats. Levels of aminoadipic acid in serum are elevated years prior to the development of insulin resistance in humans

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[122]. Experimental studies show that it augments the hypoglycemic actions of insulin,

suggesting an adaptive role in maintaining glucose homeostasis. Despite its emerging

role in metabolic disease, little is known about dietary regulation of aminoadipic acid.

Consumption of beef was shown to acutely increase plasma levels of aminoadipic acid,

compared to a meal containing fish [179]. However in our study, tissue levels were

lowest in the Japanese and Mediterranean diets, one of which (Japanese) is devoid of

bovine protein while the other (Mediterranean) derived almost 70% of its protein from

bovine sources (beef and casein). Therefore although we cannot identify a specific

dietary component that regulates aminoadipic acid levels, the significant impact of diet

in both muscle and liver in our results warrant future investigation into this relationship.

Given that the experimental diets used in this study are associated with pro- or

anti-obesogenic effects, the relatively modest impact of diet on the adipose metabolome

is somewhat surprising. Only five metabolites were significantly affected by diet, either

alone (n=3) or through interaction with sex (n=2). One of these metabolites, 3-

methylphenylacetic acid, is a metabolite of common herbicides [143-145]. Interestingly,

this metabolite was significantly more abundant in adipose tissue from mice fed the

Japanese diet, which contains the highest proportion of plant-derived ingredients (e.g.,

rice starch, soy protein). Many organic pollutants have been shown to accumulate in

the lipid-rich matrix of adipose tissue and to increase risk of obesity and metabolic

disease. Although we are not aware of such associations for 3-methylphenylacete, its

significant enrichment emphasizes the potential for unintended consequences of

experimental diets on tissue metabolomes.

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Sex influenced the adipose metabolome more so than diet, based on both the ANOVA results and unsupervised clustering. Notably, pantothenate levels differed significantly by sex, with increased levels in adipose tissue from females. Comparable differences were seen in skeletal muscle, and levels in both tissues correlated significantly with energy expenditure. Pantothenate plays a pivotal role in energy metabolism because it is the substrate from which Coenzyme A (CoA) is synthesized. This essential nutrient is provided in the diet and actively transported into tissues. Pantothenate kinase is the rate-limiting enzyme for synthesis of CoA from pantothenate, and is under nutritional, hormonal, and allosteric control [180-182]. Differences in tissue pantothenate content between males and females may therefore be due to sex effects on pantothenate uptake or on regulation of its conversion to CoA. Either way, the relationship between energy expenditure and pantothenate in both adipose tissue and muscle suggests that CoA levels contribute to metabolic differences between males and females. Although pantothenate levels were not affected by diets included in this study, according to the network analysis they were correlated with seven metabolites across tissues, including aminoadipic acid suggesting a potential relationship between pantothenate and potential predictors of disease. Collectively, these relationships suggest that pantothenate availability or uptake may be an important variable in metabolic disease. Interestingly, pantothenate supplementation was recently shown to attenuate the consequences of a high sucrose diet on lipid metabolism in Drosophila [183].

While our focus was on tissue metabolomes, effects of the ketogenic diet on adiposity were notable. Ketogenic diets are popular for weight loss, although clinical

studies do not consistently support their efficacy. Studies in rodents have shown that a ketogenic diet paradoxically increases the percentage of body fat, often at the expense of lean mass and despite increased energy expenditure [184, 185]. Our data from males are consistent with these studies, all of which also used only male mice. In contrast, ketogenic diet females in our study had the lowest adiposity of all diets, although some pairwise comparisons to other diets were not significantly different. Both males and females had the lowest RER values, suggesting that fat utilization was comparably affected in both sexes. To our knowledge, ours is the first study to demonstrate sex-specific effects of a ketogenic diet on body composition.

Several limitations of our study should be emphasized. In particular, the diets were designed to reflect common patterns of global consumption in humans. Accordingly, we cannot the range of variation in diet composition prevents us from associating specific dietary factors with metabolite patterns. We have also measured metabolites at a single time point. Effects of diet or sex on metabolite turnover therefore go undetected in our study. Furthermore, the extraction method we used targets polar metabolites at the expense of lipids, which are also likely subject to extensive sex and diet regulation. Finally, due to the specificity of the extraction method and the limited number of metabolites that can be identified using our in-house database, it is difficult to ascertain whether pathways significantly altered are affected by sex, diet or sex-by-diet interaction or because a metabolite is more easily identified to due inherent biases in the experimental procedure.

4.6 Conclusion

We profiled three tissues (adipose, muscle, and liver), which play a major role in energy metabolism to determine the effects of diet and sex on the tissue metabolome. The stratified results showed tissue-specific effects of sex and diet on tissue metabolites. This study, however, was performed in the context of a single genetic background. Including other genetic backgrounds may reveal a more complex relationship between the tissue metabolome and diet. Nonetheless, this study has shown several metabolites that could be investigated to determine their role in metabolism and disease.

CHAPTER FIVE

TISSUE LEVEL STRAIN AND SEX-BY-STRAIN INTERACTIONS REVEAL UNIQUE METABOLITE AND CLUSTERING PROFILES USING UNTARGETED LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ON ADIPOSE, SKELETAL MUSCLE, AND LIVER TISSUE IN MICE FED A STANDARD CHOW DIET

Ann Wells[1], William T. Barrington[2], Stephen Dearth[3], David W. Threadgill[2], Shawn R. Campagna[1, 3], Brynn H. Voy[1, 4]

[1]University of Tennessee-Knoxville, UT-ORNL Graduate School of Genome Science and Technology, [2] Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, [3]University of Tennessee-Knoxville, Department of Chemistry, [4]University of Tennessee-Knoxville, Department of Animal Science

Ann Wells' contribution included: tissue extractions, data analysis, and primary writing of the manuscript.

5.1 Abstract

Genetics have been shown to play an important role in the development of metabolic diseases. Despite the known role of genetics in metabolic diseases it is unknown how genetic background alters metabolism at the tissue level, particularly when fed the same diet. Using untargeted metabolomics, this study aimed to identify changes in water-soluble metabolites in A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ males and females, fed a standard mouse chow diet for seven months. Metabolite abundance was examined in liver, skeletal muscle, and adipose tissue for sex, strain, and sex-by-strain interaction. Females were lighter than males, independent of strain. A/J females had more adiposity than all other sex-by-strain combinations. ANOVA suggests that liver is most metabolically active across different genetic backgrounds, although adipose and muscle tissue were sensitive to genetic differences as well. Purine

metabolism and pathways that played a role in amino acid metabolism were significantly altered (FDR < 0.05) by strain in adipose and liver. All three tissues were largely unaffected by sex or sex-by-strain interaction relative to strain, suggesting that the tissue metabolome remains largely stable across genders consuming the same diet. This study revealed that no one strain had the predominant affect on physiology. Metabolomics revealed that strain affected the metabolome in a tissue-specific manner; however, liver was most sensitive to genetic differences.

5.2 Introduction

Genetics have been shown to play an important role in the development of metabolic diseases, such as type 2 diabetes [186, 187], cardiovascular disease [188-190], and obesity [191, 192]. Furthermore, markers for fasting glucose and insulin, body mass index, and triglycerides have been linked to genetic loci, independent of the disease state [191, 193, 194]. Despite the known role of genetics in metabolic diseases it is unknown how genetic background alters metabolism at the tissue level, particularly when fed the same diet.

Due to the inherent genetic differences in humans it can be difficult to ascertain the role of genes associated with metabolic states. Moreover, investigating the role of metabolism at the tissue level is difficult at best in humans. Mice are an ideal alternative due their genetic similarity, inherent compliance, and replicative ability. Accordingly, the severity of disease due to genetic background has been shown through phenotypic markers in mice [82, 91]. Four strains of mice (A/J, C57BL/6J, FVB/NJ, and

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NOD/ShiLtJ) were chosen to represent different metabolic states, both diseased and non-diseased. A/J mice are susceptible to cancer; however, they are resistant to diabetes, obesity, atherosclerosis, insulin resistance, and glucose intolerance [78, 84, 87]. In contrast, C57BL/6J mice are susceptible to diet-induced obesity, type 2 diabetes, and atherosclerosis [78, 82, 89-91]. FVB/NJ mice are susceptible to certain cancers, have higher basal body temperature, activity, and anxiety levels [93, 94]. NOD/ShiLtJ mice are a model for type 1 diabetes [96]. In addition NOD/ShiLtJ females are the first to show decreased in pancreatic insulin, while males express this phenotype weeks later [81].

Mass spectrometry-based metabolomic platforms now enable global, discovery-based profiling of tissue and circulating metabolomes. Metabolomics has emerged as a tool to understand the impact of genetics on metabolism and identify metabolites and pathways that are associated with disease [96, 195]. Furthermore, metabolomics studies are typically limited to samples that can be obtained noninvasively (e.g., plasma, erythrocytes or urine). Less is known, however, about the impact of genetics at the tissue level, and the extent to which tissues respond to genetics in a similar manner when fed the same diet. The objective of this study was to use metabolomics to address the fundamental question of how genetics, which can vary widely, affect tissue metabolomes and to associate tissue metabolites with systems level phenotypes of energy utilization and body composition in mice. This was accomplished by comparing the effects of genetic differences, using A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ males and females fed a standard chow diet, on metabolism in liver, skeletal muscle, and adipose tissues, in the context of a single diet. Males and females were used to

evaluate the relative effects of strain, sex, and sex-by-strain interaction on tissue metabolism. Untargeted metabolomics was used to comprehensively profile the metabolomes of liver, skeletal muscle, and adipose tissue because of their roles in energy balance [155, 156].

5.3 Materials and Methods

5.3.1 Animals and Diets

All husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Four-week old A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were allowed to acclimate for 14 days and consumed a standard mouse chow (PicoLab Mouse Diet 20, LabDiet, St. Louis, MO) with 23.2% of calories provided by protein. At 42 days of age, five male and five female mice were switched to a marginally lower protein standard chow diet (19% protein). Mice were maintained on a 12-hour light/dark cycle throughout the study. At age 18 weeks, after 12 weeks on the diets, mice were housed in Phenomaster metabolic chambers (TSE Systems, Inc.) for 48 hours for measurement of metabolic rate and activity. The chambers measured respiratory exchange rate (RER), volume of oxygen (VO_2), and heat output via heat dissipation, and activity level by laser detection. Activity levels measured voluntary movement of the mouse in the x and y plane. Mice were euthanized at seven months of age by CO_2 asphyxiation. Perigonadal adipose tissue was dissected and weighed as a measure of adiposity. Samples of adipose tissue, the

left lobe of the liver and the vastus medialis, vastus lateralis, and rectus femoris muscle were snap-frozen in liquid N and stored at -80°C for metabolomics analysis.

5.3.2 Metabolite Extraction and Liquid Chromatography Mass Spectrometry

Frozen tissue samples were pulverized under liquid N. Approximately 25 mg of pulverized tissue was extracted sequentially in methanol and then in methanol:water (4:1), as previously described [157]. Supernatants were dried under nitrogen and reconstituted in 160 µl of sterile MilliQ water. Internal standard (60 µL of a ^{13}C-labeled *E.Coli* metabolite pool) was added to each sample.

5.3.3 Preparation of ^{13}C-labelled E. Coli metabolite pool

Cultures of *Escherichia coli* NCM3722 were grown on minimal media ^{13}C-glucose agar plates and passed 5 times. Single colonies were then transferred to minimal media ^{13}C-glucose liquid cultures (0.4% w/v made from 99% U-^{13}C-glucose, Cambridge Isotope Laboratories). Liquid cultures were set back 5 times before samples were extracted and run on mass spectrometer to ensure complete ^{13}C-labeling of all metabolites.

E. coli cells were extracted by vacuum filtering three 10 mL aliquots, per culture, for three different cultures (grown to ~0.4 OD_{600}) through Magna nylon membrane filters (0.45 micron, 47 mm filter, Maine Manufacturing, Sanford, ME). The filters were transferred face down into petri dishes containing 1.3 mL of extraction solvent (40:40:20 HPLC grade methanol, acetonitrile, water with 0.1M formic acid) chilled to $-20~^{\circ}$C. The extraction was allowed to proceed for 15 min at $-20~^{\circ}$C. The following extraction was carried out in a room maintained at 4 $^{\circ}$C unless otherwise specified. The filters were

rotated so that the cell side was on top and rinsed by pipetting the extraction solvent over the face of the filters. The extraction fluid was then transferred to 1.5 mL centrifuge tubes and an additional 300 μL of new extraction fluid was used to wash the filters again. The remaining extraction solvent was also transferred to the 1.5 mL centrifuge tube and centrifuged for 5 minutes (16.1 rcf). The resulting supernatant was transferred to new vials and the pelleted cell was resuspended in 50 μL of extraction solvent. The extraction was allowed to proceed for 15 min at -20 °C at which time the samples were centrifuged for 5 min (16.1 rcf). The supernatant was transferred to the vials and another 50 μL of extraction solvent was added to the pelleted cell repeating the previous extraction once more. Vials containing all of the collected supernatant were dried under a stream of N_2 until all the extraction solvent had been evaporated. Following the resuspension of extracted *E. coli* residue in 300 μL of sterile water, samples were physically averaged and 10 mL were directly spiked into unlabeled samples.

5.3.4 Metabolomics data Processing

Raw files generated by Xcalibur were converted to mzML, an open-source format, using msconvert[139]. An open source data analyzer for metabolomics, MAVEN [140, 141] (Princeton University) was used to correct total ion chromatograms based on retention time automatically, for each sample. Known metabolites were manually chosen and peak abundance was integrated by mass (±5 ppm) and retention time. Unknown metabolites were chosen using an algorithm with the following settings: minimum peak width, 5; minimum signal/blank ratio, 3 or greater; minimum peak

intensity, 10,000; and minimum peak/baseline, 3. Unknown peaks were filtered manually to remove those that did not meet the above criteria.

5.3.5 Statistical Analysis

All statistical analyses were performed in the language R (3.1.0 and 3.2.2) [158]. An ANOVA model was used to identify significant effects of sex, strain, and sex-by-strain interaction on physiological traits and metabolites. Tukey's Honest Significant Difference (HSD) was used for post-hoc testing. Significance for physiological traits was based on raw p-values (p < 0.05).

Metabolite peak area data files were read into R using the package XLConnect [159]. Metabolites that were missing 70% or more sample measurements were removed from analysis. Missing values in the remaining metabolites were imputed using k-nearest numbers from the function impute [160] (Additional File 1 and 2). Metabolites were matched to their corresponding ^{13}C-labeled internal standard, or to a ^{13}C-labeled standard of the same compound class. Class types were identified using the Human Metabolome Database [143-145]. The actual weight of tissue that was extracted and the peak intensity of the corresponding internal standard were used as covariates in statistical analyses.

Prior to statistical analyses, a linear model was created for each metabolite using the terms sex, strain, sex* strain, tissue weight and internal standard (eq. 5.1):

Equation 5.1

$$Metabolite = sex + strain + sex * strain + tissue\ weight + \mathrm{int}\,ernal\ s\tan dard$$

Coefficients for the terms for tissue weight and internal standard were used to

adjust metabolite abundance for technical variation using eq. 5.2:

Equation 5.2

$Adjusted\ metabolite = metabolite + tissue\ weight\ coefficient(mean\ tissue\ weight -$
$tissue\ weight) + internal\ standard\ coefficient(mean\ internal\ standard -$
$internal\ standard)$

Adjusted metabolites were Pareto scaled across all mice for each metabolite

using the package MetabolAnalyze [161], normalized to the median across all

metabolites for each mouse, and cube root transformed to create a normal distribution.

Normalized metabolite values were analyzed for effects of sex, diet and sex*diet. Data

were assessed for normality using Q-Q plots, residuals, and the Shapiro-Wilks test.

False discovery rate for ANOVA and correlation analyses was set to 5% using the

method of Benjamin-Hochberg [162]. Venn diagrams were created using the package

VennDiagram [163].

5.3.6 Correlation Analysis

Associations between physiological measurements and metabolite

measurements across tissues were assessed using Pearson correlation from the

package Hmisc [164] and the package R.Utils [165]. Correlation p-values were FDR-

adjusted using the Benjamini-Hochberg procedure. Correlations were visualized using

the Cytoscape app Metscape [166].

5.3.7 Functional Pathway Analysis

Overrepresentation pathway analysis was performed using Metaboanalyst [167]. KEGG IDs were input and compared against the mouse KEGG reference metabolome. Overrepresentation was performed using the algorithm Fisher's exact test. Pathway topology was performed using relative-between centrality.

5.4 Results

5.4.1 Physiological Measurements Reveal Differences Among Strain and Sex-by-strain

Body composition and indirect calorimetry were used to define system-level effects of strain and sex on metabolism. Adiposity, oxygen consumption (VO_2), and heat output were all significantly influenced by strain, but in a sex-dependent manner ($p_{sexXstrain}<0.05$; Table 5.1). In contrast, body weight was significantly affected by sex ($p<0.001$) but not by strain or sex-by-strain interaction. Activity levels were significantly affected by sex ($p=0.009$) and strain ($p=0.020$) but not sex-by-strain interaction. Tukey's post-hoc comparisons were assessed to determine significant pairwise comparisons. Evidence of sex-by-strain interaction were clearly visible in adipose tissue. Adiposity levels in A/J females ($4.01\pm0.3\%$) were significantly higher than female FVB/NJ ($1.14\pm0.21\%$, $p<0.001$) and NOD/ShiLtJ mice ($1.71\pm0.26\%$, $p=0.004$) and numerically higher than all other female mice regardless of strain (Fig. 5.1B). Conversely, adiposity levels in C57BL/6J males ($3.8\pm0.69\%$) were significantly higher compared to male FVB/NJ mice ($1.64\pm0.7\%$, $p=0.01$) and numerically higher than the other three mice

strains. Despite differences in adiposity across sex and strain, weight was only

significant for sex. Males were heaviest compared to females, regardless of strain (Fig.

5.1A). Oxygen consumption, an indirect measurement of energy expenditure, in both

sexes was lowest in A/J mice (Fig. 5.1C). Male FVB/NJ mice had the highest VO_2 levels

compared to the other three strains, however; only A/J (p=0.003) and C57BL/6J

(p=0.020) male mice were significantly different. Female A/J mice had significantly lower

VO_2 levels than C57BL/6J (p=0.020) and NOD/ShiLtJ (p=0.026) females. Female

C57BL/6J mice produced significantly more heat than A/J (p=0.002) and NOD/ShiLtJ

(p=0.010) females (Fig. 5.1E). FVB/NJ males produced significantly more heat than

C57BL/6J (p=0.033) and NOD/ShiLtJ (p=0.037) males. Activity levels varied between

sexes and strains but in an independent manner. Females had significantly higher

levels of activity while C57BL/6J mice had the highest levels of activity relative to all

other strains. RER had no significant difference for sex, strain, or sex-by-strain, which

was expected due to all strains being fed a single diet (Fig. 5.1D).

Table 5.1: P-values for Effects of Sex, Strain, and Sex-by-strain Interaction on Weight and Metabolism.

	Sex	Strain	Sex-by-Strain
Weight	**<0.001**	0.284	0.052
Adiposity	0.338	**<0.001**	**0.015**
VO_2	**0.001**	**<0.001**	**0.023**
RER	0.148	0.594	0.100
Heat Output	**<0.001**	**0.001**	**<0.001**
Activity	**0.009**	**0.020**	0.124

P<0.05 is considered significant.

5.4.2 Significant Effects of Strain, Sex and their Interactions on Tissue Metabolomes

Liver, skeletal muscle, and adipose tissue are fundamental to systemic energy balance and play central roles in the body's response to diet, but it is unclear how genetics influences these tissues. Global metabolomic profiling of these tissues was used to characterize the effects of sex and strain on tissue metabolism. A total of 191 known compounds, those for which m/z and retention time have been mapped to specific metabolites on our platform, were detected in one or more tissues. In addition, several thousand features corresponding to unidentified metabolites (6,292, 4,877, and 4,864) were detected in liver, skeletal muscle, and adipose, respectively.

An ANOVA model was used to identify metabolites that were affected by sex, strain, and sex-by-strain interaction (Appendix C, Additional File 4, Fig. 5.2). A total of 146 metabolites (76% of all known metabolites) were significantly affected by sex, strain, or sex-by-strain interaction, in one or more tissues. Approximately ninety percent (130 of 146) of these metabolites were influenced by strain in at least one tissue. To determine which tissue was most affected by strain we compared all significant metabolites across all tissues (Fig. 5.2A). Strain affected 105 metabolites in liver with seventy-two metabolites specific to liver. Adipose and skeletal muscle were less sensitive to strain with 38 and 30 metabolites. Thirteen and eight of the total metabolites were specific to adipose and skeletal muscle. Only six metabolites were significant for strain in all three tissues.
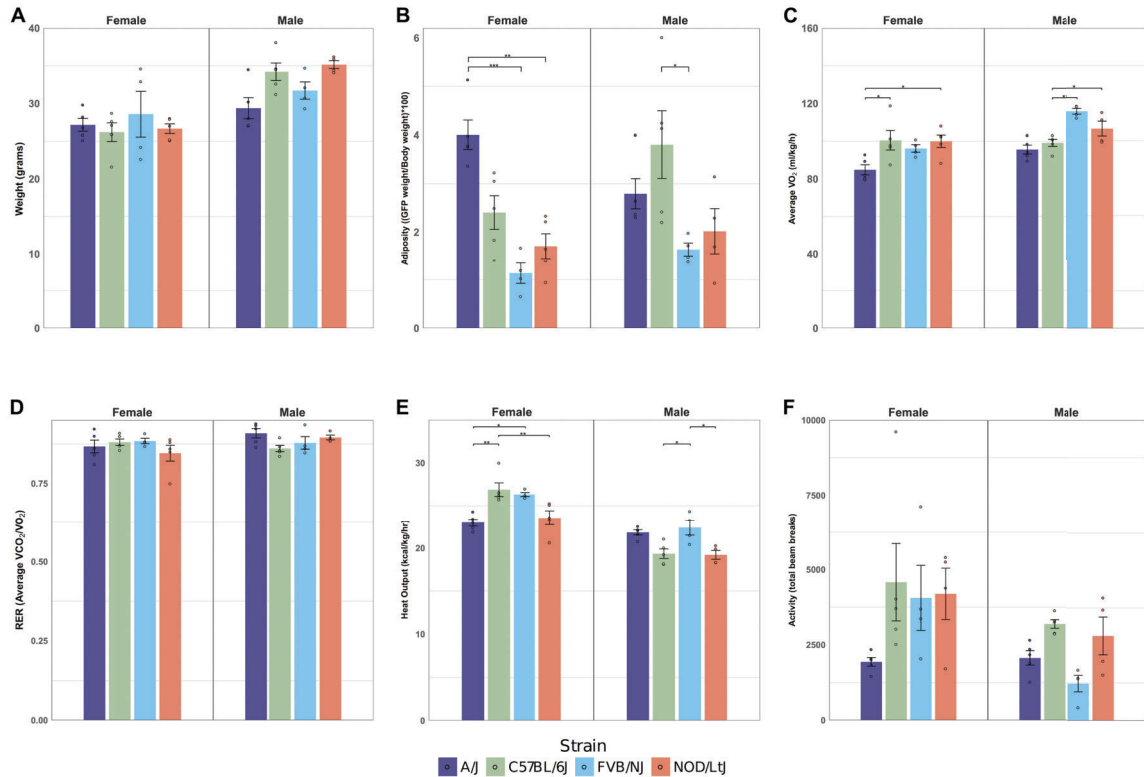
Figure 5.1: Effects of sex and strain on weight and metabolic phenotypes; N=5/ sex and strain group, avg. ± std. dev. Dots represent individual mice within sex-by-strain combination. Horizontal bars represent pairwise comparisons performed using Tukey's HSD post-hoc analysis; * p<0.05, ** p<0.01, and *** p<0.001. Body weight (A) and adiposity (B) were measured at 28 weeks of age, after 16 weeks on the experimental diets. Adiposity is expressed as the relative weight of the perigonadal adipose fat pads. Oxygen consumption (C), RER (D), heat output (E), and activity (F) were measured during a 48 hr. period when mice were housed in Phenomaster metabolic cages.

We then assessed the effects of sex, strain, and sex-by-strain in each tissue. Strain had the largest affect on the liver metabolome. Ninety-two metabolites responded to strain variation, independent of sex (Fig. 5.2B). Seventeen metabolites responded to strain in a sex dependent manner but only one metabolite (citrulline) was specific for sex-by-strain interaction only. Another 26 metabolites were significant for sex, independent of strain. Three of these metabolites (acetyllysine, glutamine, and 3-methylphenylacetic acid) were specific to sex. Strain also affected adipose and skeletal muscle (Fig. 5.2C, D). Thirty-eight metabolites were altered in adipose tissue and 29 metabolites were altered in skeletal muscle by strain, independent of sex. Only three metabolites (pantothenate, s-adenosyl-l-homocysteine, and threonine) were altered by sex, independent of strain, in adipose tissue. In contrast, no metabolites were altered by sex-by-strain interaction in adipose. Five metabolites were altered by sex, independent of strain, in skeletal muscle. Only four metabolites (shikimate, ornithine, hydroxyphenylacetic acid, and 2-isopropylmalic acid) were altered by sex-by-strain, in muscle. Assessing metabolites significant for strain using Tukey's HSD post-hoc analysis revealed no one dominant pairwise comparison for any tissue (Fig. 5.2E, F, and G). Unsupervised hierarchical clustering was performed to visualize the relatedness of each of the 8-sex/strain groups, based on similarities between hepatic metabolomes. A/J and FVB/NJ mice clustered by strain and then sex, however, C57BL/6J and NOD/ShiLtJ mice clustered by sex (Fig. 5.3). This indicates that genetic background and sex have varying levels of influence on the metabolome.

5.4.3 Functional Annotation of Strain Effects

The sets of metabolites affected by sex, strain, or sex-by-strain interaction were functionally annotated using KEGG pathways (Table 5.2). Strain had the largest effect on pathways in adipose and liver tissue. Purine metabolism was significantly altered for strain with eight and sixteen metabolites (referred to as hits) mapping onto the KEGG pathway for adipose ($p < 0.001$) and liver tissue ($p < 0.001$) (Fig. 5.4). In addition, 13 metabolites mapped onto pyrimidine metabolism ($p < 0.001$), in liver, for strain. Amino acid metabolism (alanine, aspartate, and glutamate metabolism (adipose, $p < 0.001$, hits=5) and aminoacyl-tRNA biosynthesis (adipose, $p = 0.001$, hits=7; muscle, $p = 0.002$, hits=6)) was also significantly altered by strain for both tissues (FDR < 0.05). Metabolites affected by strain, independent of sex, also mapped onto pathways that represent amino acid metabolism (aminoacyl-tRNA and lysine biosynthesis), in muscle, but were not significantly overrepresented after controlling for FDR (> 0.05). Sex, independent of strain, also significantly affected purine metabolism ($p < 0.001$) with eight metabolites mapping on to the pathway in liver tissue. Sex, in muscle, also significantly altered by sex (lysine biosynthesis, $p = 0.001$, hits=2 and aminoacyl-tRNA biosynthesis, $p = 0.001$, hits=5). Sex-by-strain interaction did not affect any pathways for any tissue.
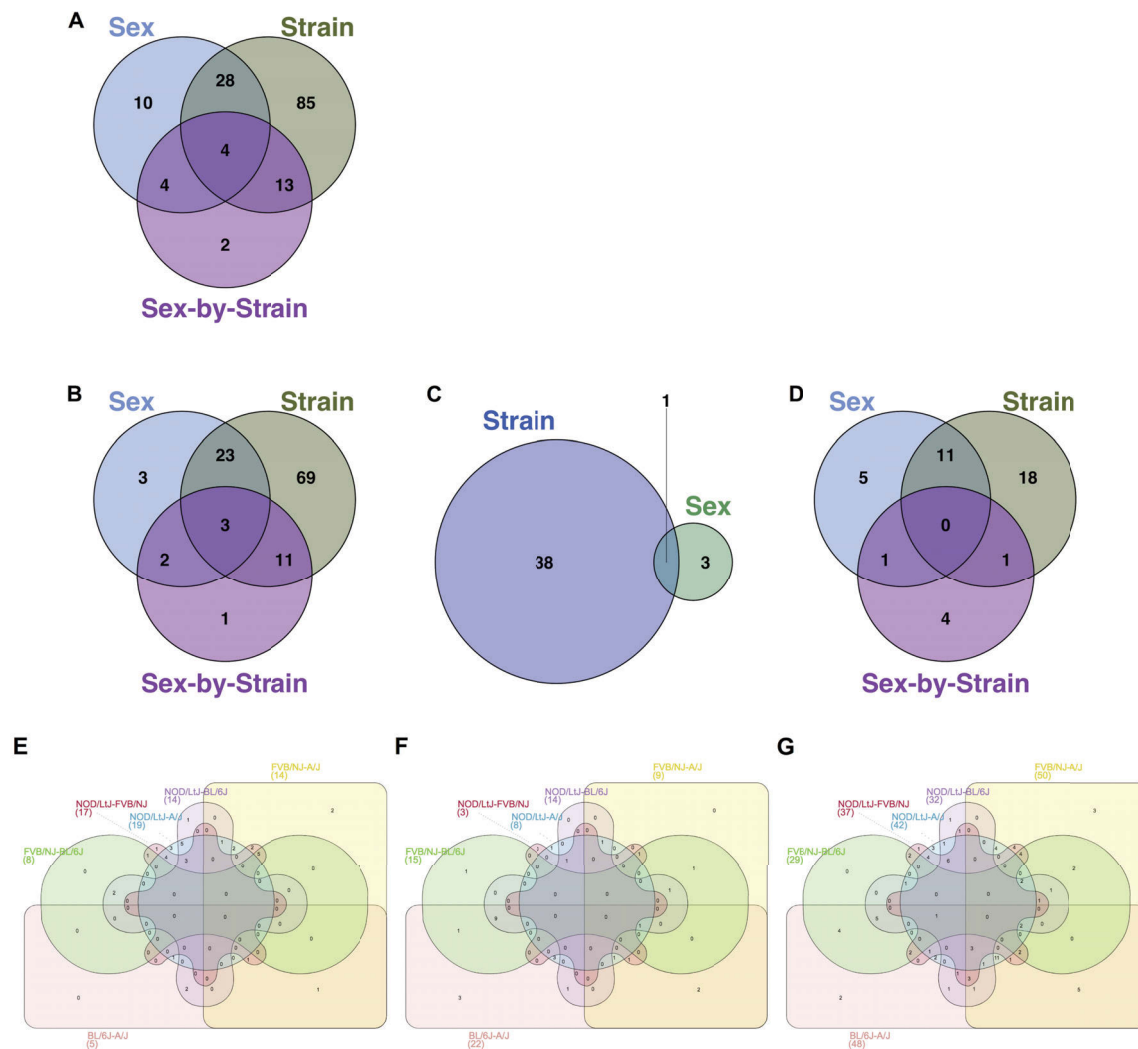
Figure 5.2: Shared and unique effects of strain on tissue metabolomes. The sets of metabolites that differed significantly, based on ANOVA (FDR < 0.05) by strain across tissues, (A), and by sex, strain, and sex-by-strain interaction in liver (B), adipose (C), and muscle (D) were visualized for shared and factor-specific effects using Venn diagrams. Metabolites that differed significantly for strain in adipose (E), muscle (F), and liver (G) were assessed for pairwise comparisons using Tukey's HSD and visualized for shared and specific effects using Venn diagrams.

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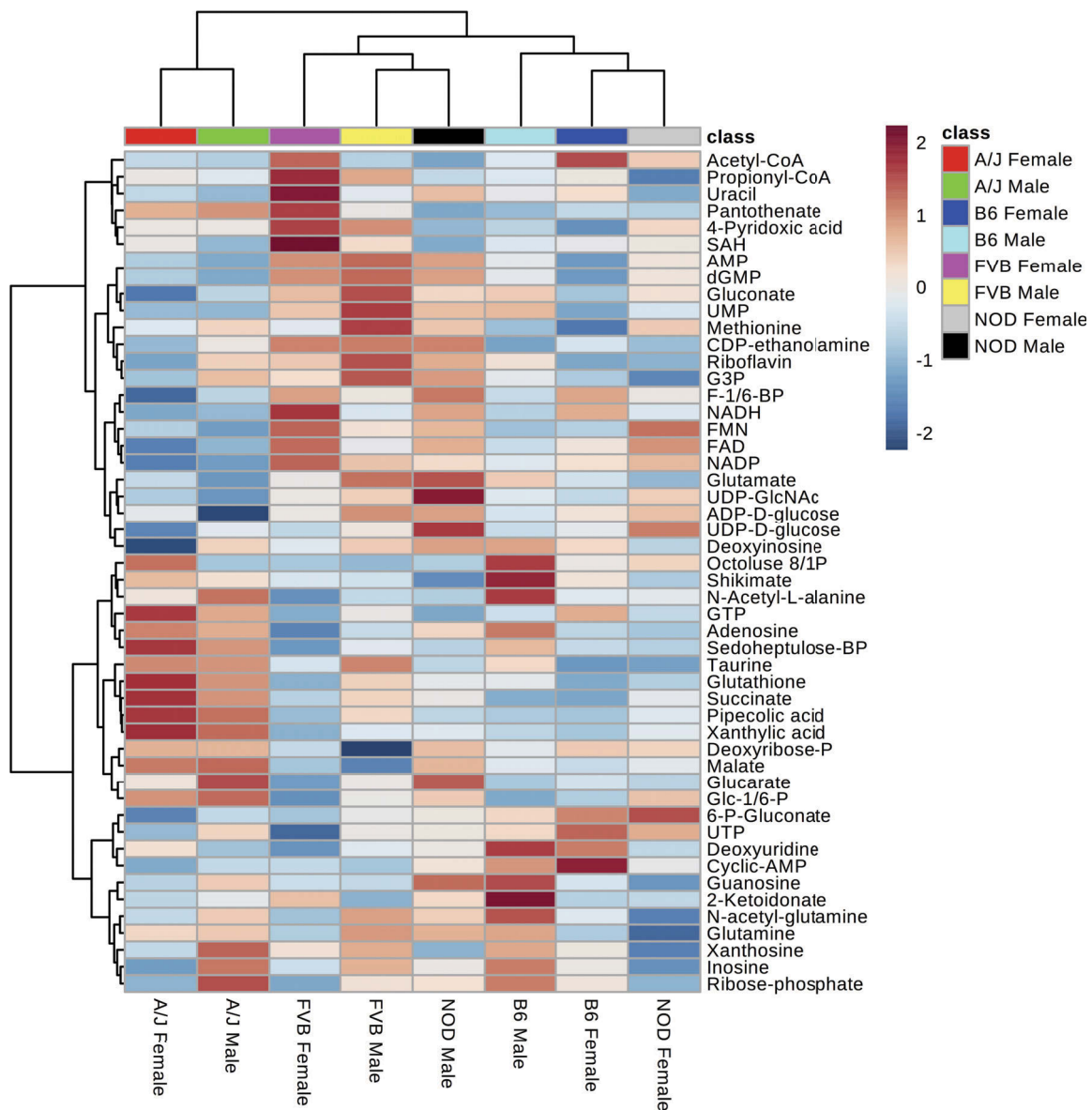
Figure 5.3: Hierarchical clustering of sex/strain groups based on metabolite abundance in liver.
Heatmaps were generated in Metaboanalyst (v3.0) using group averages of the 50 metabolites
that varied the most across sex and strain combinations. Samples were normalized to median
values and values were scaled using Pareto scaling. Z-score was used to determine scale.

5.4.4 Relationship Between Known Metabolites and Phenotypes

The relationships between metabolite profiles in each tissue and systems level energy balance traits were identified using a correlation-based approach (Fig. 5.5). Energy expenditure and adiposity were significantly correlated with 18 and 25 metabolites, respectively, across all three tissues. The majority of the associations with adiposity are due to hepatic (15) and adipose (10) metabolomes while liver (9) and muscle (5) are responsible for most of the associations with heat. Partial correlation was used to adjust for misleading correlations to body weight, after which all of the metabolites remained significantly correlated with adiposity and heat output.

Figure 5.4: Purine metabolism in adipose and liver tissue. Metabolites significant for strain were plotted for adipose and liver tissue. Dashed lines represent missing metabolites in the pathway. Horizontal bars represent pairwise comparisons performed using Tukey's HSD post-hoc analysis; * p<0.05, ** p<0.01, and *** p<0.001.
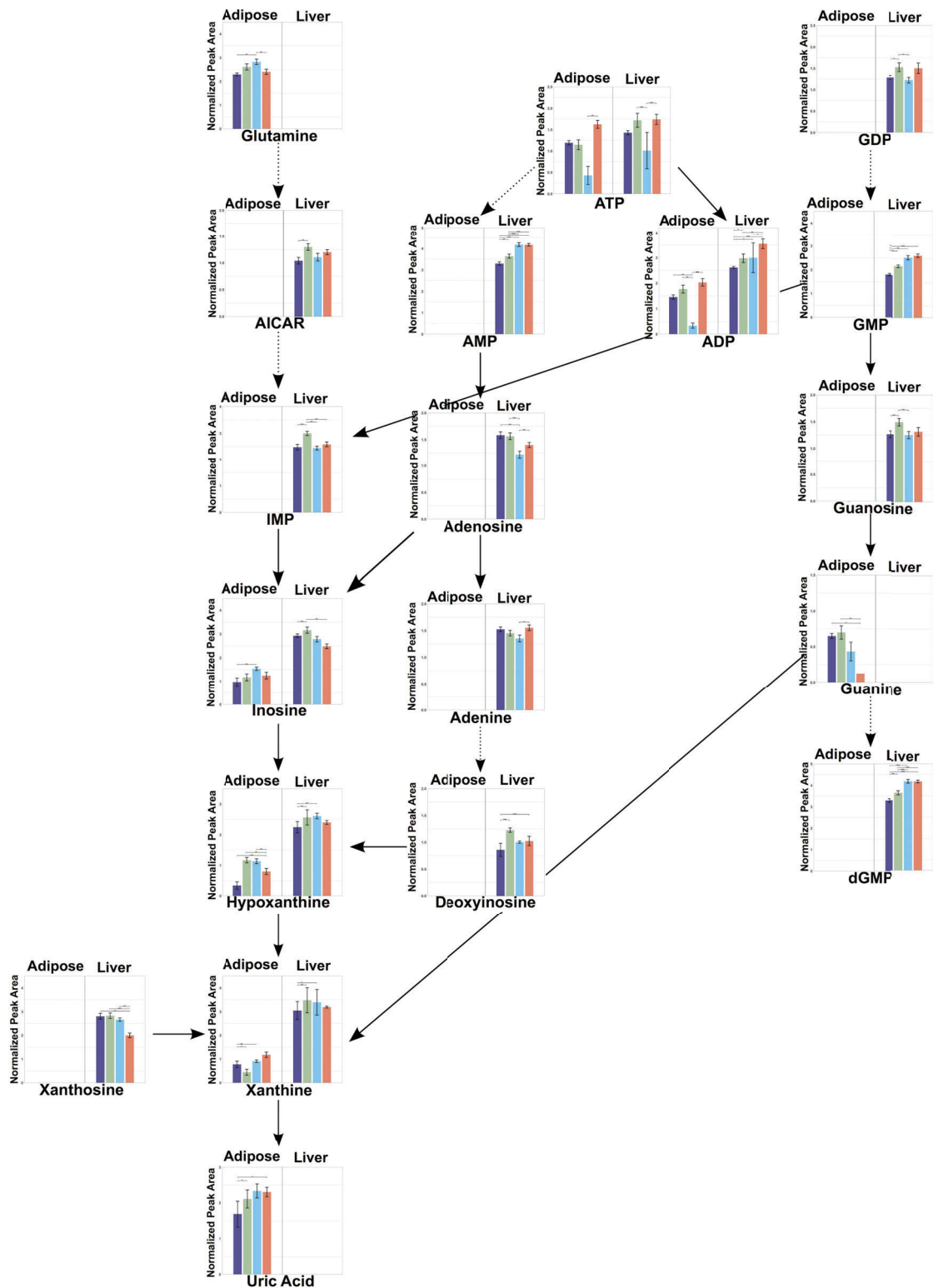
Table 5.2: KEGG Pathway Enrichment of Metabolites Affected by Sex, Strain, and Sex-By-Strain

Tissue	Factor	Pathway	Hits	P-value	(k/m)q
All Tissues	Sex	Purine metabolism	9	<0.001	0.005
		Aminoacyl-tRNA biosynthesis	7	0.002	0.005
		Riboflavin metabolism	3	0.002	0.005
		Lysine biosynthesis	2	0.004	0.005
	Strain	Purine metabolism	22	<0.001	0.007
		Pyrimidine metabolism	15	<0.001	0.007
		Alanine, aspartate and glutamate metabolism	9	<0.001	0.007
		Citrate cycle (TCA cycle)	7	0.001	0.007
		Pantothenate and CoA biosynthesis	6	0.001	0.007
		Glyoxylate and dicarboxylate metabolism	6	0.002	0.007
		Ascorbate and aldarate metabolism	4	0.004	0.007
Adipose	Sex	Cysteine and methionine metabolism	2	0.002	0.025
		Aminoacyl-tRNA biosynthesis	2	0.013	0.025
	Strain	Purine metabolism	8	<0.001	0.007
		Alanine, aspartate and glutamate metabolism	5	<0.001	0.007
		Aminoacyl-tRNA biosynthesis	7	0.001	0.007
		Pyrimidine metabolism	5	0.003	0.007
Muscle	Sex	Lysine biosynthesis	2	0.001	0.004
		Aminoacyl-tRNA biosynthesis	5	0.001	0.004
	Strain	Aminoacyl-tRNA biosynthesis	6	0.002	0.003
		Lysine biosynthesis	2	0.002	0.003
Liver	Sex	Purine metabolism	9	<0.001	0.003
		Riboflavin metabolism	3	0.001	0.003
	Strain	Purine metabolism	16	<0.001	0.004
		Pyrimidine metabolism	13	<0.001	0.004
		Glyoxylate and dicarboxylate metabolism	6	<0.001	0.004
		Citrate cycle (TCA cycle)	5	0.002	0.004

The relationships between metabolite profiles and systems level energy balance traits across all three tissues were assessed. Metabolites that were associated with one or more traits were included. Eight, 15, and 24 metabolites were associated with one or more traits and one or more metabolites within or across tissues in adipose, muscle, and liver tissue (Fig. 5.6, Appendix D). Methionine in adipose tissue was negatively correlated with the bile salt taurodeoxycholic acid (r=-0.66, p<0.001) in muscle but was not associated with taurodeoxycholic acid (r=0.12) in liver tissue. Metabolites that were significant in more than one tissue showed tissue-specific responses. Pantothenate, in skeletal muscle, was negatively correlated with glycerol-3-phosphate (r=-0.54, p=0.001) in liver while glycerol-3-phosphate in muscle was positively correlated with pantothenate (r=0.23, p=0.18) in liver tissue. Furthermore, glycerol-3-phosphate in muscle was correlated with several energy molecules in skeletal muscle and liver tissue, however, glycerol-3-phosphate, in liver tissue, was only correlated with pantothenate in muscle (Fig. 5.6). Pantothenate is critical for the biosynthesis of coenzyme A while glycerol-3-phosphate is the first step in *de novo* lipogenesis, as well as a glycolysis intermediate. Additionally, when mapping metabolites from all three tissues onto KEGG pathways pantothenate and CoA biosynthesis was significantly altered by strain (Table 5.2).
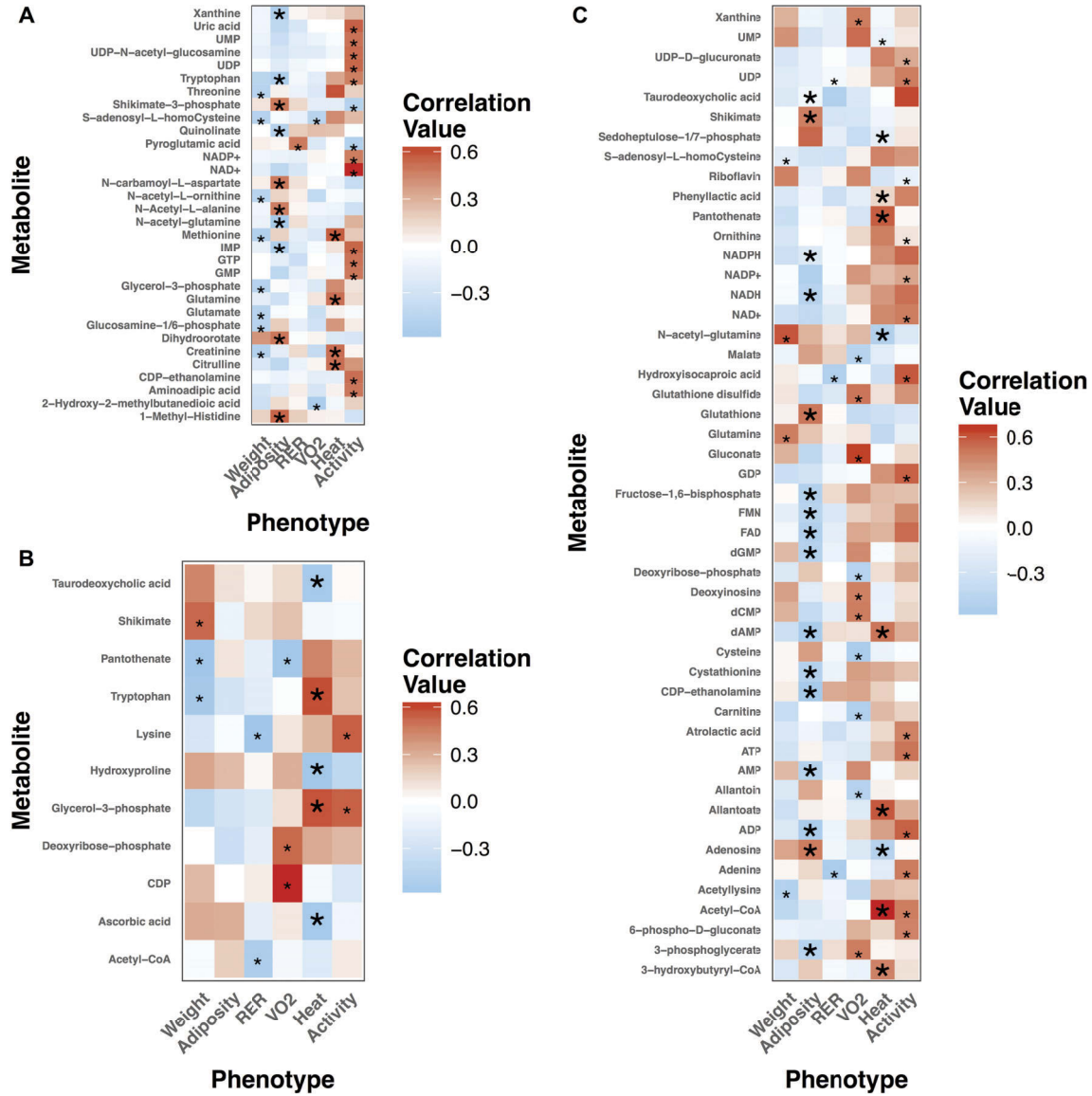
Figure 5.5: Correlations between metabolites and system phenotypes. Pearson correlation was used to associate metabolites in adipose (A), muscle (B), and liver (C) with weight, adiposity, and metabolic phenotypes. Significant correlations (FDR < 0.05) are represented by an asterisk (*). For metabolites that were significantly correlated for adiposity and/or heat output, partial correlation was used to determine if the relationship between metabolite and trait was due to spurious correlation with body weight. Correlations that remained significant are indicated by a bold asterisk (*).

Figure 5.5: Correlations between metabolites and system phenotypes. Pearson correlation was used to associate metabolites in adipose (A), muscle (B), and liver (C) with weight, adiposity, and metabolic phenotypes. Significant correlations (FDR < 0.05) are represented by an asterisk (*). For metabolites that were significantly correlated for adiposity and/or heat output, partial correlation was used to determine if the relationship between metabolite and trait was due to spurious correlation with body weight. Correlations that remained significant are indicated by a bold asterisk (*).
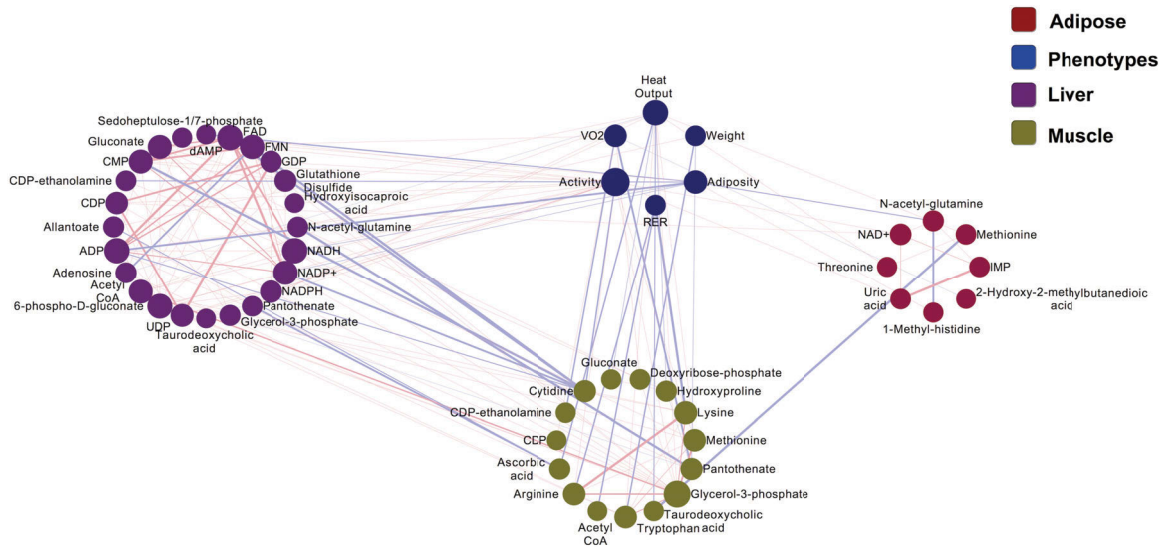
Figure 5.6: Cross-tissue metabolite and phenotype network. Pearson correlation was used to associate metabolites within and across each tissue with weight, adiposity, and metabolic phenotypes. Only significant correlations (FDR < 0.05) are shown. To assess relationships within and across tissues and phenotypes, metabolites are clustered by their respective tissue or phenotype. The size of each node represents the number of edges connected to the node. A red line represents positively correlated metabolites, while a blue line represents negatively correlated metabolites.

5.5 Discussion

Genetics has long been associated with health outcomes in an individual, but the relationship between genetics and metabolism at the tissue level are poorly understood. We used untargeted metabolomics to investigate the effects of strain and sex on tissue metabolomes, and to relate metabolites to body composition and energy expenditure. Mouse strains varied with regards to their genetic backgrounds. This gave us insight into how diverse genetic backgrounds alter the tissue metabolome when fed the same diet. Strain, in combination with sex allowed us to query the extent to which tissue metabolomes were influenced by sex, strain, and their interaction, and to characterize their relative impact on liver, adipose, and skeletal muscle. Our model, which included eight unique combinations of sex and strain, also enabled us to use correlation to relate metabolites to body composition and energy expenditure.

Since body weight was not affected by sex-by-strain interaction while adiposity, VO_2, and heat output were we expected that a modest number of metabolites would be influenced by strain in a sex-dependent manner. Few metabolites, however, were influenced by sex-by-diet for any tissue; liver (n=17), adipose (n=0), and muscle (n=6). Conversely, strain exerted substantial effects on the metabolome across all tissues; liver (n=92), adipose (n=38), and muscle (n=29) relative to sex and sex-by-strain interaction. Approximately one-third (26 of 92) of metabolites in liver that were influenced by strain are involved in purine and pyrimidine metabolism. Purine and pyrimidine metabolism consist of the breakdown and synthesis of purine and pyrimidine nucleotides, which are building blocks of nucleic acids and cofactors that are required

for energy metabolism. Although purine and pyrimidine metabolism are fundamentally important for all cells, recent metabolomics studies have shown alterations in these pathways due to differences in genetic background. C57BL/6J females had higher levels of hypoxanthine in plasma at 3 weeks compared to NOD/ShiLtJ mice while NOD/ShiLtJ mice showed higher levels of inosine over 15 weeks [96]. In contrast, our study showed no differences between NOD/ShiLtJ and C57BL/6J mice for hypoxanthine although the duration of our study was 7 months and included males. Our study also revealed significantly higher levels of inosine in C57BL/6J mice compared to NOD/ShiLtJ mice contradicting the aforementioned study. Our conflicting results could be due to the fact that our measurements were performed in liver tissue versus plasma, which represents the systemic pool of metabolites rather than tissue specific metabolite pools.

Alanine, aspartate, and glutamate metabolism was influenced by strain in liver, as well as adipose tissue with six and five metabolites significantly altered between strains. NOD/ShiLtJ mice had the lowest levels of alanine and highest levels of aspartate while glutamate levels were highest in A/J mice. Glutamine converts to glutamate via increased glutaminase activity in uncontrolled diabetes [196]. NOD/ShiLtJ mice, which develop type-1-diabetes, showed increased levels of glutamate relative to glutamine. Moreover, C57BL/6J mice can develop type 2 diabetes while ob/ob mice on a FVB/NJ background developed severe diabetes indicating that FVB/NJ can develop a diabetic phenotype [82]. C57BL/6J and FVB/NJ mice showed similar glutamate:glutamine levels to NOD/ShiLtJ mice. Although glutaminase was not measured in our study, querying single nucleotide polymorphisms (SNP) using the

mouse phenome database showed three SNPs across all four strains for glutaminase with two SNPs belonging to NOD/ShiLtJ and one belonging to FVB/NJ. In contrast, A/J mice, which are resistant to developing diabetes, only showed a slight increase in glutamate relative to glutamine despite having the highest levels of glutamate. In addition to glutamate, alanine and aspartate levels may be increased due to higher levels of alanine (ALT) and aspartate aminotransferase (AST). Both of these enzymes have been shown to be increased in individuals at risk for type 2 diabetes [197]. ALT, in particular, has been used as a marker of liver damage. Querying SNPs for ALT using the mouse phenome database showed eight SNPs across all four strains with all eight SNPs belonging to NOD/ShiLtJ and one belonging to C57BL/6J mice. ALT levels, measured in the liver in our study, were highest in A/J mice (data not shown), which also had the highest alanine levels in liver, despite their resistance to type 2 diabetes. ALT levels in the liver are also a marker of non-alcoholic fatty liver disease [60], although A/J mice have also been shown to be resistant to developing NAFLD [198]. Our study was not designed to link specific metabolites with specific genetic markers. Despite this investigating the potential association between ALT levels, alanine and the effects of A/J mice on this relationship requires further investigation.

In addition to pathways that were significantly affected in this study recent metabolomics studies have shown different metabolic signatures in plasma between C57BL/6J and NOD/ShiLtJ female mice. C57BL/6J mice had significantly increased levels of TCA cycle intermediaries (citrate, α-ketoglutarate, fumarate, malate, β-alanine, and glutamine) compared to NOD/ShiLtJ mice [96]. Although the TCA cycle pathway, in liver, was not significantly altered there were significant differences across metabolites

present in the TCA cycle. In contrast to the findings in plasma, our study revealed that NOD/ShiLtJ mice had significantly higher levels of citrate/isocitrate and fumarate compared to C57BL/6J mice; however, these results were specific to liver. When assessing differences within the TCA cycle across all strains, in liver, A/J mice had significantly higher levels of acetyl-CoA, citrate/isocitrate, coenzyme A, fumarate, malate, and succinate compared to C57BL/6J. A previous study revealed higher levels of metabolites in the TCA cycle for adipose compared to muscle and liver, in males, of two obesity mouse models, ob/ob and C57BL/6J fed a high-fat diet [199]. Our study included males and females, which may account for the differences found across the literature. Additionally, our measurements are relative. In the context of different strains metabolites abundances from C57BL/6J mice may appear to contradict previous studies. Regardless, these results emphasize that metabolite pools can change significantly based on location in the body.

Phenotypes were associated with metabolites in all three tissues. Cofactors are important for biochemical reactions to occur. NAD+ was positively correlated with activity levels in adipose and liver tissue. Previous studies have shown an increase in NAD+ during exercise [200]. Furthermore, ADP and ATP also showed a positive relationship with activity levels in liver while AMP had an inverse relationship with adiposity. AMP is necessary for the activation of AMPK, which plays an important role in energy metabolism. In the liver, activation of AMPK inhibits fatty acid synthesis through the phosphorylation of acetyl-CoA carboxylase 1 and 2 (ACC 1 and 2) [201]. Without AMP to activate AMPK fatty acid synthesis is not controlled potentially leading to NAFLD. Recent advancements have shown therapeutic potential in treating NAFLD

through modulation of AMPK levels [202]. Furthermore, metformin and salicylate have been shown to increase AMPK levels in liver tissue improving insulin sensitivity and inhibiting lipogenesis [201]. The negative association between AMP and adiposity in our study may provide an explanation for lowered AMPK levels during increased weight gain; however, we did not measure AMPK or ACC1 and 2 levels in our mice. Further studies investigating the relationship between adiposity, AMP, and AMPK levels are warranted.

Several limitations should be noted. First, our study measured metabolites in a steady state, at a single time point. Thus we are unable to assess potential turnover due to genetic differences. Second, our extraction method targets polar metabolites at the expense of lipid metabolites, so we are unable to assess the effects of genetics on the lipid metabolome, which are likely to be affected by sex and genetic differences. Furthermore, due to the specificity of the extraction method and the limited number of metabolites that can be identified using our in-house database, it is difficult to ascertain whether pathways significantly altered are affected by sex, strain or sex-by-strain interaction or because a metabolite is more easily identified to due inherent biases in the experimental procedure. Finally, although we know the strains of mice chosen have been genetically profiled we did not genetically assess the mice in this study.

5.6 Conclusion

We profiled three tissues (adipose, muscle, and liver), which play a major role in energy metabolism to determine the effects of strain and sex on the tissue metabolome.

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The stratified results showed tissue-specific effects of strain and sex on tissue metabolites. This study, however, was performed in the context of a single diet. Including other diets may reveal a more complex relationship between the tissue metabolome and genetics. Nonetheless, this study has shown several metabolites that could be investigated to determine their role in metabolism and disease.

CHAPTER SIX

TISSUE LEVEL SEX, DIET, AND GENETICS INTERACT REVEAL

UNIQUE METABOLITE AND CLUSTERING PROFILES USING

UNTARGETED LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

ON ADIPOSE, SKELETAL MUSCLE, AND LIVER TISSUE IN MICE

Ann Wells[1], William T. Barrington[2], Stephen Dearth[3], David W. Threadgill[2], Shawn R. Campagna[1, 3], Brynn H. Voy[1, 4]

[1]University of Tennessee-Knoxville, UT-ORNL Graduate School of Genome Science and Technology, [2] Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, [3]University of Tennessee-Knoxville, Department of Chemistry, [4]University of Tennessee-Knoxville, Department of Animal Science

Ann Wells' contribution included: tissue extractions, data analysis, and primary writing of the manuscript.

6.1 Abstract

The physiological consequences of diet can vary widely due to differences in genetic background and gender yet diet remains one of the most commonly used tools for weight loss or to improve health. Although the relationship between diet and health is widely accepted, and countless individuals use various diets for weight loss, relatively little is known about the fundamental impact of diet on metabolism at the tissue level. Using untargeted metabolomics, this study aimed to identify changes in water-soluble metabolites in A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ males and females, fed one of five diets (Japanese, ketogenic, Mediterranean, American, and standard mouse chow) for seven months. Metabolite abundance was examined in liver, skeletal muscle, and adipose tissue for sex, strain, diet, sex-by-strain, sex-by-diet, strain-by-diet, and sex-by-strain-by-diet interaction. ANOVA suggests that liver is the most metabolically active

when assessing all factors; however, muscle was most sensitive to strain effects. Unsupervised clustering revealed that mice clustered by strain then sex when comparing known metabolites. Pathway analysis showed that the majority of pathways affected played a role in amino acids metabolism across all three tissues. Purine metabolism, in liver tissue, however, was the only pathway altered by sex-by-strain-by-diet. The results of this study revealed that amino acid metabolism is sensitive to diet, genetic, and strain effects but not necessarily their interactions. Furthermore, metabolites were affected in a tissue specific manner.

6.2 Introduction

The physiological consequences of diet can vary widely due to differences in genetic background and gender yet diet remains one of the most commonly used tools for weight loss or to improve health. Obesity is a major contributor to the intersection between diet and disease due its effect on risk of developing type 2 diabetes, hypertension, cardiovascular disease, stroke, osteoarthritis, and certain cancers [2, 147, 148]. Obesity rates, however, stratify based on gender and race [203]. Accordingly, the prevalence of metabolic diseases such as type 2 diabetes have also shown disparities between races. Native Americans (33%) had the highest rates of type 2 diabetes while Alaskan natives (5.5%) had the lowest [204].

Previous studies investigating the efficacy of diet on health have shown marked differences in the response to certain aspects of diet. Intake of fruits and vegetables showed higher circulating levels of beta-cryptoxanthin and lutein in women compared to

men [205]. Significant effects genetic risk scores and dietary intake were identified in relation to markers of adiposity [206]. Individuals with the A allele of melanocortin-4 receptor had a positive association between total fat intake and consumption of an American diet and abdominal obesity [207]. Additionally, individuals with the TCF7L2-rs7903146 polymorphism have an increased incidence of type 2 diabetes, CVD, and stroke. Adherence to a Mediterranean diet improved fasting glucose and reduced their risk of CVD and stroke [208]. Despite the growing body of knowledge dissecting the effects of sex and diet and genetics and diet, a more comprehensive understanding of the intersection between all three is necessary.

Investigation of diet and health in humans has provided insight into the potential relationships between diet and sex, and diet and genome, however, humans are often non-compliant and under report dietary intake on food frequency questionnaires [209]. Mice are an ideal alternative due their genetic similarity, inherent compliance, and replicative ability. Accordingly, the severity of disease due to genetic background has been shown through phenotypic markers in mice [82, 91]. Although the relationship between diet and health is widely accepted, and countless individuals use various diets for weight loss, relatively little is known about the fundamental impact of diet on metabolism, especially in the context of different genetic backgrounds, at the tissue level.

Mass spectrometry-based metabolomic platforms now enable global, discovery-based profiling of tissue and circulating metabolomes. Accordingly, metabolomics has emerged as a tool with which to understand the impact of diet, genetics, and sex on metabolism and to identify metabolites and pathways that are associated with disease

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[90, 110, 154]. Metabolomics studies on the effects of diet, genetics, or sex in humans and mice frequently use samples that can be obtained noninvasively (e.g., plasma, erythrocytes or urine). Less is known, however, about the impact of diet, genetics, or sex at the tissue level, and the extent to which tissues may respond to diet, genetics, or sex in a similar manner. The objective of this study was to use metabolomics to determine the effects of diet, genetics, and sex on metabolomes at the tissue level and to associate tissue metabolites with systems level phenotypes of energy utilization and body composition in mice. Four common dietary profiles (American, Japanese, Mediterranean and ketogenic) were chosen because of their relationships to various aspects of metabolic health in humans, and because they vary widely in composition. Mouse diets were formulated to represent both the macronutrient profiles and sources that are characteristic of each of these four eating patterns, while maintaining the same caloric value and sufficient amounts of micronutrients. Four strains of mice (A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ) were chosen for their genetic diversity. Both males and females were used to evaluate the relative effects of diet, strain, sex, sex-by-strain, sex-by-diet, strain-by-diet, and sex-by-strain-diet interaction on tissue metabolism. Untargeted metabolomics was used to comprehensively profile the metabolomes of liver, skeletal muscle, and adipose tissue because of their roles in energy balance [155, 156].

6.3 Materials and Methods

6.3.1 Animals and Diets

All husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. Four-week old A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were allowed to acclimate for 14 days and consumed a standard mouse chow (PicoLab Mouse Diet 20, LabDiet, St. Louis, MO) during this period. At 42 days of age, five male and five female mice from each strain were assigned to each of five diets: American, Mediterranean, Japanese, ketogenic, or chow. Diets were designed in collaboration with Research Diets, Inc. (New Brunswick, NJ) to contain calories, macronutrient ratios, sources of ingredients, fiber content, and lipid profiles that are reflective of each diet pattern. (Table 6.1, macronutrient ratios, Appendix I and J, detailed formulations). Mice were maintained on a 12-hour light/dark cycle throughout the study. At age 18 weeks, after 12 weeks on the diets, mice were housed in Phenomaster metabolic chambers (TSE Systems, Inc.) for 48 hours for measurement of metabolic rate and activity. The chambers measured respiratory exchange rate (RER), volume of oxygen (VO_2), and heat output via heat dissipation, and activity level by laser detection. Activity levels measured voluntary movement of the mouse in the x and y plane. Mice were euthanized at seven months of age by CO_2 asphyxiation. Perigonadal adipose tissue was dissected and weighed as a measure of adiposity. Samples of adipose tissue, the left lobe of the liver and the vastus medialis,

vastus lateralis, and rectus femoris muscle were snap-frozen in liquid N_2 and stored at -80°C for metabolomics analysis.

Table 6.1: Dietary Composition

	Ketogenic D12052706	American D12052705	Mediterranean D12052702	Japanese D12052703	chow D12052701
Fat	84% Butter, Lard	35% Corn oil, butter, Olive oil	42.6% Olive oil	11% Soybean oil, Olive oil	18% Soybean oil
Carbohydrate	None	50% Corn starch, Wheat starch, Sucrose, potato starch	44.69% Wheat starch, Sucrose, Fructose	76% Rice starch, Sucrose	63% Corn starch
Protein	16% Casein	15% Soy, Beef, Egg white	12.71% Soy, Fish, Beef	13% Soy, Fish	19% Casein

6.3.2 Metabolite Extraction and Liquid Chromatography Mass Spectrometry

Frozen tissue samples were pulverized under liquid N_2. Approximately 25 mg of pulverized tissue were weighed and extracted sequentially in methanol and then in methanol:water (4:1), as previously described [157]. Supernatants were dried under nitrogen and reconstituted in 160 µL of sterile MilliQ water. Internal standard (60 µL of a ^{13}C-labeled *E.Coli* metabolite pool) was added to each sample.

6.3.3 Preparation of 13C-labelled E. Coli metabolite pool

Cultures of Escherichia coli NCM3722 were grown on minimal media ^{13}C-glucose agar plates and passed five times. Single colonies were then transferred to minimal media ^{13}C-glucose liquid cultures (0.4% w/v made from 99% U-^{13}C-glucose, Cambridge Isotope Laboratories). Liquid cultures were set back five times before samples were

extracted and run on mass spectrometer to ensure complete 13C-labeling of all metabolites.

E. Coli cells were extracted by vacuum filtering three 10 mL aliquots, per culture, for three different cultures (grown to ~0.4 OD600) through Magna nylon membrane filters (0.45 micron, 47 mm filter, Maine Manufacturing, Sanford, ME). The filters were transferred face down into petri dishes containing 1.3 mL of extraction solvent (40:40:20 HPLC grade methanol, acetonitrile, water with 0.1M formic acid) chilled to -20 °C. The extraction was allowed to proceed for 15 min at -20°C. The following extraction was carried out in a room maintained at 4°C unless otherwise specified. The filters were rotated so that the cell side was on top and rinsed by pipetting the extraction solvent over the face of the filters. The extraction fluid was then transferred to 1.5 mL centrifuge tubes and an additional 300 µL of new extraction fluid was used to wash the filters again. The remaining extraction solvent was also transferred to the 1.5 mL centrifuge tube and centrifuged for 5 minutes (16.1 rcf). The resulting supernatant was transferred to new vials and the pelleted cell was resuspended in 50 µL of extraction solvent. The extraction was allowed to proceed for 15 min at -20°C at which time the samples were centrifuged for 5 min (16.1 rcf). The supernatant was transferred to the vials and another 50 µL of extraction solvent was added to the pelleted cell repeating the previous extraction once more. Vials containing all of the collected supernatant were dried under a stream of N_2 until all the extraction solvent had been evaporated. Following the resuspension of extracted E. coli residue in 300 µL of sterile water, samples were physically averaged and 10 mL were directly spiked into unlabeled samples.

6.3.4 Metabolomics Data Processing

Raw files generated by Xcalibur were converted to mzML, an open-source format, using msconvert [139]. An open source data analyzer for metabolomics, MAVEN [140, 141] (Princeton University) was used to correct total ion chromatograms based on retention time automatically, for each sample. Known metabolites were manually chosen and peak abundance was integrated by mass (±5 ppm) and retention time. Unknown metabolites were chosen using an algorithm with the following settings: minimum peak width, 5; minimum signal/blank ratio, 3 or greater; minimum peak intensity, 10,000; and minimum peak/baseline, 3. Unknown peaks were filtered manually to remove those that did not meet the above criteria.

6.3.5 Statistical Analysis

All statistical analyses were performed in the language R (3.1.0 and 3.2.2) [158]. An ANOVA model was used to identify significant effects of sex, strain, diet, sex-by-diet, sex-by-strain, strain-by-diet, and sex-by-diet interaction on physiological traits and metabolites. Tukey's Honest Significant Difference (HSD) was used for post-hoc testing. Significance for physiological traits was based on raw p-values ($p < 0.05$).

Metabolite peak area data files were read into R using the package XLConnect [159]. Metabolites that were missing 70% or more sample measurements were removed from analysis. Missing values in the remaining metabolites were imputed using k-nearest numbers from the function impute [160] (Additional File 1 and 2). Metabolites were matched to their corresponding ^{13}C-labeled internal standard, or to a ^{13}C-labeled standard of the same compound class. Class types were identified using the Human

Metabolome Database [143-145]. Prior to statistical analyses, a linear model was created for each metabolite using the terms sex, strain, diet, sex*diet, sex*strain, strain*diet, sex*strain*diet tissue weight and internal standard (eq. 6.1):

Equation 6.1

$$Metabolite = sex + strain + diet + sex * diet + sex * strain$$
$$+ strain * diet + sex * strain * diet + tissue\ weight + \operatorname{int}ernal\ s\tan dard$$

Coefficients for the terms for tissue weight and internal standard were used to adjust metabolite abundance for technical variation using eq. 6.2:

Equation 6.2

$$Adjusted\ metabolite = metabolite + tissue\ weight\ coefficient(mean\ tissue\ weight -$$
$$tissue\ weight) + \operatorname{int}ernal\ s\tan dard\ coefficient(mean\ \operatorname{int}ernal\ s\tan dard -$$
$$\operatorname{int}ernal\ s\tan dard)$$

Adjusted metabolites were Pareto scaled across all mice for each metabolite using the package MetabolAnalyze [161], normalized to the median across all metabolites for each mouse, and cube root transformed to create a normal distribution. Normalized metabolite values were analyzed for effects of sex, strain, diet, sex*diet, sex*strain, strain*diet, and sex*strain*diet. Data were assessed for normality using Q-Q plots, residuals, and the Shapiro-Wilks test. False discovery rate for ANOVA and correlation analyses was set to 5% using the method of Benjamin-Hochberg [162]. Venn diagrams were created using the package VennDiagram [163].

6.3.6 Correlation Analysis

Associations between physiological measurements and metabolite measurements across tissues were assessed using Pearson correlation from the package Hmisc [164] and the package R.Utils [165]. Correlation p-values were FDR-adjusted using the Benjamini-Hochberg procedure. Correlations were visualized using the Cytoscape app Metscape [166].

6.3.7 Functional Pathway Analysis

Overrepresentation pathway analysis was performed using Metaboanalyst [167]. KEGG IDs were input and compared against the mouse KEGG reference metabolome. Statistical significance of pathway overrepresentation was evaluated using Fisher's exact test. Pathway topology was performed using relative-between centrality.

6.4 Results

6.4.1 Physiological Measurements Reveal Difference Among Sex-by-strain-by-diet

Body composition and indirect calorimetry were used to define system-level effects of sex, strain, and diet on metabolism. Body weight, adiposity, VO_2, RER, and heat output were significant for sex-by-strain-by-diet (Table 6.2). In contrast, activity was significant for sex-by-strain, independent of diet. Tukey's post-hoc comparisons were assessed to determine significant pairwise comparisons. Male and female C57BL/6J mice had the highest body weights relative to the other strains (Fig. 6.1A). Female A/J mice fed a ketogenic diet had the lowest bodyweight compared to all other mice.

Female FVB/NJ mice fed a Japanese diet were significantly different from all other diets fed to female FVB/NJ mice (Fig. 6.1A). Male C57BL/6J mice fed a Japanese diet were the lightest compared to all other Male C57BL/6J mice. Female C57BL/6J mice fed a ketogenic diet were lightest compared to all other female C57BL/6J mice. Although adiposity is significantly different for sex-by-strain-by-diet interaction there were very few significant pairwise comparisons (Fig. 6.1B). Female A/J mice fed a Mediterranean diet (4.91±0.76%, p<0.001) had significantly more adiposity then female A/J mice fed a ketogenic diet (1.38±0.13%). Likewise, Female C57BL/6J mice fed a Mediterranean diet (5.19±0.77%) had significantly more adiposity then female C57BL/6J mice fed a ketogenic (1.85±0.53%, p<0.001) or chow diet (2.4±0.35%, p=0.15). Female FVB/NJ mice were lightest relative to all other mice; however, they were not significantly different. VO_2 levels were used to measure energy expenditure. Female A/J mice fed a ketogenic diet had the highest VO_2 levels relative to all other female A/J mice (Fig. 6.1C). RER responded in a similar manner for each strain and sex across diets. The Japanese diet, which is high in carbohydrates was represented by a high RER value (~0.9) while the ketogenic diet, which is high in fat was represented by a low RER value (~0.73) (Fig. 6.1D). Heat output was highest in male and female A/J mice relative to all other diets (Fig. 6.1E). Furthermore, female FVB/NJ mice had the highest heat output compared to female FVB/NJ fed a Japanese diet or chow diet. Activity levels were not affected by diet but rather by sex-by-strain interaction (Fig.6.1F). Female A/J mice (1855 beam breaks) had significantly lower activity levels compared to female FVB/NJ (3620, p<0.001), NOD/ShiLtJ (7135, p<0.001), and C57BL/6J mice (3638, p<0.001). Conversely, male A/J mice showed no difference in activity levels. Male FVB/NJ mice

(1520) had significantly lower activity levels compared to male C57BL/6J mice (2782, p=0.040).

Table 6.2: P-values of Effects of Sex, Strain, Diet, and Sex-by-Strain, Sex-by-Diet, Strain-by-Diet, and Sex-by-Strain-by-Diet Interaction on Weight and Metabolism.

	Sex	Strain	Diet	Sex by Strain	Sex by Diet	Strain by Diet	Sex by Strain by Diet
Weight	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	0.022
Adiposity	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003
RER	0.539	<0.001	<0.001	0.003	0.417	0.533	0.008
VO2	<0.001	<0.001	<0.001	0.001	0.080	<0.001	0.009
Heat Output	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	0.001
Activity	<0.001	<0.001	0.137	0.004	0.857	0.978	0.750

6.4.2 Significant Effects of Sex, Diet, Strain, and Their Interactions on Tissue Metabolomes

An ANOVA model was used to identify metabolites that were robustly affect by sex, strain, diet, and their interactions. A total of 178 metabolites (98% of all known metabolites detected) were significantly affected by sex, strain, diet, or their interactions in one or more tissues (Appendix E, Additional File 5, Fig. 6.2). One hundred and fifty-four metabolites (84% of total effected metabolites) were influenced by strain in at least one tissue. Twenty of these metabolites were significant for strain independent of all other factors. Only one metabolite was significant for each factor; sex (thymine), diet (dAMP), strain-by-diet (citrate/isocitrate), and sex-by-strain-by-diet (nicotinamide ribotide), independent of all other factors.

Figure 6.1: Effects of sex, strain, and diet on weight and metabolic phenotypes; N=5/ sex, strain, and diet group, avg. ± std. dev. Dots represent individual mice within sex-by-strain-by-diet combination. Horizontal bars represent pairwise comparisons performed using Tukey's HSD post-hoc analysis; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ Body weight (A) and adiposity (B) were measured at 28 weeks of age, after 16 weeks on the experimental diets. Adiposity is expressed as the relative weight of the perigonadal adipose fat pads. Oxygen consumption (C), RER (D), heat output (E), and activity (F) were measured during a 48 hr. period when mice were housed in Phenomaster metabolic cages.

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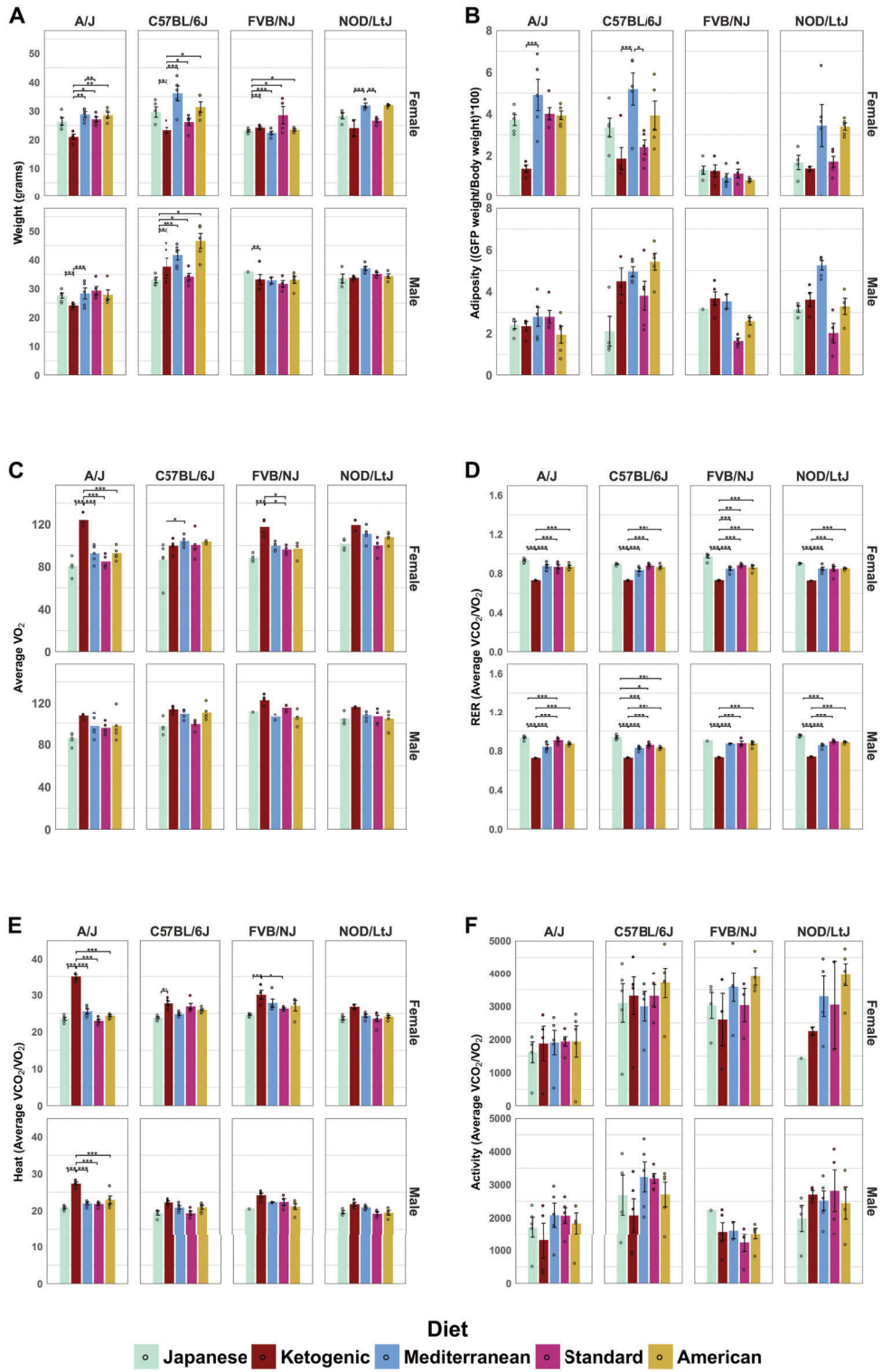
Strain, independent of all other factors, affected the largest number of metabolites in muscle (n=40); however, adipose (n=34) and liver (n=19) were also affected. In contrast, adipose tissue was most sensitive to sex (n=12), independent of other factors, relative to liver (n=0) and skeletal muscle (n=3). Only liver tissue was affected by diet (n=3), independent of all other factors.

Unsupervised hierarchical clustering was performed to visualize the relatedness of each of the 40-sex/strain/diet combinations, based on similarities between metabolomes (Fig. 6.3). In liver, A/J and C57BL/6J mice clustered separately, while NOD/ShiLtJ and FVB/NJ mice were predominantly independently clustered with one NOD/ShiLtJ mouse clustered with FVB/NJ mice. Additionally, mice predominantly clustered by sex secondarily to genetic background. Skeletal muscle primarily followed the same pattern, however, A/J and C57BL/6J mice did not cluster together perfectly as in liver. Conversely, adipose tissue did not show a specific clustering pattern for any factor.

6.4.3 Functional Annotation of Sex, Strain, and Diet Effects

The sets of metabolites affected by each factor and their interactions were functionally annotated using KEGG pathways (Table 6.3). Strain, sex, and diet interacted to significantly affect purine metabolism in liver (FDR, $p<0.001$), with seven metabolites (referred to as hits) mapping onto this pathway. Purine metabolism was also significantly affected by diet (hits=12), strain (hits=18), and sex-by-strain (hits=12) interaction in liver. Strain affected pathways involved in energy metabolism (pantothenate and CoA biosynthesis and TCA cycle). Additionally, diet affected amino
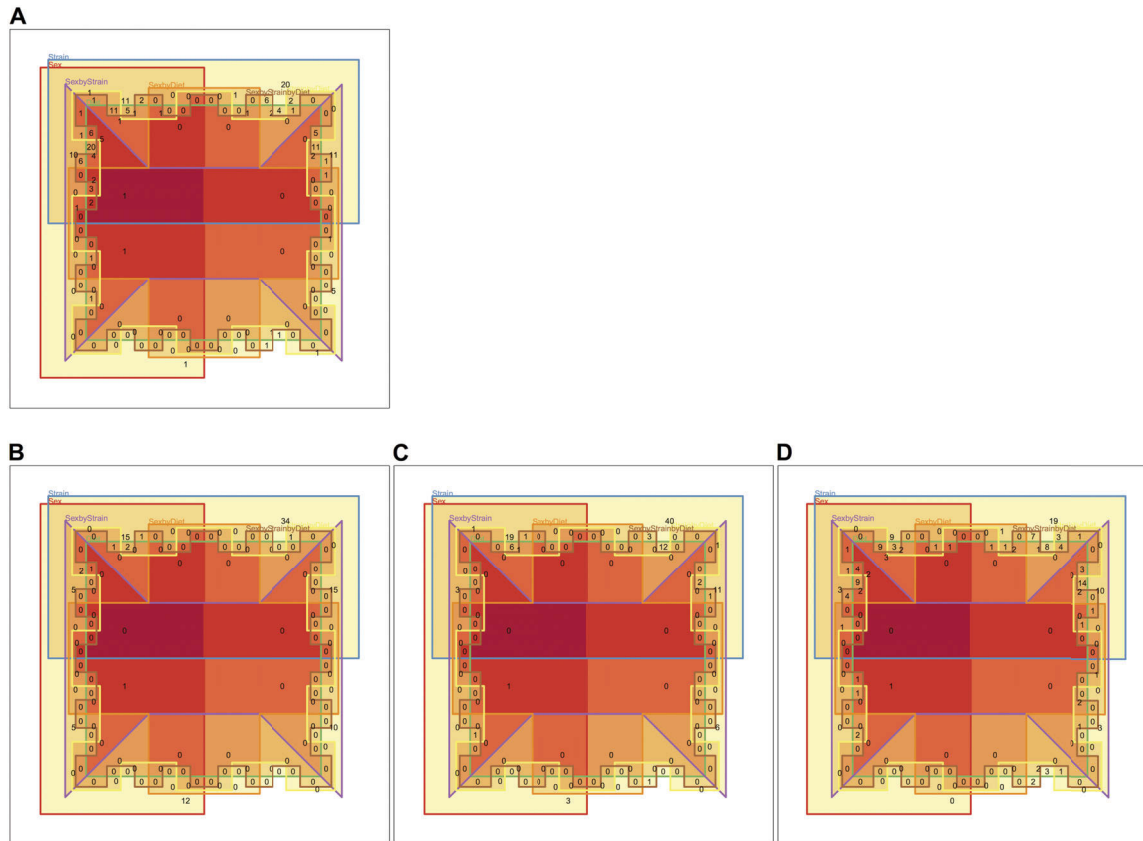
Figure 6.2: Shared and unique effects of sex, strain, diet, sex-by-diet, sex-by-strain, strain-by-diet, and sex-by-strain-by-diet interactions on tissue metabolomes. The sets of metabolites that differed significantly, based on ANOVA (FDR < 0.05) by sex, diet, strain, sex-by-diet, strain-by-diet, or sex-by-strain-by-diet interaction across tissues, (A), and in adipose (B), muscle (C), and liver (D) were visualized for shared and factor-specific effects using Venn diagrams.
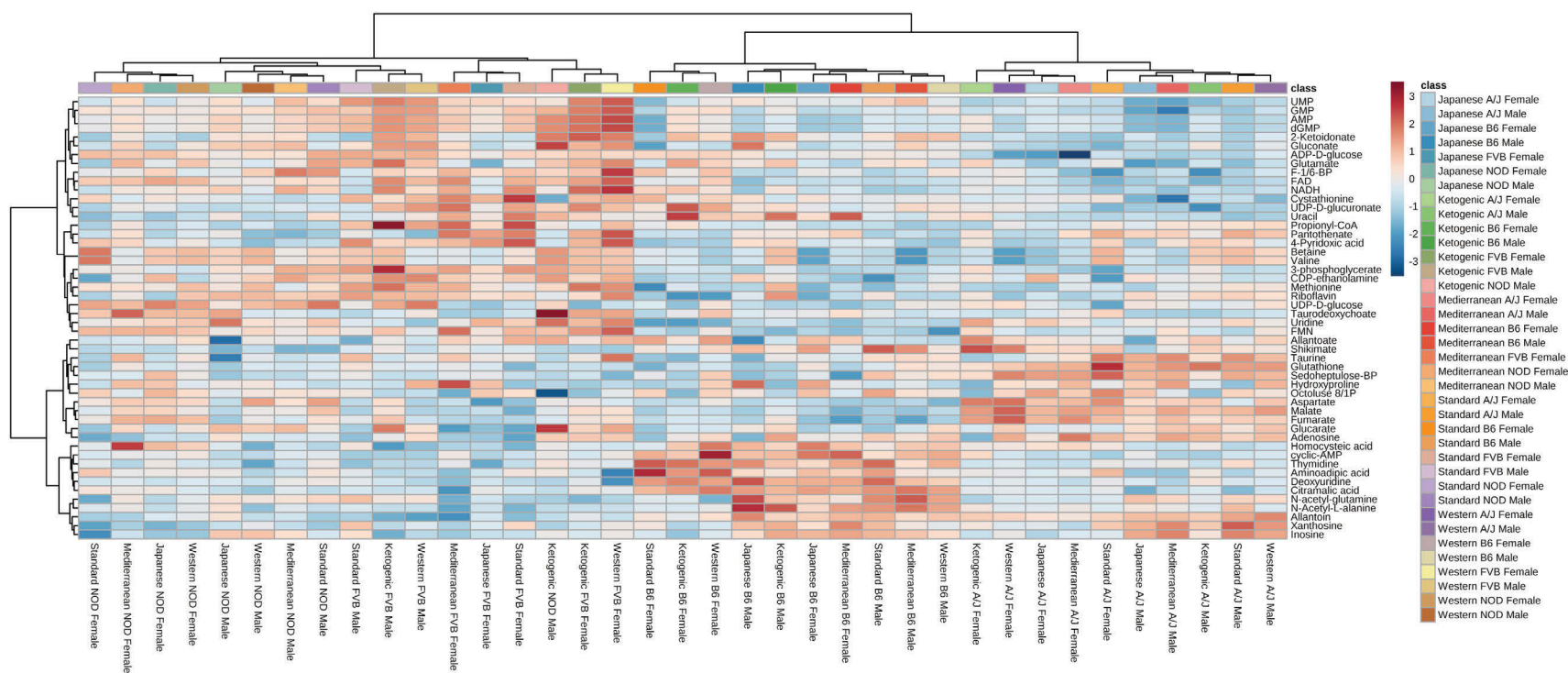
Figure 6.3: Hierarchical clustering of sex/diet groups based on metabolite abundance in liver. Heatmaps were generated in Metaboanalyst (v3.0) using group averages of the 50 metabolites that varied the most across diet/genetic/sex combinations. Samples were normalized to median values and values were scaled using Pareto scaling. Z-score was used to determine scale. Abbreviations: F-1/6-BP: Fructose-1,6-bisphosphate, Sedoheptulose-BP: Sedoheptulose-bisphosphate

acid metabolism (aminoacyl-tRNA biosynthesis and arginine and proline metabolism). In muscle, sex, strain, and diet all affected amino acid metabolism (aminoacyl-tRNA biosynthesis, lysine biosynthesis, arginine and proline biosynthesis, and glutamate and glutamine metabolism, and alanine, aspartate, and glutamate metabolism) (Fig. 6.4). Additionally, strain affected the TCA cycle (hits=6). Likewise, sex and strain affected amino acid metabolism (aminoacyl-tRNA biosynthesis, arginine and proline metabolism, and alanine, aspartate, and glutamate metabolism) in adipose tissue.

6.4.4 Relationship Between Known Metabolites and Phenotypes

The relationships between metabolite profiles in each tissue and systems level energy balance traits were identified using a correlation-based approach (Fig. 6.5). Energy expenditure and adiposity were significantly correlated with 58 and 84 metabolites, respectively, across the three tissues. Hepatic (26 heat, 33 adiposity), adipose (16 heat, 33 adiposity), and skeletal muscle (16 heat, 18 adiposity) metabolomes contributed the associations in a similar manner. Partial correlation was used to adjust for spurious correlations to body weight, after which 75 (33 in adipose, 33 in liver, and 9 in muscle) metabolites remained significantly correlated with adiposity and 54 (16 in adipose, 26 in liver, and 12 in muscle) with heat output.
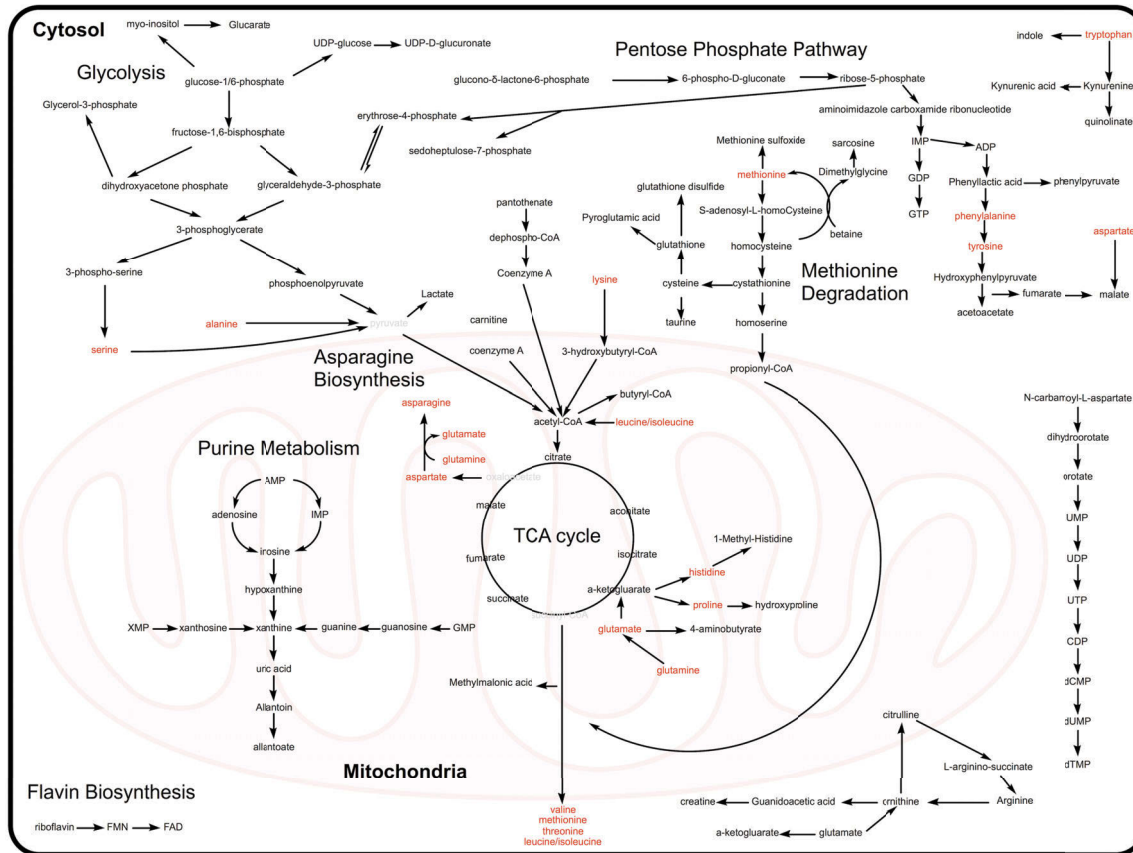
Figure 6.4: Metabolites mapped onto pathways in a cell. Metabolites identified in adipose, muscle, and liver were mapped onto pathways in a cell. Amino acids are highlighted in red.

Table 6.3:KEGG Pathway Enrichment of Metabolites Affected by Sex, Strain, Diet, Sex-by-diet, Sex-by-strain, Strain-by-diet, and Sex-strain-by-diet Interaction.

Tissue	Effect	Pathway	Hits	P-value	(k/m)q
All Tissues	**Sex**	Pyrimidine metabolism	**11**	**<0.001**	**0.004**
		Purine metabolism	**14**	**<0.001**	**0.004**
		Arginine and proline metabolism	**9**	**<0.001**	**0.004**
		Aminoacyl-tRNA biosynthesis	**11**	**0.001**	**0.004**
	Strain	Pyrimidine metabolism	**18**	**<0.001**	**0.011**
		Purine metabolism	**20**	**<0.001**	**0.011**
		Alanine, aspartate and glutamate metabolism	**9**	**<0.001**	**0.011**
		Glycine, serine and threonine metabolism	**10**	**<0.001**	**0.011**
		Arginine and proline metabolism	**12**	**<0.001**	**0.011**
		Ascorbate and aldarate metabolism	**5**	**0.001**	**0.011**
		Aminoacyl-tRNA biosynthesis	**15**	**0.001**	**0.011**
		Citrate cycle (TCA cycle)	**7**	**0.001**	**0.011**
		Pantothenate and CoA biosynthesis	**6**	**0.001**	**0.011**
		Phenylalanine, tyrosine and tryptophan biosynthesis	**3**	**0.003**	**0.011**
		D-Glutamine and D-glutamate metabolism	**3**	**0.007**	**0.011**
		Glutathione metabolism	**7**	**0.007**	**0.011**
	Diet	Aminoacyl-tRNA biosynthesis	**13**	**<0.001**	**0.005**
		Arginine and proline metabolism	**10**	**<0.001**	**0.005**
		Purine metabolism	**12**	**<0.001**	**0.005**
		Pyrimidine metabolism	**9**	**<0.001**	**0.005**
		Ascorbate and aldarate metabolism	**4**	**0.001**	**0.005**
	Sex-by-strain	Purine metabolism	**14**	**<0.001**	**0.002**
		Pyrimidine metabolism	**9**	**<0.001**	**0.002**
	Sex-by-diet	Glycine, serine and threonine metabolism	**4**	**<0.001**	**0.003**
	Sex-by-strain-by-diet	Purine metabolism	**8**	**<0.001**	**0.002**
Adipose	**Sex**	Aminoacyl-tRNA biosynthesis	**11**	**<0.001**	**0.007**
		Arginine and proline metabolism	**8**	**<0.001**	**0.007**
		Alanine, aspartate and glutamate metabolism	**4**	**0.003**	**0.007**
		Lysine biosynthesis	**2**	**0.004**	**0.007**
		Pantothenate and CoA biosynthesis	**3**	**0.006**	**0.007**

Table 6.3 continued

Tissue	Effect	Pathway	Hits	P-value	(i/m)q
	Strain	Arginine and proline metabolism	10	**<0.001**	**0.011**
		Aminoacyl-tRNA biosynthesis	12	**<0.001**	**0.011**
		Pyrimidine metabolism	7	**0.002**	**0.011**
		Glycine, serine and threonine metabolism	6	**0.002**	**0.011**
		Alanine, aspartate and glutamate metabolism	5	**0.003**	**0.011**
		Ascorbate and aldarate metabolism	3	**0.006**	**0.011**
		Purine metabolism	8	**0.008**	**0.011**
		Citrate cycle (TCA cycle)	4	**0.01**	**0.011**
	Sex-by-Strain	Purine metabolism	8	**<0.001**	**0.005**
		Pyrimidine metabolism	3	**0.002**	**0.005**
		Pantothenate and CoA biosynthesis	2	**0.004**	**0.005**
Muscle	**Sex**	Pyrimidine metabolism	6	**<0.001**	**0.008**
		Arginine and proline metabolism	5	**0.002**	**0.008**
		Aminoacyl-tRNA biosynthesis	6	**0.002**	**0.008**
		Lysine biosynthesis	2	**0.002**	**0.008**
	Strain	Pyrimidine metabolism	11	**<0.001**	**0.011**
		Purine metabolism	13	**<0.001**	**0.011**
		Aminoacyl-tRNA biosynthesis	13	**<0.001**	**0.011**
		Arginine and proline metabolism	10	**<0.001**	**0.011**
		Citrate cycle (TCA cycle)	6	**0.001**	**0.011**
		Ascorbate and aldarate metabolism	4	**0.001**	**0.011**
		D-Glutamine and D-glutamate metabolism	3	**0.002**	**0.011**
		Alanine, aspartate and glutamate metabolism	6	**0.002**	**0.011**
		Glycine, serine and threonine metabolism	6	**0.009**	**0.011**
		Pantothenate and CoA biosynthesis	4	**0.01**	**0.011**
	Diet	Aminoacyl-tRNA biosynthesis	10	**<0.001**	**0.005**
		Lysine biosynthesis	2	**0.001**	**0.005**
Liver	**Strain**	Pyrimidine metabolism	16	**<0.001**	**0.007**
		Purine metabolism	18	**<0.001**	**0.007**
		Alanine, aspartate and glutamate metabolism	9	**<0.001**	**0.007**

Table 6.3 continued

Tissue	Effect	Pathway	Hits	P-value	(i/m)q
		Phenylalanine, tyrosine and tryptophan biosynthesis	3	**0.002**	**0.007**
		Citrate cycle (TCA cycle)	6	**0.003**	**0.007**
		Ascorbate and aldarate metabolism	4	**0.003**	**0.007**
		Pantothenate and CoA biosynthesis	5	**0.004**	**0.007**
	Diet	Purine metabolism	12	**<0.001**	**0.005**
		Aminoacyl-tRNA biosynthesis	12	**<0.001**	**0.005**
		Pyrimidine metabolism	9	**<0.001**	**0.005**
		Arginine and proline metabolism	9	**<0.001**	**0.005**
		Ascorbate and aldarate metabolism	4	**<0.001**	**0.005**
	Sex-by-Strain	Purine metabolism	12	**<0.001**	**0.002**
		Pyrimidine metabolism	7	**0.001**	**0.002**
	Sex-by-Diet	Glycine, serine and threonine metabolism	4	**<0.001**	**0.003**
	Sex-by-Strain-by-Diet	Purine metabolism	7	**<0.001**	**0.002**

The relationships between tissue metabolites and systems level energy balance traits were assessed by constructing a correlation-based network. The network consists of metabolites (21, 19 and 13 from liver, adipose and muscle, respectively) that were significantly correlated with at least one energy balance trait and with one or more metabolites in any of the three tissues (Fig. 6.6, Appendix F). Adiposity was correlated with all 53 metabolites identified. A-ketoglutarate was positively correlated with adiposity in adipose (r=0.34,p<0.001) and muscle tissue (r=0.27). Conversely, α-ketoglutarate was negatively correlated with weight (r=-0.29,p<0.001) in adipose tissue indicating that increased body weight due to increased muscle mass may influence a-ketoglutarate levels. Pantothenate, in adipose, a precursor for CoA biosynthesis, was also negatively correlated with adiposity (r=-0.40,p<0.001). Furthermore, pantothenate was negatively correlated with metabolites identified and muscle and liver, but positively correlated with metabolites identified in adipose tissue with the exception of cysteine. Pantothenate converts to coenzyme A via an enzymatic reaction requiring cysteine, which may explain the inverse relationship. Quinolinate, a potential marker of obesity, was positively correlated with adiposity (r=-.35,p<0.001) in liver tissue and negatively correlated with adiposity in adipose tissue (r=-.33,p<0.001), suggesting a complex relationship between adiposity and tissue type.

6.5 Discussion

Genetics and diet play a major role in health; however, the fundamental relationship between genetics, dietary intake, and metabolism at the tissue level remain

poorly understood. We used an untargeted metabolomics approach to investigate the effects of diet, genetics, and sex on tissue metabolomes, and to relate metabolites to body composition and energy expenditure. Experimental diets were designed to model eating patterns (American, Japanese, Mediterranean, and ketogenic) that are known to affect various aspects of metabolic health in humans. Diet composition varied widely in terms of the sources of ingredients, relative contribution of animal- and plant-based ingredients, and macronutrient and micronutrient composition. Furthermore, differences in genetic background could result in various predispositions to metabolic disease. The combination of diet, genetics, and sex produced a stratified response on the tissue metabolome. This allowed us to query the extent to which tissue metabolomes were influenced by diet, genetics, sex, and their various interactions, and to characterize their relative impact on liver, adipose, and skeletal muscle. Our model, which included 40 unique combinations of diet, genetics, and sex, also enable us to use correlation to relate metabolites to body composition and energy expenditure.

Although sex-by-strain-by-diet exerted effects on weight and energy expenditure phenotypes, a considerable proportion of metabolites were influenced by strain alone. Surprisingly, skeletal muscle was most sensitive to strain (n=40); however, strain exerted effects on adipose (n=34) and liver (n=19) tissue, independent of all other treatments or combinations.
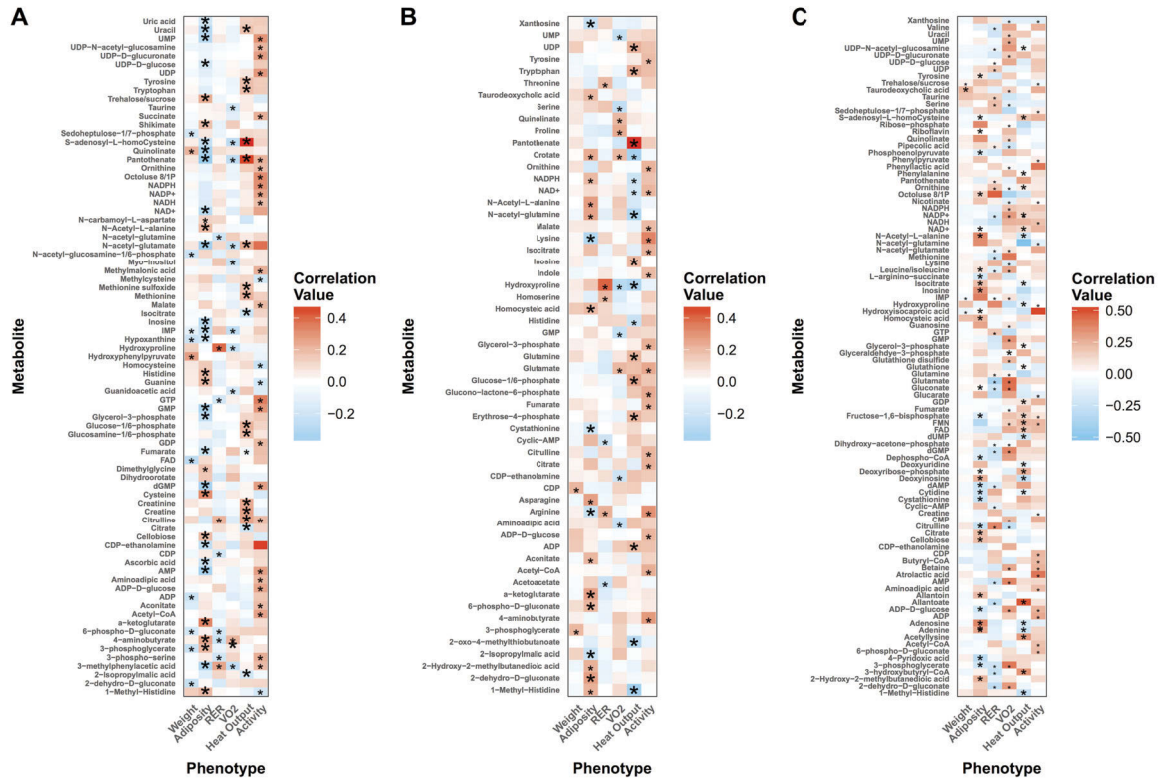
Figure 6.5: Correlations between metabolites and system phenotypes. Pearson correlation was used to associate metabolites in adipose (A), muscle (B), and liver (C) with weight, adiposity, and metabolic phenotypes. Significant correlations (FDR < 0.05) are represented by an asterisk (*). For metabolites that were significantly correlated for adiposity and/or heat output, partial correlation was used to determine if the relationship between metabolite and trait was due to spurious correlation with body weight. Correlations that remained significant are indicated by a bold asterisk (*).
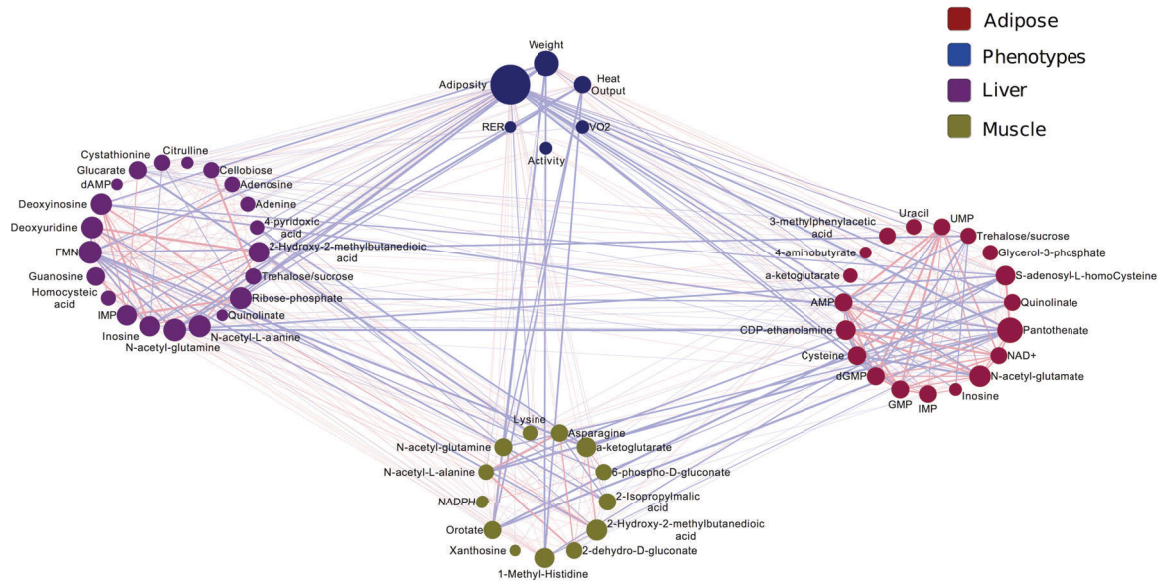
Figure 6.6: Cross-tissue metabolite and phenotype network. Pearson correlation was used to associate metabolites within and across each tissue with weight, adiposity, and metabolic phenotypes. Only significant correlations (FDR < 0.05) are shown. To assess relationships within and across tissues and phenotypes, metabolites are clustered by their respective tissue or phenotype. The size of each node represents the number of edges connected to the node. A red line represents positively correlated metabolites, while a blue line represents negatively correlated metabolites.

Metabolites affected by strain and diet, in muscle, were involved in several aspects of amino acid metabolism (aminoacyl-tRNA biosynthesis, arginine and proline metabolism, glutamine and glutamate metabolism, alanine, aspartate, and glutamate metabolism). Strain altered 15 of the 20 amino acids while diet altered 11 in muscle. The percentage of protein in each diet varied considerably (~13-19%), which may be attributable to amino acid differences seen across diets. Additionally, variations in carbohydrate and fat levels may also contribute to diet-associated differences in amino acid profiles. Genetic effects on amino acid metabolism have been linked to metabolic diseases, such as type 2 diabetes. Additionally, GWAS studies have attributed genetic variants to BCAA levels. Specifically PPM1K, which activates the branched-chain alpha-ketoacid complex via mitochondrial phosphatase and serves as the rate limiting to BCAA catabolism was positively associated with BCAA levels [210]. Although we cannot comment specifically about PPM1K in our study since we did not perform genetic sequencing this relationship warrants further investigation. Furthermore, insulin resistance in humans has been attributable to alterations in BCAA levels in muscle, as well as TCA cycle intermediates [211]. Methylmalonyl CoA mutase may play a role in BCAA availability for use in the TCA cycle and is linked to altered lipid metabolism in muscle. Methylmalonyl CoA is converted to succinyl CoA via this enzyme, which then enters the TCA cycle. FVB/NJ mice showed 434 SNP variations compared to the other strains, using the mouse phenome database, for methylmalonyl-CoA mutase. Homozygous mutations in this gene can cause increased levels of methylmalonic acid in plasma potentially resulting in methylmalonic aciduria, a severe metabolic disease [211]. While we did not measure metabolites in plasma we did find significantly

increased levels methylmalonic acid between strains for muscle. Whether or not tissue levels of methylmalonic acid play a role in metabolic dysfunction cannot be determined in the current study. Further studies are needed to determine the role of methylmalonic acid on disease. Additionally, amino acid metabolism was also affected by sex and strain in adipose, sex in muscle, and strain, diet, and sex-by-diet in liver tissue.

In contrast to strain, few metabolites responded to sex-by-strain-by-diet interaction. Despite this, sex-by-strain-by-diet interaction influenced purine metabolism in liver. Purine metabolism includes the synthesis and breakdown of purine nucleotides, which are building blocks of nucleic acids and cofactors that are required for energy metabolism. While these functions are fundamentally important for all cells, recent studies have associated alterations in this pathway with gene mutations and metabolic disease. Gout is a disease characterized by a build up of uric acid in the blood. Genome wide association studies have related genetic variants in GLUT9, URAT1, and ABCG2 to prevention or progression of gout [212]. Assessing the role of GLUT9 in males and females from Sardinia and Chianti, Tuscany revealed that SNP rs6855911 had the strongest association with uric acid levels, lowering uric acid levels in individuals from both cohorts with this allele [213]. Furthermore, gender contributed to differences in uric acid levels. Women with this allele had even lower levels of uric acid than men [213]. Although this study did not investigate the role of dietary components on metabolism it has been well established that diet contributes purine metabolite levels [214-216]. Diets containing animal products contributed to higher purine levels compared to other diets [216]. Furthermore, men have a higher risk of developing gout due to higher blood

levels of uric acid compared to women [216]. Seven metabolites in our study were mapped onto purine metabolism; however, uric acid levels were not significantly altered.

Tryptophan is an essential amino acid important for protein synthesis and the biosynthesis of serotonin and melatonin, two metabolites that aid in the regulation of mood and sleep [217]. Furthermore, tryptophan can be broken down into kynurenine, kynurenic acid, and quinolinate. The obesogenic state in humans has been associated with these three metabolites [218]. Our data identified tryptophan, kynurenine, kynurenic acid, and quinolinate in liver tissue displaying differences due to diet. Furthermore, associations with phenotypic markers of metabolism showed a positive correlation between quinolinate and adiposity, independent of body weight for both adipose and liver tissue. In contrast, kynurenine and kynurenic acid were not significantly correlated with any phenotype. Correlation network analysis also revealed a positive associate between quinolinate, in liver, and a negative correlation, in adipose tissue, with adiposity. Although this dual relationship with adiposity cannot be completely explained previous studies have associated quinolinate levels in the liver with toxicity [219]. Additionally, quinolinate is the precursor to NAD+ formation [220]. NAD+ is necessary for the TCA cycle to function, however, both the TCA cycle and NAD+ levels have been shown to decrease as adiposity increases [221], so the inverse relationship between quinolinate and adiposity in adipose tissue cannot be explained by increased NAD+ levels. Furthermore, our data showed no correlation between NAD+ and quinolinate for the correlation network analysis or between quinolinate and NAD+ in adipose tissue. It is possible that quinolinate was converted to nicotinamide, the intermediary metabolite

between quinolinate and NAD+; however, we did not measure this metabolite in our system. Measuring this metabolite may provide critical information regarding this relationship. Additionally, quinolinate phosphoribosyltransferase (QPRT) is necessary for the conversion of quinolinate to NAD+. Previous studies have shown a negative relationship between QPRT expression and markers of diabetes [218]. It is possible that QPRT expression was altered in our study; however, gene expression was not measured. Further investigation into the relationship between adiposity, quinolinate, and QPRT are warranted to further define this relationship. In addition to diet, quinolinate was significant for strain in all three tissues. All four strains differentially respond to diet-induced obesity. Diet-induced obesity resistant A/J mice are had the lowest levels of quinolinate.

Several limitations of our study should be emphasized. In particular, the diets were designed to reflect common patterns of global consumption in humans. Accordingly, the range of variation in diet composition prevents us from associating specific dietary factors with metabolite patterns. We have also measured metabolites in a steady state, at a single time point. Effects of diet, genetics, or sex on metabolite turnover therefore go undetected in our study. Furthermore, the extraction method we used targets polar metabolites at the expense of lipids, which are also likely subject to extensive sex, genetic, and diet regulation. Finally, due to the specificity of the extraction method and the limited number of metabolites that can be identified using our in-house database, it is difficult to ascertain whether pathways significantly altered are

affected by sex, strain, diet, or their interactions or because a metabolite is more easily identified to due inherent biases in the experimental procedure.

6.6 Conclusion

We profiled three tissues (adipose, muscle, and liver), which play a major role in energy metabolism to determine the effects of genetics, diet, and sex on the tissue metabolome. The stratified results showed many tissue specific effects, however, amino acids metabolism was universally affected. Although this study comprehensively profiles the interactions between genetic, sex, and diet, serum metabolites were not measured. Profiling serum metabolites may provide insight into the global metabolic profile. Nonetheless, this study has shown several metabolites that could be investigated to determine their role in metabolism and disease.

CHAPTER SEVEN

CONCLUSION

Mass spectrometry based metabolomics is in its infancy but is quickly becoming a powerful tool to elucidate the metabolic effects of small perturbations in a system. Many studies have shown that this method is able identify potential biomarkers of disease. This dissertation had three aims: 1.) characterize the relative effects of genetics, sex, diet, and their interactions on tissue metabolomes, 2.) identify tissue-level metabolic effects of diets that are commonly recommended for health, and 3.) link tissue metabolite profiles to systems phenotypes relevant to metabolic syndrome. To accomplish these aims adipose, skeletal muscle, and liver tissue were collected from males and females of four strains of mice (A/J, C57BL/6J, FVB/NJ, and NOD/ShiLtJ) fed one of five diets (Japanese, ketogenic, Mediterranean, American, and standard chow). The tissues were chosen due to their known role in energy metabolism, while the diets were chosen for their known roles in health. The strains of mice were chosen for their genetic diversity. The first aim allowed us to identify metabolites sensitive to differences in the system due to discrepancies in diet, sex, or genetic background. This allowed us to query the extent to which diet, in the presence or absence of genetic or sex differences, influenced the tissue metabolome. The second aim allowed us to map altered metabolites onto KEGG pathways to query the biological role on individual metabolites and their role in metabolic pathways, which may influence health status. The third aim allowed us find associations between phenotypic metabolic data and altered metabolites in the body, which provides insight into the mechanism through which metabolites influence physiological responses.

In all three studies we examined each tissue metabolome across genetic, sex and/or diet effects. In addition to metabolome measurements we were provided

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measurements pertaining to body weight, adiposity, RER, VO$_2$, heat output, and activity levels. In the first study, we examined each tissue metabolome in C57BL/6J males and females fed all five diets for 7 months. In the second study, we examined each tissue metabolome in males and females for all four strains of mice fed a standard chow diet for 7 months. In the third study, we examined each tissue metabolome in males and females for all four strains of mice fed all five diets for 7 months.

Metabolism is fundamental to life and plays a critical role in the health and the well being of cells, tissues, and overall biological systems. Fluctuations in metabolism can have profound physiological impacts contributing to metabolic diseases. Moreover, metabolism is often sensitive to environmental stimuli, such as diet and lifestyle [112, 222-225] while also being sensitive to genetic differences and gender [177, 210, 226, 227]. Genetic mutations, immunological responses, and protein conformation changes have been much of the focus over the last 50 years when assessing disease and potential treatments, however, alterations in the metabolome have gained increasing attention in recent years. Furthermore, the ability to integrate of genomics, transcriptomics, proteomics, and metabolomics, provides a means with which to develop a comprehensive understanding of how the body may change at the cellular, tissue, and systems-level to promote a disease state. While the genetic information can provide information regarding an individual's potential for developing a disease, the metabolome can provide information with respect to the beginning stages of a disease. Aminoadipic acid, for example, has been linked to the development of type 2 diabetes with higher levels circulating in the blood up to 12 years prior to the disease state [122]. Additionally, while this dissertation has shown tissue specific effects on the

metabolome, which should be taken into consideration, serum and urine metabolomes can be non-invasively profiled making them an ideal candidate for long-term surveillance for disease risk. Most metabolomics studies currently aim to identify metabolic differences between the diseased and non-diseased state, however, few have aimed to identify baseline differences between, diet, genetic, or sex differences. The work presented in this dissertation contributes to the later body of work. A disease state is difficult to interpret without understanding the "normal" state, therefore a basic understanding of how diet influences the metabolome under normal physiological conditions can aid in the understanding of which diet may improve symptoms and which may exacerbate symptoms. Furthermore, the environment can influence genetic predisposition to certain diseases. Understanding how basic genetic differences translate to the metabolome can provide a basic understanding of how the metabolome is influenced due to inherent genetic differences. Finally, many studies that have studied the disease state have primarily investigated these effects in men. Recently, an influx of studies have included women, unsurprisingly discovering that while women develop the same diseases as men their symptoms, treatments, and outcomes can be vastly different. While much of this has been attributed to sex hormones, investigating how the metabolome differs between genders is critical to identifying inherent differences. It may be discovered that a metabolite biomarker may be appropriate for one gender but not the other. Our research has demonstrated that specific metabolites differentiate by sex, while others are perturb through differences in diet (presented in chapter 3). Furthermore, there is a subset of metabolites that are influenced by the interaction between diet and gender. When we considered genetic background we demonstrated

that genetic background was far more influential to alterations in the metabolome than either gender or the interaction between gender and genetics underscoring the importance of genetics on metabolism (presented in chapter 4). Finally, when we considered the influence of diet, genetics, and sex simultaneously we demonstrated that genetics had the largest impact across all potential combinations with gender playing a secondary role, however, sex-by-strain-by diet interaction influenced a subset of metabolites (presented in chapter 5). It is important to note that metabolites had a stratified response across all factors and their interactions emphasizing the complex relationship between diet, genetics, and sex. It must also be pointed out, however, that diet had the least impact on the metabolome; however, differences in diet still impacted the metabolome in combination with gender and genetic background.

Metabolomics has become a powerful tool to investigate systems-level alterations that are the result of many biochemical reactions. While metabolomics is still in its infancy technological improvements have allowed for significant advancements in recent years. However, metabolomics studies still remain expensive ant time consuming. Furthermore, no one extraction method can extract all the metabolites present in a system requiring multiple extraction methods and a large quantity of sample to obtain comprehensive cover. Additionally, there are thousands of metabolites that have yet to be identified creating a large gap in knowledge regarding the biological role these metabolites have in the body. Accordingly, unknown metabolites also present a challenge because they can only be identified by their mass-to-charge ratio making it difficult to separate whole metabolites from fragments. Finally, since metabolomics is a young field, repositories and databases are still being formed to aid in the identification

and functional analysis of metabolites. The lack of databases and repositories has made it difficult to share raw spectral data and made it necessary for researchers to build their own internal databases and has made it nearly impossible to identify unknown analytes.

Metabolomics has flourished since the development of liquid chromatography, allowing for the identification of larger molecules. As the field continues to mature researchers will be able to investigate more complex biological questions. This dissertation shows the capacity with which metabolomics has to address complex relationships in a biological system. Diet has long been a complex topic to investigate due to the complex nature of diet composition and the many confounding variable, such as diet and sex, as well as potential lifestyle components. This dissertation has contributed to understanding the complex relationship between diet, genetics, and sex and their influence on the tissue metabolome. This has highlighted the potential of metabolomics to dissect nuanced differences among the interactions. As metabolomic analyses continue to increased in popularity, our understanding of the metabolome's contribution to disease and will continue to deepen.

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APPENDIX

APPENDIX A

Table A.1: Significant ANOVA p-values (FDR < 0.05) for Adipose, Muscle, and Liver Tissue in C57BL/6J Mice

		Sex	(i/m)q	Diet	(i/m)q	Sex-by-Diet	(i/m)q
Liver	1-Methyl-Histidine	**0.0001**	**0.0021**	0.1715	0.0291	0.0807	0.0166
	1-Methyladenosine	0.0962	0.0179	**0.0011**	**0.0064**	0.7719	0.0454
	2-dehydro-D-gluconate	**0.0001**	**0.0021**	**0.0006**	**0.0055**	0.0651	0.0148
	3-hydroxybutyryl-CoA	0.0118	0.0080	**0.0031**	**0.0086**	0.0867	0.0172
	3-S-methylthiopropionate	**0.0054**	**0.0059**	0.0081	0.0114	0.1148	0.0195
	4-Pyridoxic acid	0.1508	0.0215	**0.0049**	**0.0099**	**0.0008**	**0.0018**
	6-phospho-D-gluconate	**0.0000**	**0.0012**	**0.0000**	**0.0001**	0.0705	0.0154
	Acetyl-CoA	**0.0048**	**0.0056**	**0.0063**	**0.0107**	0.2313	0.0264
	Acetyllysine	**0.0000**	**0.0007**	0.6248	0.0441	0.0652	0.0149
	Adenosine	0.1108	0.0189	**0.0019**	**0.0076**	**0.0007**	**0.0017**
	ADP-D-glucose	**0.0056**	**0.0060**	0.2238	0.0317	0.2064	0.0252
	Allantoate	**0.0000**	**0.0002**	0.0283	0.0165	0.0751	0.0160
	Aminoadipic acid	**0.0000**	**0.0011**	**0.0007**	**0.0057**	0.0735	0.0157
	AMP	0.9185	0.0479	**0.0014**	**0.0069**	**0.0038**	**0.0039**
	Asparagine	0.0103	0.0077	**0.0084**	**0.0116**	**0.0006**	**0.0016**
	Betaine	0.6662	0.0413	**0.0002**	**0.0042**	**0.0000**	**0.0004**
	Carnitine	0.5131	0.0365	0.8455	0.0480	**0.0034**	**0.0037**
	Citrulline	0.0062	0.0061	**0.0042**	**0.0094**	**0.0034**	**0.0037**
	Coenzyme A	**0.0047**	**0.0056**	0.0207	0.0151	0.1875	0.0241
	Creatine	0.5157	0.0367	**0.0020**	**0.0078**	0.0048	0.0043
	Cyclic-AMP	**0.0006**	**0.0030**	0.0710	0.0221	0.1013	0.0184
	Deoxyinosine	0.2198	0.0254	**0.0047**	**0.0097**	**0.0003**	**0.0011**
	Deoxyribose-phosphate	**0.0000**	**0.0014**	**0.0010**	**0.0062**	0.2634	0.0280
	Deoxyuridine	0.5261	0.0370	**0.0001**	**0.0034**	**0.0001**	**0.0009**
	Dephospho-CoA	0.9076	0.0477	**0.0000**	**0.0031**	0.0097	0.0059
	dGMP	0.9185	0.0479	**0.0014**	**0.0069**	**0.0038**	**0.0039**
	Dihydroxy-acetone-phosphate	**0.0002**	**0.0024**	0.7020	0.0455	0.0043	0.0042
	FAD	**0.0005**	**0.0029**	0.0279	0.0164	0.5207	0.0389
	Fructose-1/6-bisphosphate	**0.0004**	**0.0028**	0.1198	0.0259	0.2956	0.0294
	Fumarate	0.2785	0.0280	**0.0001**	**0.0033**	0.1204	0.0198
	Glucarate	0.0083	0.0069	**0.0018**	**0.0076**	0.0362	0.0110
	Gluconate	**0.0000**	**0.0006**	**0.0013**	**0.0068**	**0.0013**	**0.0023**
	Glucose-1/6-phosphate	0.5329	0.0372	**0.0000**	**0.0023**	0.0079	0.0054

Table A continued

	Sex	(i/m)q	Diet	(i/m)q	Sex-by-Diet	(i/m)q
Glutamate	0.6724	0.0415	**0.0000**	**0.0030**	0.0156	0.0073
Glutamine	**0.0000**	**0.0013**	**0.0003**	**0.0048**	0.0185	0.0079
Glutathione	0.5254	0.0369	0.4719	0.0403	**0.0006**	**0.0017**
Glyceraldehdye-3-phosphate	**0.0002**	**0.0024**	0.7020	0.0455	0.0043	0.0042
Glycerol-3-phosphate	0.2279	0.0258	**0.0009**	**0.0060**	0.0077	0.0053
GMP	0.7821	0.0442	**0.0004**	**0.0050**	0.6924	0.0434
Guanine	**0.0055**	**0.0059**	0.9039	0.0488	0.0540	0.0134
Histidine	0.0451	0.0132	**0.0015**	**0.0071**	0.8798	0.0478
Homocysteic acid	**0.0021**	**0.0044**	**0.0000**	**0.0022**	0.1993	0.0247
Homoserine	**0.0001**	**0.0020**	0.5854	0.0431	0.0079	0.0054
Hydroxyisocaproic acid	**0.0001**	**0.0019**	0.0872	0.0236	0.0784	0.0163
Hydroxyproline	0.1762	0.0231	**0.0000**	**0.0021**	0.0886	0.0174
Hypoxanthine	0.7102	0.0422	**0.0000**	**0.0023**	**0.0003**	**0.0012**
IMP	**0.0043**	**0.0055**	0.7088	0.0456	**0.0007**	**0.0018**
Inosine	0.0404	0.0124	0.0591	0.0209	**0.0003**	**0.0012**
Leucine/isoleucine	0.0733	0.0160	0.0158	0.0140	**0.0002**	**0.0010**
Lysine	0.1135	0.0191	**0.0007**	**0.0057**	0.0019	0.0028
Methionine	**0.0053**	**0.0059**	**0.0000**	**0.0015**	0.0169	0.0076
N-acetyl-glutamine	**0.0000**	**0.0007**	**0.0015**	**0.0071**	0.0465	0.0125
N-Acetyl-L-alanine	**0.0000**	**0.0010**	0.0604	0.0210	0.0128	0.0067
N-acetyl-L-ornithine	0.3087	0.0291	**0.0034**	**0.0089**	0.7569	0.0451
NADH	**0.0005**	**0.0029**	**0.0083**	**0.0116**	0.4639	0.0368
Octulose 8/1P	0.6719	0.0414	**0.0000**	**0.0004**	0.0722	0.0155
Ornithine	0.2097	0.0249	0.0638	0.0213	**0.0015**	**0.0025**
Pantothenate	0.0511	0.0138	0.0232	0.0156	**0.0026**	**0.0032**
Proline	0.0078	0.0067	**0.0036**	**0.0090**	0.0024	0.0031
Riboflavin	**0.0000**	**0.0017**	0.0769	0.0227	0.0068	0.0050
Ribose-phosphate	**0.0001**	**0.0021**	0.2206	0.0316	0.0102	0.0061
Sedoheptulose bisphosphate	**0.0010**	**0.0035**	0.0637	0.0213	0.0633	0.0145
Sedoheptulose-1/7-phosphate	0.1508	0.0215	**0.0000**	**0.0015**	0.0199	0.0082
Serine	0.0077	0.0067	**0.0000**	**0.0019**	**0.0008**	**0.0019**
Taurine	0.1122	0.0190	0.0450	0.0191	**0.0024**	**0.0031**
Taurodeoxycholic acid	**0.0017**	**0.0041**	0.0422	0.0187	**0.0006**	**0.0016**
Threonine	**0.0001**	**0.0020**	0.5854	0.0431	0.0079	0.0054
Tryptophan	0.0758	0.0162	**0.0024**	**0.0080**	0.0670	0.0150
UDP-D-glucuronate	0.1355	0.0206	**0.0051**	**0.0101**	0.0703	0.0154
Uracil	**0.0029**	**0.0049**	**0.0056**	**0.0104**	0.3383	0.0316
Uridine	**0.0000**	**0.0005**	0.1519	0.0279	0.0165	0.0075
Valine	0.6864	0.0417	**0.0002**	**0.0041**	**0.0000**	**0.0004**

Table A continued

		Sex	(i/m)q	Diet	(i/m)q	Sex-by-Diet	(i/m)q
	Xanthine	0.8622	0.0466	**0.0034**	**0.0089**	**0.0000**	**0.0006**
	Xanthosine	0.1669	0.0225	**0.0014**	**0.0070**	**0.0001**	**0.0007**
	Xanthurenic acid	0.0230	0.0101	**0.0122**	**0.0130**	0.1089	0.0190
Muscle	**Acetyllysine**	**0.0028**	**0.0032**	**0.0005**	**0.0009**	0.5030	0.0315
	Aminoadipic acid	**0.0000**	**0.0012**	**0.0000**	**0.0004**	0.0349	0.0048
	Deoxyribose-phosphate	**0.0008**	**0.0022**	0.0450	0.0072	0.3385	0.0242
	Deoxyuridine	**0.0002**	**0.0017**	0.3868	0.0285	0.0028	0.0007
	Erythrose-4-phosphate	**0.0005**	**0.0020**	**0.0006**	**0.0010**	0.0079	0.0016
	Glucose-1/6-phosphate	**0.0007**	**0.0021**	0.0066	0.0026	0.0310	0.0045
	Histidine	0.6679	0.0383	**0.0008**	**0.0011**	0.0009	0.0003
	Homocysteic acid	0.0058	0.0038	**0.0000**	**0.0002**	0.0009	0.0003
	Homoserine	0.6309	0.0369	**0.0000**	**0.0004**	0.0139	0.0025
	Hydroxyphenylacetic acid	**0.0011**	**0.0024**	0.5603	0.0359	0.1858	0.0157
	Hydroxyproline	0.0059	0.0038	**0.0000**	**0.0000**	0.0003	0.0001
	Kynurenine	**0.0004**	**0.0018**	0.6971	0.0411	0.8638	0.0457
	Methionine	**0.0026**	**0.0031**	0.0084	0.0029	0.2760	0.0210
	N-acetyl-glutamine	**0.0002**	**0.0016**	0.9717	0.0493	0.0400	0.0053
	N-Acetyl-L-alanine	**0.0029**	**0.0032**	0.7999	0.0443	0.3076	0.0227
	Orotate	**0.0000**	**0.0009**	0.9987	0.0500	0.0750	0.0082
	Pantothenate	**0.0000**	**0.0003**	0.0116	0.0032	0.0969	0.0099
	Proline	0.5518	0.0338	**0.0013**	**0.0014**	0.0993	0.0101
	Threonine	0.5957	0.0356	**0.0000**	**0.0004**	0.0126	0.0023
	Tryptophan	**0.0007**	**0.0021**	**0.0006**	**0.0010**	0.0091	0.0018
	Valine	0.6530	0.0377	**0.0006**	**0.0010**	0.0482	0.0060
Adipose	**1-Methyladenosine**	**0.0008**	**0.0010**	0.2280	0.0226	0.0649	0.0063
	a-ketoglutarate	0.0196	0.0035	**0.0007**	**0.0009**	0.5926	0.0336
	Adenosine	**0.0001**	**0.0006**	0.0244	0.0061	0.1696	0.0127
	Citrulline	**0.0008**	**0.0010**	0.0958	0.0134	0.1980	0.0142
	Glucosamine-1/6-phosphate	0.2739	0.0197	**0.0008**	**0.0010**	**0.0001**	**0.0001**
	Glutamine	0.8048	0.0424	0.002	0.0017	**0.0000**	**0.0000**
	Guanosine	**0.0001**	**0.0005**	0.0819	0.0121	0.1541	0.0119
	Hydroxyproline	0.8013	0.0422	**0.0000**	**0.0001**	0.3356	0.0219
	Inosine	**0.0007**	**0.0010**	0.1627	0.0183	0.8314	0.0438
	N-acetyl-glutamate	**0.0000**	**0.0004**	0.0150	0.0046	0.1040	0.0088
	Pantothenate	**0.0000**	**0.0001**	0.1033	0.0140	0.5085	0.0300
	Proline	0.0053	0.0018	0.0011	0.0011	**0.0000**	**0.0001**
	S-adenosyl-L-homoCysteine	**0.0000**	**0.0002**	0.0798	0.0118	0.3760	0.0239

All known metabolites considered significant in the ANOVA were identified in liver, skeletal muscle, and adipose tissue. Bold values indicate that the metabolite is statistically significant. Bold metabolites are not tissue specific. Bold and italicized metabolites are shared between all three tissues. Benjamini-Hochberg procedure was performed to determine significance.

APPENDIX B

Table B.2: Number of Edges for Each Node in Correlation Network Analysis in C57BL/6J Mice

Metabolite	Tissue	Total Degree (+/-)	Degree (Adipose) (+/-)	Degree (Muscle) (+/-)	Degree (Liver) (+/-)	Degree (Phenotype) (+/-)
α-ketoglutarate	Adipose	6 (2/4)	1 (0/1)	0	3 (0/3)	2 (2/0)
Adenosine	Adipose	6 (3/3)	0	1 (0/1)	4 (3/1)	1 (0/1)
ADP	Adipose	3 (2/1)	1 (1/0)	0	1 (1/0)	1 (0/1)
IMP	Adipose	3 (3/0)	1 (1/0)	0	1 (1/0)	1 (1/0)
Myo-inositol	Adipose	3 (2/1)	1 (1/0)	0	2 (1/1)	0
N-acetyl-glutamate	Adipose	9 (5/4)	4 (3/1)	1 (1/0)	2 (1/1)	2 (0/2)
Pantothenate	Adipose	14 (9/5)	2 (2/0)	2 (1/1)	8 (5/3)	2 (1/1)
S-adenosyl-L-homoCysteine	Adipose	6 (5/1)	1 (1/0)	1 (1/0)	2 (1/1)	2 (2/0)
3-S-methylthiopropionate	Liver	5 (1/4)	0	0	4 (1/3)	1 (0/1)
6-phospho-D-gluconate	Liver	9 (7/2)	0	0	8 (6/2)	1 (1/0)
Acetyllysine	Liver	6 (5/1)	1 (1/0)	1 (1/0)	3 (2/1)	1 (1/0)
Adenosine	Liver	4 (2/2)	0	0	2 (1/1)	2 (1/1)
ADP-D-glucose	Liver	12 (10/2)	0	0	10 (9/1)	2 (1/1)
Allantoate	Liver	11 (8/3)	3 (2/1)	1 (1/0)	6 (4/2)	1 (1/0)
Aminoadipic acid	Liver	13 (9/4)	2 (2/0)	1 (1/0)	8 (5/3)	2 (1/1)
AMP	Liver	4 (3/1)	0	0	3 (3/0)	1 (0/1)
ATP	Liver	11 (9/2)	0	0	10 (7/2)	1 (1/0)
Butyryl-CoA	Liver	11 (8/3)	2 (2/0)	1 (1/0)	7 (4/3)	1 (1/0)
CDP	Liver	7 (7/0)	0	0	6 (6/0)	1 (1/0)
Citrulline	Liver	5 (2/3)	1 (0/1)	0	2 (1/1)	2 (1/1)
Creatine	Liver	3 (3/0)	0	0	2 (2/0)	1 (1/0)
Cytidine	Liver	5 (2/3)	2 (1/1)	0	2 (1/1)	1 (0/1)
Deoxyuridine	Liver	5 (2/3)	1 (0/1)	0	2 (2/0)	2 (0/2)
Dephospho-CoA	Liver	6 (3/3)	0	1 (0/1)	4 (3/1)	1 (0/1)
dGMP	Liver	4 (3/1)	0	0	3 (3/0)	1 (0/1)
FAD	Liver	13 (10/3)	0	0	12 (9/3)	1 (1/0)
Fructose-1,6-bisphosphate	Liver	11 (9/2)	0	0	10 (8/2)	1 (1/0)
GDP	Liver	10 (10/0)	0	1 (1/0)	8 (8/0)	1 (1/0)
Glucarate	Liver	2 (1/1)	0	1 (0/1)	0	1 (1/0)
Gluconate	Liver	8 (4/4)	1 (1/0)	1 (0/1)	5 (3/2)	1 (0/1)
Glutamine	Liver	12 (6/6)	1 (1/0)	0	10 (5/5)	1 (0/1)
GMP	Liver	5 (4/1)	0	0	4 (4/0)	1 (0/1)
Guanosine	Liver	6 (5/1)	0	0	4 (3/1)	2 (2/0)

Table B continued

Metabolite	Tissue	Total Degree (+/-)	Degree (Adipose) (+/-)	Degree (Muscle) (+/-)	Degree (Liver) (+/-)	Degree (Phenotype) (+/-)
Hydroxyisocaproic acid	Liver	3 (2/1)	0	0	2 (2/0)	1 (0/1)
IMP	Liver	3 (3/0)	0	0	2 (2/0)	1 (1/0)
Inosine	Liver	8 (8/0)	0	0	7 (7/0)	1 (1/0)
Methionine	Liver	11 (3/8)	1 (0/1)	2 (0/2)	7 (3/4)	1 (0/1)
N-acetyl-glutamine	Liver	13 (5/8)	4 (1/3)	1 (0/1)	7 (4/3)	1 (0/1)
N-Acetyl-L-alanine	Liver	13 (10/3)	1 (0/1)	2 (1/1)	8 (8/0)	2 (1/1)
NADH	Liver	8 (7/1)	1 (1/0)	0	6 (5/1)	1 (1/0)
Octulose 8/1P	Liver	4 (2/2)	1 (1/0)	0	2 (0/2)	1 (1/0)
Riboflavin	Liver	9 (9/0)	0	0	8 (8/0)	1 (1/0)
Ribose-phosphate	Liver	6 (6/0)	0	0	5 (5/0)	1 (1/0)
Sedoheptulose bisphosphate	Liver	10 (9/1)	0	0	9 (8/1)	1 (1/0)
Taurine	Liver	3 (3/0)	0	0	2 (2/0)	1 (1/0)
UDP-N-acetyl-glucosamine	Liver	9 (9/0)	0	0	8 (8/0)	1 (1/0)
Uridine	Liver	10 (5/5)	1 (0/1)	1 (1/0)	7 (4/3)	1 (0/1)
Arginine	Muscle	5 (3/2)	0	2 (2/0)	2 (0/2)	1 (1/0)
Histidine	Muscle	3 (3/0)	0	2 (2/0)	0	1 (1/0)
Lysine	Muscle	5 (2/3)	0	2 (2/0)	2 (0/2)	1 (0/1)
Orotate	Muscle	4 (2/2)	1 (0/1)	0	2 (2/0)	1 (0/1)
Pantothenate	Muscle	12 (8/4)	4 (3/1)	0	7 (4/3)	1 (1/0)
Activity	Phenotype	7 (6/1)	0	0	7 (6/1)	0
Adiposity	Phenotype	15 (9/6)	2 (1/1)	1 (0/1)	11 (7/4)	1 (1/0)
Heat Output	Phenotype	19 (10/9)	3 (2/1)	2 (1/1)	13 (7/6)	1 (0/1)
RER	Phenotype	12 (6/6)	1 (1/0)	2 (2/0)	9 (3/6)	0
Weight	Phenotype	11 (5/6)	4 (1/3)	0	5 (3/2)	2 (1/1)

APPENDIX C

Table C.3: Significant ANOVA p-values (FDR < 0.05) for Adipose, Muscle, and Liver Tissue in Mice Fed a Standard Chow Diet

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Sex-by-Strain	(i/m)q
Liver	1-Methyladenosine	0.3736	0.0292	**0.0143**	0.0265	0.2465	0.0262
	2-dehydro-D-gluconate	0.7035	0.0408	**0.0000**	0.0050	**0.0001**	0.0008
	2-Hydroxy-2-methylbutanedioic acid	**0.0044**	0.0049	**0.0001**	0.0076	0.1543	0.0209
	2-Isopropylmalic acid	0.1751	0.0204	**0.0008**	0.0141	0.0861	0.0157
	3-hydroxybutyryl-CoA	0.0089	0.0063	**0.0007**	0.0135	0.7803	0.0447
	3-methylphenylacetic acid	**0.0033**	0.0045	0.4894	0.0467	0.3394	0.0305
	3-phosphoglycerate	0.5168	0.0345	**0.0000**	0.0053	0.7237	0.0434
	3-S-methylthiopropionate	0.2107	0.0223	**0.0298**	0.0305	0.2288	0.0252
	4-Pyridoxic acid	0.0377	0.0108	**0.0002**	0.0096	0.0235	0.0089
	6-phospho-D-gluconate	0.0739	0.0140	**0.0000**	0.0020	**0.0030**	0.0038
	Acetyl-CoA	**0.0000**	0.0002	**0.0000**	0.0070	0.0094	0.0060
	Acetyllysine	**0.0019**	0.0038	0.4096	0.0456	0.5880	0.0397
	Aconitate	0.2581	0.0243	**0.0264**	0.0299	0.4087	0.0330
	Adenine	0.8649	0.0459	**0.0225**	0.0290	0.2669	0.0272
	Adenosine	0.0219	0.0089	**0.0000**	0.0072	0.0554	0.0131
	Adenosine 5-phosphosulfate	0.3379	0.0279	**0.0142**	0.0264	0.9104	0.0479
	ADP	**0.0002**	0.0018	**0.0000**	0.0037	0.0064	0.0052
	ADP-D-glucose	0.0098	0.0066	**0.0000**	0.0042	0.0235	0.0089
	Alanine	0.1970	0.0215	**0.0003**	0.0111	0.1201	0.0186
	Allantoate	**0.0001**	0.0016	0.0516	0.0337	**0.0042**	0.0044
	Allantoin	0.3929	0.0298	**0.0007**	0.0136	0.2942	0.0286
	Aminoadipic acid	**0.0010**	0.0030	**0.0004**	0.0116	0.8011	0.0453
	Aminoimidazole carboxamide ribonucleotide	0.8912	0.0467	**0.0057**	0.0220	0.0334	0.0104
	AMP	0.9086	0.0472	**0.0000**	0.0005	0.0387	0.0110
	Aspartate	0.0267	0.0094	**0.0001**	0.0084	0.0803	0.0153
	ATP	**0.0017**	0.0037	**0.0002**	0.0099	0.1085	0.0175
	Atrolactic acid	**0.0004**	0.0023	**0.0026**	0.0186	0.4953	0.0364
	Betaine	**0.0005**	0.0024	**0.0008**	0.0141	0.1546	0.0210
	CDP	0.0196	0.0085	**0.0004**	0.0116	**0.0032**	0.0040
	CDP-ethanolamine	0.5596	0.0361	**0.0001**	0.0091	**0.0009**	0.0024
	Citrate	0.6513	0.0392	**0.0085**	0.0240	0.2581	0.0268
	Citrate/isocitrate	0.6513	0.0393	**0.0085**	0.0240	0.2581	0.0268

Table C continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Sex-by-Strain	(i/m)q
	Citrulline	0.0284	0.0097	0.0809	0.0363	**0.0042**	0.0044
	CMP	0.1913	0.0211	**0.0000**	0.0015	0.4067	0.0330
	Coenzyme A	0.1205	0.0174	**0.0018**	0.0171	0.1290	0.0191
	Creatine	0.3737	0.0292	**0.0000**	0.0073	0.8408	0.0463
	Cyclic-AMP	0.0374	0.0108	**0.0000**	0.0006	0.0390	0.0111
	Cystathionine	0.1029	0.0162	**0.0002**	0.0096	0.3140	0.0294
	dCMP	0.0385	0.0109	**0.0020**	0.0175	0.2021	0.0238
	Deoxyinosine	**0.0003**	0.0021	**0.0006**	0.0131	0.0426	0.0115
	Deoxyribose-phosphate	**0.0036**	0.0046	**0.0000**	0.0026	0.0159	0.0075
	Deoxyuridine	0.3580	0.0286	**0.0000**	0.0013	0.0132	0.0069
	Dephospho-CoA	0.9282	0.0479	**0.0167**	0.0273	0.7065	0.0429
	dGMP	0.9086	0.0472	**0.0000**	0.0005	0.0387	0.0111
	dTMP	0.9898	0.0496	**0.0001**	0.0075	0.7052	0.0428
	FAD	**0.0001**	0.0016	**0.0000**	0.0002	**0.0004**	0.0016
	FMN	**0.0002**	0.0017	**0.0000**	0.0023	0.3379	0.0304
	Fructose-1,6-bisphosphate	0.3541	0.0284	**0.0000**	0.0018	**0.0000**	0.0007
	Fumarate	0.2344	0.0233	**0.0036**	0.0200	0.8579	0.0467
	GDP	**0.0017**	0.0037	**0.0099**	0.0247	0.0175	0.0079
	Glucarate	0.0225	0.0090	**0.0083**	0.0238	0.0193	0.0082
	Gluconate	0.0363	0.0107	**0.0000**	0.0004	0.0695	0.0144
	Glucosamine-1/6-phosphate	0.6601	0.0395	**0.0005**	0.0124	0.3160	0.0295
	Glucose-1/6-phosphate	0.5012	0.0340	**0.0000**	0.0036	0.9431	0.0487
	Glutamate	0.0681	0.0136	**0.0000**	0.0053	**0.0024**	0.0035
	Glutamine	**0.0003**	0.0022	0.1385	0.0394	0.0349	0.0106
	Glutathione	0.6965	0.0407	**0.0000**	0.0068	0.0053	0.0050
	Glutathione disulfide	0.8430	0.0453	**0.0009**	0.0146	0.0580	0.0134
	Glycerate	0.1059	0.0164	**0.0079**	0.0236	0.7164	0.0432
	Glycerol-3-phosphate	0.0070	0.0058	**0.0030**	0.0192	0.0290	0.0098
	GMP	0.2765	0.0251	**0.0001**	0.0082	0.2461	0.0261
	GTP	0.0203	0.0086	**0.0010**	0.0148	0.1005	0.0169
	Guanosine	**0.0029**	0.0043	**0.0010**	0.0149	0.0152	0.0074
	Homocysteic acid	0.8481	0.0454	**0.0049**	0.0212	**0.0002**	0.0012
	Hypoxanthine	0.0607	0.0129	**0.0017**	0.0168	0.1584	0.0212
	IMP	0.4582	0.0324	**0.0002**	0.0094	0.0477	0.0121
	Indole	0.0939	0.0156	**0.0283**	0.0303	0.1126	0.0179
	Inosine	**0.0000**	0.0010	**0.0007**	0.0138	0.1031	0.0171
	Isocitrate	0.6513	0.0393	**0.0085**	0.0240	0.2581	0.0268
	Kynurenine	0.1595	0.0195	**0.0206**	0.0284	0.1971	0.0236
	Lysine	0.0548	0.0124	**0.0021**	0.0176	0.1371	0.0197

Table C continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Sex-by-Strain	(i/m)q
	Malate	0.6725	0.0400	**0.0000**	0.0052	0.1698	0.0219
	Methionine	0.2327	0.0232	**0.0000**	0.0050	0.2343	0.0255
	N-acetyl-glucosamine-1/6-phosphate	0.2406	0.0236	**0.0013**	0.0157	0.1749	0.0223
	N-acetyl-glutamine	**0.0000**	0.0000	**0.0000**	0.0014	0.0419	0.0115
	N-Acetyl-L-alanine	0.1237	0.0176	**0.0001**	0.0080	0.0334	0.0105
	N-carbamoyl-L-aspartate	0.3423	0.0280	**0.0001**	0.0094	0.0943	0.0164
	NAD+	**0.0012**	0.0032	**0.0034**	0.0198	0.0667	0.0141
	NADH	**0.0003**	0.0021	**0.0000**	0.0025	**0.0000**	0.0005
	NADP+	0.0291	0.0098	**0.0000**	0.0020	0.1978	0.0236
	NADPH	**0.0002**	0.0017	**0.0037**	0.0200	0.0979	0.0166
	Octulose 8/1P	**0.0025**	0.0041	**0.0000**	0.0055	0.0106	0.0064
	Orotate	0.5941	0.0372	**0.0105**	0.0249	0.0049	0.0048
	Pantothenate	**0.0000**	0.0012	**0.0070**	0.0229	**0.0030**	0.0038
	Phenylalanine	0.6681	0.0398	**0.0048**	0.0211	0.1199	0.0185
	Phenyllactic acid	**0.0004**	0.0022	**0.0026**	0.0186	0.4953	0.0364
	Pipecolic acid	0.0750	0.0141	**0.0001**	0.0082	0.1335	0.0194
	Propionyl-CoA	0.5425	0.0355	**0.0000**	0.0064	0.0411	0.0114
	Riboflavin	**0.0003**	0.0021	**0.0017**	0.0169	0.3796	0.0319
	Ribose-phosphate	**0.0009**	0.0029	**0.0024**	0.0182	0.1765	0.0224
	S-adenosyl-L-homoCysteine	**0.0000**	0.0010	**0.0003**	0.0115	0.1292	0.0192
	Sarcosine	0.2018	0.0217	**0.0002**	0.0096	0.1304	0.0193
	Sedoheptulose bisphosphate	**0.0010**	0.0030	0.1659	0.0405	**0.0005**	0.0017
	Sedoheptulose-1/7-phosphate	0.5927	0.0371	**0.0003**	0.0114	0.1719	0.0220
	Shikimate	0.7653	0.0427	**0.0000**	0.0015	**0.0004**	0.0016
	Succinate	0.0611	0.0129	**0.0118**	0.0256	0.7422	0.0437
	Thymidine	0.0867	0.0150	**0.0001**	0.0085	0.1432	0.0201
	Tyrosine	0.6965	0.0407	**0.0009**	0.0144	0.3278	0.0300
	UDP	0.0310	0.0100	**0.0005**	0.0127	0.0144	0.0072
	UDP-D-glucose	0.2803	0.0252	**0.0000**	0.0002	**0.0031**	0.0039
	UDP-D-glucuronate	0.0096	0.0066	**0.0014**	0.0162	0.0997	0.0168
	UDP-N-acetyl-glucosamine	0.5977	0.0373	**0.0000**	0.0009	0.0740	0.0148
	UMP	0.0677	0.0135	**0.0000**	0.0026	0.1308	0.0193
	Uracil	0.1856	0.0208	**0.0016**	0.0167	**0.0008**	0.0022
	Uridine	0.4525	0.0322	**0.0002**	0.0102	0.0183	0.0081
	UTP	0.6797	0.0402	**0.0001**	0.0091	**0.0018**	0.0031
	Valine	**0.0005**	0.0024	**0.0008**	0.0141	0.1546	0.0210
	Xanthine	0.0158	0.0078	**0.0022**	0.0179	0.1751	0.0223
	Xanthosine	**0.0032**	0.0044	**0.0000**	0.0053	0.0656	0.0139
	Xanthosine-5-phosphate	0.0502	0.0120	**0.0001**	0.0087	0.7525	0.0440

Table C continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Sex-by-Strain	(i/m)q
	Xanthurenic acid	0.2755	0.0251	**0.0030**	0.0193	0.2835	0.0280
Muscle	*2-Hydroxy-2-methylbutanedioic acid*	0.0624	0.0128	**0.0000**	0.0008	0.1713	0.0194
	2-Isopropylmalic acid	0.9837	0.0494	0.0282	0.0135	**0.0002**	0.0011
	3-phosphoglycerate	0.1845	0.0202	**0.0004**	0.0030	0.1584	0.0184
	a-ketoglutarate	0.2860	0.0247	**0.0001**	0.0020	0.0078	0.0041
	Acetyllysine	0.2039	0.0211	**0.0032**	0.0060	0.6132	0.0397
	ADP	0.0237	0.0087	**0.0013**	0.0044	0.5862	0.0388
	Allantoin	0.2424	0.0228	**0.0030**	0.0059	0.5276	0.0370
	Aminoadipic acid	**0.0035**	0.0038	**0.0066**	0.0081	0.3078	0.0273
	Arginine	0.0041	0.0041	**0.0009**	0.0038	0.0165	0.0058
	Ascorbic acid	**0.0007**	0.0022	**0.0049**	0.0072	0.0027	0.0026
	Asparagine	0.3617	0.0279	**0.0031**	0.0059	0.1990	0.0212
	Betaine	**0.0027**	0.0035	0.0897	0.0216	0.3606	0.0298
	CDP-ethanolamine	**0.0013**	0.0026	0.0167	0.0111	0.1918	0.0207
	Cholic acid	0.0333	0.0101	**0.0012**	0.0043	**0.0004**	0.0013
	Citrulline	0.0530	0.0118	**0.0002**	0.0025	0.0079	0.0042
	Cytidine	**0.0007**	0.0022	**0.0002**	0.0026	0.1365	0.0170
	Deoxyribose-phosphate	0.6841	0.0396	**0.0025**	0.0055	0.0511	0.0104
	Deoxyuridine	0.9631	0.0489	**0.0000**	0.0000	0.0400	0.0091
	Erythrose-4-phosphate	**0.0002**	0.0014	**0.0000**	0.0012	0.2968	0.0267
	Fructose-1,6-bisphosphate	0.5162	0.0342	**0.0009**	0.0037	0.1225	0.0161
	Glucose-1/6-phosphate	**0.0008**	0.0022	**0.0000**	0.0010	0.2604	0.0247
	Glutamine	**0.0000**	0.0005	0.0392	0.0154	0.0097	0.0045
	Glycerol-3-phosphate	**0.0000**	0.0006	**0.0000**	0.0008	0.0040	0.0030
	Histidine	0.2052	0.0212	**0.0000**	0.0014	0.0399	0.0091
	Homocysteic acid	0.0075	0.0053	**0.0000**	0.0002	0.1424	0.0173
	Hydroxyphenylacetic acid	0.1237	0.0168	0.0114	0.0095	**0.0004**	0.0013
	Hydroxyproline	**0.0000**	0.0006	0.0900	0.0216	0.0541	0.0106
	Lysine	**0.0003**	0.0016	**0.0009**	0.0037	0.0073	0.0040
	Methionine	**0.0005**	0.0019	**0.0008**	0.0036	0.5136	0.0365
	Myo-inositol	0.2198	0.0219	**0.0001**	0.0016	0.2633	0.0249
	N-Acetyl-L-alanine	0.0053	0.0045	**0.0053**	0.0074	0.1231	0.0162
	NAD+	0.9950	0.0499	**0.0064**	0.0080	0.0158	0.0057
	Ornithine	0.7939	0.0431	0.0742	0.0199	**0.0022**	0.0024
	Orotate	**0.0009**	0.0023	0.4797	0.0394	0.0485	0.0101
	Pantothenate	**0.0000**	0.0001	**0.0027**	0.0057	0.1678	0.0192
	Shikimate	0.2798	0.0245	0.0334	0.0145	**0.0021**	0.0024
	Taurodeoxycholic acid	**0.0035**	0.0038	**0.0017**	0.0048	0.0026	0.0026

Table C continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Sex-by-Strain	(i/m)q
	Thymidine	0.9708	0.0491	**0.0004**	0.0031	0.7468	0.0439
	Tryptophan	**0.0000**	0.0004	0.0213	0.0121	**0.0003**	0.0012
	Tyrosine	**0.0012**	0.0026	**0.0079**	0.0085	0.3490	0.0293
Adipose	*2-Hydroxy-2-methylbutanedioic acid*	0.1418	0.0143	**0.0006**	0.0031	0.1427	0.0147
	ADP	0.5012	0.0323	**0.0005**	0.0029	0.9951	0.0499
	ADP-D-glucose	0.1443	0.0145	**0.0003**	0.0019	0.8726	0.0471
	Asparagine	0.0431	0.0068	**0.0015**	0.0049	0.4399	0.0330
	ATP	0.1201	0.0127	**0.0037**	0.0072	0.5823	0.0391
	Citrulline	0.0012	0.0010	**0.0014**	0.0046	0.9339	0.0487
	Creatine	0.6733	0.0390	**0.0012**	0.0045	0.1488	0.0153
	Cysteine	0.2436	0.0203	**0.0010**	0.0041	0.4288	0.0325
	Dihydroxy-acetone-phosphate	0.2887	0.0228	**0.0102**	0.0115	0.6172	0.0401
	Fumarate	0.7827	0.0427	**0.0067**	0.0096	0.3973	0.0311
	Gluconate	0.8164	0.0439	**0.0102**	0.0114	0.1695	0.0170
	Glucosamine-1/6-phosphate	0.0131	0.0034	**0.0117**	0.0122	0.3021	0.0262
	Glutamine	0.0084	0.0026	**0.0037**	0.0072	0.0679	0.0080
	Glyceraldehdye-3-phosphate	0.2887	0.0228	**0.0102**	0.0115	0.6172	0.0401
	Guanine	0.7760	0.0425	**0.0068**	0.0096	0.6298	0.0406
	Histidine	0.8567	0.0454	**0.0029**	0.0065	0.2742	0.0244
	Hypoxanthine	0.3840	0.0274	**0.0001**	0.0012	0.6710	0.0419
	Inosine	0.2890	0.0228	**0.0018**	0.0052	0.1552	0.0157
	Isocitrate	0.8999	0.0467	**0.0032**	0.0068	0.0624	0.0075
	Methionine	**0.0001**	0.0005	**0.0000**	0.0003	0.0039	0.0012
	Methionine sulfoxide	0.0216	0.0043	**0.0031**	0.0068	0.1092	0.0118
	N-Acetyl-L-alanine	0.0838	0.0103	**0.0005**	0.0026	0.0708	0.0083
	N-carbamoyl-L-aspartate	0.0957	0.0110	**0.0086**	0.0107	0.6035	0.0397
	Octulose 8/1P	0.5446	0.0342	**0.0057**	0.0089	0.4822	0.0348
	Orotate	0.6533	0.0383	**0.0053**	0.0087	0.7551	0.0443
	Pantothenate	**0.0001**	0.0005	0.1836	0.0319	0.0896	0.0100
	Pyroglutamic acid	0.1618	0.0156	**0.0058**	0.0090	0.0817	0.0094
	Pyrophosphate	0.0332	0.0057	**0.0054**	0.0088	0.0265	0.0038
	Quinolinate	0.4481	0.0302	**0.0019**	0.0055	0.0314	0.0044
	Ribose-phosphate	0.4764	0.0313	**0.0121**	0.0123	0.8438	0.0464
	S-adenosyl-L-homoCysteine	**0.0000**	0.0001	0.0751	0.0241	0.1993	0.0190
	Shikimate-3-phosphate	0.0586	0.0081	**0.0050**	0.0084	0.5047	0.0358
	Taurine	0.7024	0.0401	**0.0022**	0.0057	0.1799	0.0178
	Taurodeoxycholic acid	0.7332	0.0412	**0.0002**	0.0014	0.0687	0.0081
	Threonine	**0.0006**	0.0008	0.0457	0.0203	0.4334	0.0327

Table C continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Sex-by-Strain	(i/m)q
	Thymidine	0.0876	0.0106	**0.0052**	0.0086	0.3717	0.0298
	Thymine	0.7119	0.0404	**0.0107**	0.0117	0.0693	0.0082
	Tyrosine	0.0021	0.0013	**0.0060**	0.0092	0.5122	0.0361
	Uric acid	0.3619	0.0263	**0.0111**	0.0118	0.4138	0.0318
	Valine	0.4149	0.0289	**0.0000**	0.0006	0.6908	0.0424
	Xanthine	0.4980	0.0322	**0.0051**	0.0085	0.9289	0.0485

All known metabolites considered significant in the ANOVA were identified in liver, skeletal muscle, and adipose tissue. Bold values indicate that the metabolite is statistically significant. Bold metabolites are not tissue specific. Bold and italicized metabolites are shared between all three tissues. Benjamini-Hochberg procedure was performed to determine significance.

APPENDIX D

Table D.4: Number of Edges for Each Node in Correlation Network Analysis in Mice Fed a Standard Chow Diet

Metabolite	Tissue	Total Degree (+/-)	Degree (Adipose) (+/-)	Degree (Muscle) (+/-)	Degree (Liver) (+/-)	Degree (Phenotype) (+/-)
1-Methyl-Histidine	Adipose	2 (1/1)	1 (0/1)	0	0	1 (1/0)
2-Hydroxy-2-methylbutanedioic acid	Adipose	1 (0/1)	0	0	0	1 (0/1)
Methionine	Adipose	3 (2/1)	1 (1/0)	1 (0/1)	0	1 (1/0)
N-acetyl-glutamine	Adipose	3 (1/2)	2 (1/1)	0	0	1 (0/1)
NAD+	Adipose	4 (4/0)	3 (3/0)	0	1	1 (1/0)
Threonine	Adipose	2 (2/0)	1 (1/0)	0	0	1 (1/0)
Uric acid	Adipose	2 (2/0)	1 (1/0)	0	0	1 (1/0)
6-phospho-D-gluconate	Liver	11 (11/0)	0	2 (2/0)	8 (8/0)	1 (1/0)
ADP	Liver	13 (11/2)	0	2 (1/1)	9 (9/0)	2 (1/1)
CDP	Liver	7 (7/0)	1 (1/0)	0	5 (5/0)	1 (1/0)
CDP-ethanolamine	Liver	3 (2/1)	0	0	2 (2/0)	1 (0/1)
CMP	Liver	9 (8/1)	0	1 (0/1)	7 (7/0)	1 (1/0)
Gluconate	Liver	9 (9/0)	0	1 (1/0)	7 (7/0)	1 (1/0)
Sedoheptulose-1/7-phosphate	Liver	2 (2/0)	0	0	1 (1/0)	1 (1/0)
FAD	Liver	12 (10/2)	0	2 (1/1)	8 (8/0)	2 (1/1)
FMN	Liver	10 (8/2)	0	1 (0/1)	8 (7/1)	1 (1/0)
GDP	Liver	6 (6/0)	0	1 (1/0)	4 (4/0)	1 (1/0)
Hydroxyisocaproic acid	Liver	1 (1/0)	0	0	0	1 (1/0)
N-acetyl-glutamine	Liver	3 (2/1)	0	2 (1/1)	0	1 (1/0)
NADH	Liver	13	0	2 (2/0)	9 (9/0)	2 (1/1)
NADP+	Liver	10 (8/2)	0	2 (1/1)	7 (7/0)	1 (0/1)
NADPH	Liver	7 (7/0)	0	1 (1/0)	5 (5/0)	1 (1/0)
Taurodeoxycholic acid	Liver	1 (1/0)	0	0	0	1 (1/0)
UDP	Liver	8 (8/0)	0	2 (2/0)	5 (5/0)	1 (1/0)
Acetyl-CoA	Liver	10 (10/0)	0	4 (4/0)	4 (4/0)	2 (2/0)
Adenosine	Liver	4 (2/2)	0	1 (1/0)	2 (1/1)	1 (0/1)
Allantoate	Liver	4 (3/1)	0	1 (0/1)	2 (2/0)	1 (1/0)
dAMP	Liver	1 (0/1)	0	0	0	1 (0/1)
Glutathione disulfide	Liver	6	0	0	6	0
Pantothenate	Liver	3 (2/1)	0	1 (0/1)	1 (1/0)	1 (1/0)
Glycerol-3-phosphate	Liver	4 (3/1)	0	1 (0/1)	2 (2/0)	1 (1/0)
Ascorbic acid	Muscle	4 (1/3)	0	0	3 (1/2)	1 (0/1)

Metabolite	Tissue	Total Degree (+/-)	Degree (Adipose) (+/-)	Degree (Muscle) (+/-)	Degree (Liver) (+/-)	Degree (Phenotype) (+/-)
CDP	Muscle	1 (1/0)	0	0	0	1 (1/0)
CDP-ethanolamine	Muscle	2 (1/1)	0	1 (1/0)	0	1 (0/1)
Gluconate	Muscle	2 (1/1)	0	1 (1/0)	0	1 (0/1)
Taurodeoxycholic acid	Muscle	2 (0/2)	1 (0/1)	0	0	1 (0/1)
Acetyl-CoA	Muscle	1 (0/1)	0	0	0	1 (0/1)
Arginine	Muscle	7 (6/1)	0	4 (4/0)	2 (2/0)	1 (0/1)
Cytidine	Muscle	5 (0/5)	0	0	4 (0/4)	1 (0/1)
Deoxyribose-phosphate	Muscle	3 (3/0)	0	1 (1/0)	1 (1/0)	1 (1/0)
Hydroxyproline	Muscle	4 (2/2)	0	2 (1/1)	1 (1/0)	1 (0/1)
Lysine	Muscle	8 (7/1)	0	4 (4/0)	2 (2/0)	2 (1/1)
Methionine	Muscle	7 (7/0)	0	4 (4/0)	2 (2/0)	1 (1/0)
Pantothenate	Muscle	6 (1/5)	0	2 (1/1)	2 (0/2)	2 (0/2)
Glycerol-3-phosphate	Muscle	16 (16/0)	0	5 (5/0)	9 (9/0)	2 (2/0)
Tryptophan	Muscle	7 (6/1)	0	4 (4/0)	1 (1/0)	2 (1/1)
RER	Phenotype	3 (0/3)	0	3 (0/3)	0	0
VO2	Phenotype	7 (4/3)	1 (0/1)	4 (2/2)	2 (2/0)	0
Weight	Phenotype	3 (1/2)	0	2 (0/2)	1 (1/0)	0
Activity	Phenotype	19 (18/1)	2 (2/0)	4 (4/0)	13 (12/1)	0
Adiposity	Phenotype	9 (7/2)	2 (1/1)	0	7 (6/1)	0
Heat	Phenotype	13 (8/5)	2 (2/0)	7 (3/4)	4 (3/1)	0

APPENDIX E

Table E.5: Significant ANOVA p-values (FDR < 0.05) for Adipose, Muscle, and Liver Tissue

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
Adipose	1-Methyl-Histidine	**0.0000**	0.0049	**0.0014**	0.0299	**0.0041**	0.0128	0.0627	0.0250	**0.0019**	0.0034	0.3293	0.0340	0.5772	0.0424
	1-Methyladenosine	0.2433	0.0296	**0.0056**	0.0335	0.1824	0.0292	**0.0163**	**0.0177**	0.3079	0.0292	0.0658	0.0179	0.9080	0.0487
	2-dehydro-D-gluconate	**0.0004**	0.0068	**0.0000**	0.0013	**0.0000**	0.0017	**0.0001**	**0.0053**	0.1366	0.0200	**0.0000**	0.0013	0.0984	0.0215
	2-Hydroxy-2-methylbutanedioic acid	**0.0037**	0.0100	**0.0000**	0.0003	0.7897	0.0458	0.0292	0.0207	0.0607	0.0137	0.0629	0.0176	0.8399	0.0477
	2-Isopropylmalic acid	0.2764	0.0308	**0.0000**	0.0083	0.7094	0.0440	**0.0002**	**0.0065**	0.1719	0.0222	0.1519	0.0253	0.3395	0.0349
	2,3-Diphosphoglyceric acid	0.1154	0.0235	**0.0004**	0.0276	0.9770	0.0496	0.0201	0.0187	0.7688	0.0443	0.8603	0.0485	**0.0067**	0.0071
	3-hydroxybutyryl-CoA	**0.0000**	0.0037	**0.0000**	0.0187	**0.0000**	0.0048	0.0599	0.0247	0.1278	0.0194	**0.0043**	0.0065	0.5031	0.0404
	3-phosphoglycerate	0.0559	0.0188	**0.0000**	0.0009	**0.0000**	0.0067	0.0877	0.0273	0.9886	0.0497	0.1055	0.0217	0.1134	0.0227
	3-S-methylthiopropionate	0.2506	0.0298	**0.0030**	0.0319	0.0302	0.0186	0.3150	0.0381	0.8472	0.0464	0.6414	0.0442	**0.0035**	0.0053
	4-aminobutyrate	0.5791	0.0399	**0.0043**	0.0329	0.2506	0.0319	**0.0000**	**0.0044**	0.0987	0.0171	0.2452	0.0305	0.2371	0.0306
	4-Pyridoxic acid	**0.0077**	0.0117	**0.0000**	0.0051	0.0180	0.0167	**0.0024**	**0.0111**	0.4661	0.0354	0.0542	0.0166	0.1770	0.0272
	6-phospho-D-gluconate	0.9790	0.0495	**0.0000**	0.0127	**0.0080**	0.0143	**0.0000**	0.0038	0.0066	0.0054	**0.0000**	0.0009	0.3010	0.0335
	a-ketoglutarate	0.9399	0.0487	0.1187	0.0432	0.8911	0.0479	**0.0000**	**0.0028**	0.0131	0.0070	0.1668	0.0262	0.2957	0.0332
	Acetyl-CoA	**0.0000**	0.0042	**0.0080**	0.0344	**0.0010**	0.0101	**0.0046**	**0.0132**	0.1633	0.0217	0.1027	0.0215	0.0184	0.0107
	Acetyllysine	**0.0000**	0.0011	**0.0012**	0.0295	**0.0048**	0.0131	**0.0142**	**0.0172**	0.1849	0.0230	0.2044	0.0282	0.4267	0.0379
	Aconitate	0.8617	0.0470	**0.0001**	0.0235	0.3599	0.0356	0.0446	0.0230	0.1676	0.0219	0.1349	0.0240	0.4453	0.0387
	Adenine	0.0711	0.0202	**0.0000**	0.0179	0.5298	0.0403	0.9940	0.0499	0.0664	0.0143	0.2755	0.0319	0.0120	0.0090
	Adenosine	**0.0130**	0.0131	**0.0000**	0.0195	**0.0009**	0.0099	0.0443	0.0229	0.1035	0.0175	**0.0000**	0.0007	**0.0027**	0.0049
	ADP	0.0331	0.0163	**0.0000**	0.0065	0.1513	0.0277	0.1241	0.0301	0.1123	0.0183	**0.0017**	0.0045	**0.0067**	0.0071
	ADP-D-glucose	0.5662	0.0396	**0.0000**	0.0003	**0.0004**	0.0091	**0.0004**	**0.0077**	0.0260	0.0095	0.3260	0.0339	0.1772	0.0272

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Alanine	0.9207	0.0483	**0.0033**	0.0322	0.4595	0.0385	0.1873	0.0335	0.3180	0.0297	0.9851	0.0498	**0.0001**	0.0017
	Allantoate	**0.0000**	0.0004	0.0567	0.0407	**0.0000**	0.0035	**0.0004**	**0.0078**	**0.0001**	0.0013	**0.0037**	0.0061	0.0178	0.0106
	Allantoin	0.6372	0.0417	**0.0000**	0.0011	0.0431	0.0201	0.0832	0.0269	0.2847	0.0282	0.1440	0.0246	0.0689	0.0187
	Aminoadipic acid	**0.0000**	0.0030	**0.0000**	0.0009	**0.0000**	0.0035	**0.0005**	**0.0082**	0.1753	0.0224	0.0465	0.0157	0.0264	0.0125
	Aminoimidazole carboxamide ribonucleotide	0.0142	0.0134	**0.0000**	0.0064	**0.0003**	0.0085	**0.0018**	**0.0105**	0.1174	0.0187	0.3551	0.0351	0.1158	0.0228
	AMP	0.0735	0.0204	**0.0000**	0.0004	**0.0000**	0.0049	**0.0000**	**0.0039**	0.4048	0.0332	0.0607	0.0174	0.1822	0.0275
	Asparagine	0.1322	0.0247	**0.0027**	0.0316	**0.0093**	0.0148	0.1709	0.0325	0.0177	0.0080	0.0640	0.0177	0.0299	0.0132
	Aspartate	**0.0002**	0.0061	**0.0000**	0.0048	**0.0142**	0.0160	**0.0002**	**0.0065**	0.6120	0.0399	0.0205	0.0112	0.3001	0.0334
	ATP	**0.0011**	0.0080	**0.0000**	0.0151	0.4052	0.0370	0.6667	0.0456	0.1717	0.0222	0.0469	0.0157	0.0279	0.0128
	Atrolactic acid	**0.0004**	0.0069	**0.0000**	0.0129	**0.0000**	0.0070	0.0714	0.0260	0.1390	0.0202	**0.0012**	0.0040	0.4139	0.0375
	Betaine	0.5131	0.0382	**0.0000**	0.0024	**0.0000**	0.0021	0.5178	0.0430	**0.0002**	0.0017	0.2061	0.0284	**0.0011**	0.0035
	Butyryl-CoA	**0.0001**	0.0051	0.8310	0.0495	**0.0000**	0.0050	**0.0019**	**0.0107**	**0.0000**	0.0009	0.0645	0.0178	0.3372	0.0349
	Carnitine	0.6484	0.0420	0.0856	0.0420	**0.0154**	0.0163	0.0235	0.0195	0.0787	0.0154	0.6097	0.0432	**0.0012**	0.0036
	CDP	0.0529	0.0185	**0.0000**	0.0130	**0.0009**	0.0099	0.1673	0.0324	0.0111	0.0066	**0.0017**	0.0046	0.0117	0.0089
	CDP-ethanolamine	0.0455	0.0178	**0.0000**	0.0044	**0.0000**	0.0031	**0.0007**	**0.0087**	0.9762	0.0494	0.0911	0.0205	**0.0000**	0.0007
	Cellobiose	0.3134	0.0322	0.1345	0.0436	**0.0159**	0.0164	0.9630	0.0496	0.6496	0.0411	0.5528	0.0419	**0.0033**	0.0052
	Cholic acid	0.3126	0.0322	0.0846	0.0420	0.3382	0.0350	**0.0000**	**0.0012**	**0.0006**	0.0022	**0.0037**	0.0061	0.4155	0.0375
	Citrate	0.1837	0.0272	**0.0004**	0.0275	0.6178	0.0423	0.2009	0.0341	0.0505	0.0125	**0.0080**	0.0080	0.5600	0.0420
	Citrate/isocitrate	0.1837	0.0273	**0.0004**	0.0275	0.6178	0.0423	0.2009	0.0341	0.0505	0.0125	**0.0080**	0.0080	0.5600	0.0420
	Citrulline	0.0180	0.0141	**0.0004**	0.0276	**0.0000**	0.0049	**0.0002**	**0.0067**	0.0049	0.0048	0.2557	0.0310	0.1376	0.0244
	CMP	0.6122	0.0410	**0.0000**	0.0059	0.0630	0.0219	0.0560	0.0243	0.0846	0.0160	0.0302	0.0131	0.1564	0.0259
	Coenzyme A	0.0156	0.0136	**0.0000**	0.0202	**0.0161**	0.0165	0.0683	0.0255	0.3280	0.0302	0.1970	0.0278	0.2300	0.0302
	Creatine	0.2576	0.0302	**0.0009**	0.0290	**0.0000**	0.0069	0.1717	0.0326	**0.0000**	0.0011	**0.0029**	0.0056	0.4198	0.0377

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Creatinine	0.2695	0.0305	**0.0185**	0.0369	0.6998	0.0439	0.7552	0.0470	0.5329	0.0375	0.2867	0.0323	0.0319	0.0135
	Cyclic-AMP	**0.0000**	0.0030	**0.0000**	0.0002	0.1362	0.0269	0.0784	0.0266	0.2582	0.0271	0.1199	0.0228	0.0186	0.0108
	Cystathionine	**0.0000**	0.0010	**0.0000**	0.0017	**0.0001**	0.0076	0.1691	0.0324	0.1739	0.0223	0.7153	0.0458	0.0846	0.0203
	Cytidine	0.9220	0.0483	**0.0003**	0.0264	**0.0001**	0.0073	0.3281	0.0385	0.0474	0.0122	0.0968	0.0211	0.2085	0.0291
	dAMP	0.0568	0.0189	0.3762	0.0472	**0.0000**	0.0047	0.3938	0.0401	0.0094	0.0062	0.4501	0.0386	0.3035	0.0336
	dCMP	0.6813	0.0427	**0.0000**	0.0185	0.0389	0.0196	0.0900	0.0276	0.1788	0.0227	0.2338	0.0298	0.2613	0.0316
	Deoxyinosine	**0.0000**	0.0022	**0.0000**	0.0101	0.9754	0.0496	**0.0081**	**0.0151**	0.3619	0.0314	0.0088	0.0083	**0.0043**	0.0059
	Deoxyribose-phosphate	**0.0000**	0.0029	**0.0000**	0.0121	**0.0083**	0.0145	0.1144	0.0296	0.6289	0.0404	0.1437	0.0246	0.8076	0.0471
	Deoxyuridine	0.7644	0.0447	**0.0000**	0.0001	**0.0009**	0.0099	0.3970	0.0402	0.1155	0.0186	**0.0080**	0.0080	0.0228	0.0117
	Dephospho-CoA	0.8525	0.0468	**0.0000**	0.0044	**0.0018**	0.0111	0.7060	0.0462	0.2807	0.0281	0.2897	0.0324	0.7622	0.0464
	dGMP	0.0735	0.0204	**0.0000**	0.0004	**0.0000**	0.0049	**0.0000**	**0.0039**	0.4048	0.0332	0.0607	0.0174	0.1822	0.0275
	Dihydroorotate	**0.0071**	0.0115	**0.0002**	0.0261	0.8219	0.0466	0.2042	0.0342	0.2537	0.0269	0.0657	0.0179	0.5978	0.0429
	Dihydroxy-acetone-phosphate	**0.0000**	0.0025	**0.0000**	0.0161	0.0236	0.0176	**0.0089**	**0.0155**	0.7658	0.0442	0.4219	0.0377	**0.0067**	0.0071
	dTMP	0.1007	0.0225	**0.0000**	0.0086	0.1277	0.0264	0.4300	0.0410	0.0775	0.0154	0.4457	0.0385	0.1908	0.0280
	dUMP	0.6505	0.0420	**0.0000**	0.0179	**0.0000**	0.0062	**0.0028**	**0.0116**	0.0077	0.0057	0.2889	0.0324	0.0397	0.0148
	dUTP	0.3649	0.0340	**0.0062**	0.0338	0.3503	0.0354	**0.0043**	**0.0130**	0.7209	0.0431	0.5208	0.0410	0.0312	0.0134
	FAD	**0.0000**	0.0034	**0.0000**	0.0014	**0.0078**	0.0143	0.9817	0.0498	0.5971	0.0395	0.1043	0.0216	**0.0062**	0.0069
	FMN	**0.0000**	0.0032	**0.0000**	0.0023	0.7111	0.0441	0.1752	0.0328	0.2651	0.0275	0.2949	0.0326	0.1235	0.0234
	Fructose-1,6-bisphosphate	**0.0030**	0.0097	**0.0000**	0.0050	0.1295	0.0265	**0.0000**	**0.0047**	0.4141	0.0336	0.0311	0.0132	**0.0025**	0.0047
	Fumarate	**0.0001**	0.0051	**0.0000**	0.0047	**0.0015**	0.0108	**0.0001**	**0.0053**	0.3089	0.0293	0.0556	0.0168	0.0842	0.0202
	GDP	0.0440	0.0176	**0.0195**	0.0372	0.9181	0.0485	0.6257	0.0450	0.0741	0.0150	0.0240	0.0120	**0.0025**	0.0047
	Glucarate	**0.0000**	0.0022	**0.0000**	0.0198	**0.0000**	0.0020	0.2185	0.0348	0.0517	0.0127	**0.0013**	0.0042	0.5061	0.0405
	Gluconate	**0.0000**	0.0035	**0.0000**	0.0027	**0.0000**	0.0042	**0.0078**	**0.0150**	0.0165	0.0078	0.0371	0.0142	0.1732	0.0269
	Glucosamine-1/6-phosphate	0.4592	0.0366	**0.0234**	0.0378	0.9812	0.0497	0.3864	0.0399	0.7252	0.0432	0.3038	0.0330	0.0767	0.0195

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Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Glucose-1/6-phosphate	**0.0130**	0.0131	**0.0000**	0.0230	**0.0003**	0.0088	**0.0028**	**0.0115**	0.1450	0.0206	0.0265	0.0125	0.0403	0.0150
	Glutamate	0.3158	0.0323	**0.0000**	0.0022	**0.0000**	0.0024	**0.0000**	**0.0030**	0.0052	0.0049	0.0749	0.0189	0.0612	0.0178
	Glutamine	**0.0000**	0.0033	**0.0062**	0.0337	**0.0102**	0.0150	0.2142	0.0347	0.0090	0.0060	**0.0030**	0.0056	0.0381	0.0145
	Glutathione	0.2494	0.0297	**0.0000**	0.0029	0.4916	0.0392	0.0465	0.0232	0.1787	0.0226	0.4340	0.0381	**0.0069**	0.0072
	Glutathione disulfide	0.3952	0.0348	**0.0004**	0.0274	0.1783	0.0290	0.8891	0.0488	0.3757	0.0319	0.1594	0.0257	0.0677	0.0185
	Glyceraldehdye-3-phosphate	**0.0000**	0.0025	**0.0000**	0.0161	0.0236	0.0176	**0.0089**	**0.0155**	0.7658	0.0442	0.4219	0.0377	**0.0067**	0.0071
	Glycerate	0.2982	0.0317	**0.0000**	0.0214	0.0352	0.0192	0.0284	0.0205	0.0402	0.0113	0.4576	0.0389	0.0308	0.0133
	Glycerol-3-phosphate	**0.0017**	0.0086	**0.0000**	0.0164	**0.0003**	0.0087	0.1050	0.0288	0.0048	0.0048	**0.0008**	0.0034	**0.0059**	0.0068
	GMP	0.0633	0.0195	**0.0000**	0.0008	**0.0000**	0.0067	**0.0010**	**0.0093**	0.9425	0.0487	0.1123	0.0223	0.6920	0.0450
	GTP	**0.0121**	0.0128	**0.0000**	0.0091	**0.0084**	0.0145	0.1819	0.0332	0.0686	0.0145	**0.0074**	0.0078	0.0316	0.0135
	Guanine	**0.0009**	0.0077	**0.0000**	0.0152	0.7370	0.0446	**0.0086**	**0.0153**	0.7175	0.0430	0.5445	0.0416	0.2093	0.0292
	Guanosine	0.0156	0.0136	**0.0000**	0.0126	0.0542	0.0211	**0.0000**	**0.0010**	0.4558	0.0350	0.0364	0.0141	0.2387	0.0306
	Histidine	0.1014	0.0226	**0.0000**	0.0183	**0.0135**	0.0159	**0.0005**	**0.0082**	0.2804	0.0281	0.0982	0.0212	0.0280	0.0128
	Homocysteic acid	**0.0000**	0.0020	**0.0000**	0.0016	**0.0022**	0.0115	**0.0016**	**0.0102**	0.0145	0.0074	**0.0075**	0.0078	**0.0041**	0.0058
	Homoserine	0.2124	0.0285	0.0517	0.0404	**0.0083**	0.0145	**0.0041**	**0.0127**	0.0002	0.0015	0.5412	0.0415	**0.0007**	0.0029
	Hydroxyisocaproic acid	0.0418	0.0174	**0.0024**	0.0313	**0.0038**	0.0126	**0.0001**	**0.0054**	0.8997	0.0478	0.3197	0.0337	0.0953	0.0212
	Hydroxyproline	0.2524	0.0299	0.3878	0.0473	**0.0000**	0.0010	0.1415	0.0312	0.3256	0.0301	**0.0005**	0.0029	0.0528	0.0169
	Hypoxanthine	0.4370	0.0360	**0.0000**	0.0121	**0.0005**	0.0092	**0.0027**	**0.0115**	0.0206	0.0086	**0.0014**	0.0043	0.0168	0.0104
	IMP	0.0321	0.0163	**0.0000**	0.0190	**0.0065**	0.0138	0.2426	0.0358	0.2016	0.0240	0.0209	0.0113	0.0670	0.0184
	Indole	0.9572	0.0491	**0.0008**	0.0287	0.2731	0.0328	0.4842	0.0423	0.4810	0.0358	0.0462	0.0156	0.0806	0.0198
	Inosine	**0.0000**	0.0020	**0.0000**	0.0086	**0.0148**	0.0161	**0.0001**	**0.0062**	0.4932	0.0361	0.0484	0.0159	**0.0002**	0.0018
	Isocitrate	0.1837	0.0273	**0.0004**	0.0275	0.6178	0.0423	0.2009	0.0341	0.0505	0.0125	**0.0080**	0.0080	0.5600	0.0420
	Kynurenic acid	0.1045	0.0229	**0.0072**	0.0341	**0.0000**	0.0038	**0.0041**	**0.0128**	0.1434	0.0205	0.0231	0.0118	0.6396	0.0438
	Kynurenine	0.3775	0.0343	**0.0000**	0.0206	**0.0028**	0.0119	**0.0157**	**0.0176**	0.6743	0.0417	**0.0053**	0.0069	0.4986	0.0403

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	L-arginino-succinate	**0.0102**	0.0124	**0.0000**	0.0119	**0.0000**	0.0060	**0.0000**	**0.0011**	0.1986	0.0238	0.1626	0.0259	0.2104	0.0292
	Leucine/isoleucine	0.7346	0.0440	**0.0000**	0.0158	**0.0011**	0.0103	0.1409	0.0312	0.0297	0.0099	0.1848	0.0271	**0.0000**	0.0004
	Lipoate	0.1032	0.0228	**0.0065**	0.0339	0.0308	0.0187	0.2490	0.0360	0.7897	0.0449	0.5105	0.0406	0.8632	0.0481
	Lysine	**0.0000**	0.0039	**0.0004**	0.0271	**0.0000**	0.0066	0.3996	0.0403	0.4134	0.0335	**0.0005**	0.0030	0.0154	0.0101
	Malate	**0.0023**	0.0092	**0.0000**	0.0019	**0.0057**	0.0135	0.1372	0.0309	0.2643	0.0274	**0.0018**	0.0046	0.5116	0.0407
	Methionine	0.1683	0.0265	**0.0000**	0.0007	**0.0000**	0.0050	0.1057	0.0289	0.1452	0.0206	**0.0001**	0.0018	0.4681	0.0394
	Myo-inositol	0.7449	0.0443	**0.0000**	0.0065	**0.0000**	0.0047	**0.0057**	**0.0139**	0.5057	0.0365	0.1321	0.0238	0.0764	0.0195
	N-acetyl-glucosamine-1/6-phosphate	0.8722	0.0473	**0.0000**	0.0140	0.7675	0.0453	**0.0002**	**0.0069**	0.2602	0.0271	0.4178	0.0376	0.3569	0.0356
	N-acetyl-glutamate	0.0178	0.0141	**0.0000**	0.0087	**0.0002**	0.0083	0.6038	0.0446	**0.0045**	0.0046	0.3751	0.0359	0.7214	0.0457
	N-acetyl-glutamine	**0.0000**	0.0001	**0.0000**	0.0020	**0.0000**	0.0038	**0.0000**	0.0029	0.0530	0.0128	**0.0002**	0.0021	0.0126	0.0092
	N-Acetyl-L-alanine	**0.0066**	0.0113	**0.0000**	0.0010	0.0836	0.0234	**0.0000**	**0.0036**	0.0633	0.0140	0.0695	0.0183	0.0185	0.0107
	N-acetyl-L-ornithine	0.4949	0.0377	0.1667	0.0443	**0.0000**	0.0021	0.0412	0.0227	0.7817	0.0447	0.2177	0.0290	0.4354	0.0383
	N-carbamoyl-L-aspartate	**0.0000**	0.0032	**0.0004**	0.0271	0.5260	0.0402	0.7269	0.0466	0.2267	0.0254	0.0236	0.0119	0.4638	0.0393
	NAD+	**0.0005**	0.0070	**0.0000**	0.0165	0.8303	0.0468	0.1113	0.0294	0.0186	0.0082	0.1112	0.0222	0.1227	0.0234
	NADH	**0.0001**	0.0056	**0.0000**	0.0045	**0.0012**	0.0104	**0.0008**	**0.0090**	0.0874	0.0162	**0.0035**	0.0060	0.0075	0.0074
	NADP+	0.0159	0.0137	**0.0000**	0.0037	0.0375	0.0195	0.0234	0.0195	0.3396	0.0306	0.4952	0.0401	0.1758	0.0271
	NADPH	**0.0043**	0.0104	**0.0010**	0.0294	0.0221	0.0175	0.0215	0.0190	0.6927	0.0422	0.5423	0.0416	0.0232	0.0117
	Nicotinate	0.0272	0.0155	**0.0131**	0.0358	0.2339	0.0313	0.5890	0.0443	0.3560	0.0312	0.6546	0.0445	0.0818	0.0199
	Octulose 8/1P	**0.0020**	0.0090	**0.0075**	0.0342	**0.0000**	0.0008	0.0381	0.0222	0.1601	0.0215	0.0730	0.0187	0.2585	0.0314
	Ornithine	**0.0067**	0.0113	0.5801	0.0485	**0.0131**	0.0158	**0.0002**	**0.0066**	0.0275	0.0096	0.7049	0.0456	0.0213	0.0113
	Orotate	0.9861	0.0497	0.0635	0.0411	0.7142	0.0441	0.7072	0.0463	0.0797	0.0155	0.0340	0.0137	**0.0049**	0.0062
	Pantothenate	**0.0000**	0.0020	**0.0004**	0.0273	0.0211	0.0173	**0.0000**	**0.0018**	**0.0022**	0.0036	0.3535	0.0350	0.1215	0.0233
	Phenylalanine	0.3649	0.0340	**0.0000**	0.0164	0.7544	0.0450	0.0223	0.0192	0.4711	0.0355	0.0320	0.0134	0.0303	0.0133

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Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Phenyllactic acid	**0.0004**	0.0069	**0.0000**	0.0129	**0.0000**	0.0070	0.0714	0.0260	0.1390	0.0202	**0.0012**	0.0040	0.4139	0.0375
	Phenylpyruvate	0.0210	0.0147	**0.0001**	0.0246	0.6360	0.0426	0.7831	0.0474	0.6779	0.0418	0.6907	0.0453	0.2464	0.0310
	Phosphoenolpyruvate	0.8485	0.0468	**0.0000**	0.0112	**0.0001**	0.0074	0.0576	0.0244	0.0732	0.0149	0.7217	0.0459	0.0156	0.0102
	Pipecolic acid	0.3104	0.0321	**0.0000**	0.0131	0.5129	0.0397	0.5350	0.0433	0.0225	0.0089	0.4605	0.0390	0.0441	0.0156
	Proline	0.0589	0.0191	0.7651	0.0493	**0.0001**	0.0078	0.2964	0.0375	0.0125	0.0069	0.1588	0.0257	0.5870	0.0426
	Propionyl-CoA	0.9113	0.0481	**0.0000**	0.0019	0.0712	0.0225	**0.0119**	**0.0165**	**0.0026**	0.0038	0.1564	0.0256	**0.0011**	0.0035
	Pyroglutamic acid	0.0621	0.0194	**0.0007**	0.0287	0.9677	0.0494	**0.0024**	**0.0112**	0.0319	0.0102	0.6115	0.0433	0.7806	0.0467
	Quinolinate	0.5871	0.0402	**0.0000**	0.0154	**0.0083**	0.0144	0.0741	0.0262	0.6131	0.0399	**0.0021**	0.0049	0.0381	0.0145
	Riboflavin	**0.0000**	0.0022	**0.0000**	0.0054	0.6711	0.0433	**0.0053**	**0.0137**	0.0181	0.0081	**0.0004**	0.0028	**0.0008**	0.0031
	Ribose-phosphate	**0.0000**	0.0024	**0.0000**	0.0148	0.1216	0.0260	0.9830	0.0498	0.6742	0.0417	0.0994	0.0213	0.2324	0.0304
	S-adenosyl-L-homoCysteine	**0.0000**	0.0042	**0.0000**	0.0049	**0.0003**	0.0087	0.1997	0.0341	**0.0038**	0.0043	**0.0047**	0.0067	0.1892	0.0280
	Sarcosine	0.9575	0.0491	**0.0133**	0.0358	0.1507	0.0277	0.2366	0.0356	0.1413	0.0203	0.9072	0.0490	**0.0001**	0.0016
	Sedoheptulose bisphosphate	0.0286	0.0158	**0.0000**	0.0173	0.2539	0.0320	**0.0014**	**0.0099**	0.3370	0.0305	0.4439	0.0384	**0.0083**	0.0076
	Sedoheptulose-1/7-phosphate	**0.0025**	0.0093	**0.0000**	0.0067	**0.0000**	0.0066	**0.0027**	**0.0114**	0.0565	0.0132	**0.0006**	0.0031	0.0685	0.0187
	Serine	0.0889	0.0217	**0.0000**	0.0218	**0.0000**	0.0045	**0.0038**	**0.0125**	**0.0007**	0.0023	0.2802	0.0321	0.0905	0.0209
	Shikimate	**0.0077**	0.0117	**0.0000**	0.0039	0.5618	0.0410	**0.0000**	**0.0022**	0.2331	0.0258	**0.0001**	0.0017	0.2360	0.0305
	Succinate	0.4517	0.0364	**0.0003**	0.0268	0.5160	0.0398	0.1563	0.0318	**0.0032**	0.0040	0.1942	0.0276	0.0205	0.0112
	Taurine	0.0560	0.0188	0.0431	0.0398	**0.0000**	0.0038	**0.0029**	**0.0116**	0.0504	0.0124	0.2293	0.0296	**0.0037**	0.0055
	Taurodeoxycholic acid	**0.0003**	0.0065	**0.0000**	0.0038	**0.0122**	0.0156	0.7355	0.0467	0.0091	0.0061	**0.0000**	0.0011	**0.0000**	0.0005
	Threonine	0.2124	0.0285	0.0517	0.0404	**0.0083**	0.0145	**0.0041**	**0.0127**	**0.0002**	0.0015	0.5412	0.0415	**0.0007**	0.0029
	Thymidine	0.2656	0.0304	**0.0000**	0.0011	0.0315	0.0188	0.4946	0.0425	0.2291	0.0256	0.1184	0.0227	0.3366	0.0348
	Trehalose-6-Phosphate	0.3551	0.0337	0.8403	0.0496	0.0878	0.0237	**0.0046**	**0.0132**	0.9271	0.0484	0.5500	0.0418	0.4569	0.0391
	Trehalose/sucrose	0.3462	0.0334	0.1309	0.0435	0.0184	0.0168	0.9609	0.0496	0.6495	0.0410	0.5262	0.0412	**0.0037**	0.0055
	Tryptophan	**0.0008**	0.0076	0.5920	0.0486	**0.0004**	0.0090	**0.0000**	**0.0016**	0.2766	0.0279	0.1695	0.0264	0.0136	0.0096

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Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Tyrosine	0.3830	0.0345	**0.0004**	0.0272	**0.0000**	0.0025	0.5846	0.0442	0.6903	0.0422	0.4933	0.0401	0.0521	0.0168
	UDP	0.0428	0.0174	**0.0001**	0.0236	**0.0028**	0.0120	**0.0180**	**0.0182**	0.3909	0.0325	0.1094	0.0221	0.0327	0.0137
	UDP-D-glucose	0.0239	0.0151	**0.0000**	0.0019	**0.0026**	0.0118	**0.0000**	**0.0021**	0.6455	0.0409	0.0270	0.0126	**0.0001**	0.0012
	UDP-D-glucuronate	**0.0013**	0.0082	**0.0000**	0.0035	**0.0019**	0.0112	0.0956	0.0280	0.3813	0.0322	0.1785	0.0268	**0.0019**	0.0043
	UDP-N-acetyl-glucosamine	0.9251	0.0484	**0.0000**	0.0060	0.0805	0.0232	**0.0008**	**0.0089**	0.4819	0.0358	0.1317	0.0237	0.0425	0.0153
	UMP	0.8893	0.0476	**0.0000**	0.0010	0.8932	0.0479	**0.0002**	**0.0064**	0.5044	0.0365	0.1319	0.0237	0.6774	0.0447
	Uracil	**0.0094**	0.0121	**0.0000**	0.0055	**0.0001**	0.0073	**0.0001**	**0.0063**	0.0347	0.0106	0.0823	0.0197	0.0536	0.0170
	Uric acid	0.1273	0.0244	0.1748	0.0445	0.4596	0.0385	**0.0169**	**0.0179**	0.7108	0.0428	0.0807	0.0195	0.2923	0.0331
	Uridine	0.0738	0.0204	**0.0000**	0.0023	**0.0003**	0.0087	**0.0000**	**0.0008**	0.1862	0.0230	0.2376	0.0300	0.0212	0.0113
	UTP	**0.0000**	0.0046	**0.0000**	0.0087	**0.0092**	0.0148	**0.0042**	**0.0129**	0.4238	0.0339	0.0100	0.0086	**0.0040**	0.0057
	Valine	0.5131	0.0382	**0.0000**	0.0025	**0.0000**	0.0021	0.5178	0.0430	**0.0002**	0.0017	0.2061	0.0284	**0.0011**	0.0035
	Xanthine	0.7840	0.0451	**0.0000**	0.0198	0.0741	0.0227	0.0251	0.0198	0.0180	0.0081	0.0647	0.0178	**0.0001**	0.0012
	Xanthosine	**0.0000**	0.0043	**0.0000**	0.0029	**0.0154**	0.0163	**0.0137**	**0.0171**	0.7029	0.0425	**0.0014**	0.0043	**0.0000**	0.0004
	Xanthosine-5-phosphate	0.3373	0.0330	**0.0000**	0.0112	0.8075	0.0463	0.0539	0.0240	0.1144	0.0185	0.3032	0.0329	0.1579	0.0260
	Xanthurenic acid	**0.0019**	0.0089	**0.0004**	0.0274	**0.0000**	0.0037	0.0187	0.0184	0.0380	0.0110	**0.0016**	0.0045	0.5376	0.0414
Muscle	1-Methyl-Histidine	**0.0000**	0.0010	**0.0000**	0.0091	0.1049	0.0092	**0.0072**	0.0074	0.3358	0.0247	0.5765	0.0368	0.7318	0.0410
	2-dehydro-D-gluconate	0.0958	0.0156	**0.0004**	0.0160	0.3677	0.0204	0.0140	0.0090	0.6572	0.0383	0.5187	0.0344	0.3209	0.0235
	2-Hydroxy-2-methylbutanedioic acid	0.1363	0.0180	**0.0000**	0.0006	0.4680	0.0255	0.0219	0.0106	0.1395	0.0140	0.3105	0.0253	0.2310	0.0189
	2-Isopropylmalic acid	0.6523	0.0384	**0.0000**	0.0018	0.0038	0.0031	**0.0000**	0.0023	0.9280	0.0475	0.0582	0.0086	**0.0000**	0.0004
	2-oxo-4-methylthiobutanoate	**0.0000**	0.0020	**0.0002**	0.0141	0.6761	0.0350	0.8607	0.0464	0.5314	0.0332	0.5685	0.0365	0.6895	0.0395
	3-methylphenylacetic acid	0.8050	0.0437	**0.0002**	0.0142	0.9067	0.0460	0.0662	0.0157	0.1628	0.0155	0.2122	0.0199	**0.0008**	0.0015
	3-phosphoglycerate	0.8040	0.0437	**0.0005**	0.0164	0.9271	0.0469	0.0227	0.0108	0.7060	0.0400	0.3006	0.0247	0.0919	0.0105

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Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	3-S-methylthiopropionate	**0.0019**	0.0063	0.3632	0.0429	0.6688	0.0347	0.4249	0.0333	0.6377	0.0375	0.9120	0.0480	0.9900	0.0496
	6-phospho-D-gluconate	0.4584	0.0316	**0.0000**	0.0073	0.1411	0.0109	0.0895	0.0176	0.2677	0.0213	0.0164	0.0037	0.6982	0.0398
	a-ketoglutarate	0.7424	0.0417	**0.0000**	0.0010	0.3584	0.0198	**0.0026**	0.0056	0.4361	0.0292	0.1653	0.0168	0.0445	0.0066
	Acetyllysine	0.0584	0.0132	**0.0182**	0.0273	**0.0021**	0.0027	0.2508	0.0264	0.2824	0.0220	0.3239	0.0260	0.4650	0.0300
	Aconitate	0.1821	0.0202	**0.0025**	0.0204	0.7792	0.0400	0.3865	0.0318	0.3349	0.0247	0.8509	0.0464	0.8551	0.0451
	Adenosine	0.1083	0.0165	**0.0000**	0.0111	0.5141	0.0275	0.0302	0.0119	0.5743	0.0349	0.0767	0.0100	0.2417	0.0195
	ADP	**0.0027**	0.0069	**0.0000**	0.0105	0.4054	0.0223	0.4164	0.0330	0.8162	0.0439	0.1025	0.0121	0.2074	0.0177
	ADP-D-glucose	0.0646	0.0137	**0.0065**	0.0234	0.2443	0.0152	0.0878	0.0174	0.4774	0.0309	0.1173	0.0132	0.6014	0.0360
	Allantoate	0.1254	0.0174	**0.0028**	0.0207	0.1079	0.0095	0.0724	0.0161	0.0435	0.0061	0.4781	0.0328	**0.0004**	0.0014
	Allantoin	0.6491	0.0383	**0.0000**	0.0041	**0.0009**	0.0024	0.0572	0.0148	0.3027	0.0232	0.2750	0.0237	0.5184	0.0324
	Aminoadipic acid	**0.0000**	0.0013	**0.0000**	0.0046	**0.0003**	0.0021	0.6086	0.0390	0.7523	0.0416	0.1263	0.0141	0.0409	0.0064
	AMP	**0.0003**	0.0044	**0.0000**	0.0111	0.3490	0.0194	0.4544	0.0343	0.2681	0.0214	0.6529	0.0396	0.2143	0.0181
	Arginine	0.0286	0.0113	**0.0001**	0.0132	**0.0000**	0.0010	0.1300	0.0201	0.1641	0.0156	0.2201	0.0205	0.8818	0.0461
	Ascorbic acid	0.4139	0.0298	**0.0056**	0.0229	0.7306	0.0377	0.0482	0.0140	0.0822	0.0095	0.0571	0.0084	0.5782	0.0350
	Asparagine	0.0364	0.0119	**0.0004**	0.0155	0.4692	0.0256	0.0480	0.0139	0.0860	0.0098	0.1930	0.0188	0.5943	0.0356
	Aspartate	**0.0034**	0.0073	**0.0000**	0.0045	0.7005	0.0361	0.0305	0.0119	0.7209	0.0405	0.0298	0.0055	0.1171	0.0123
	ATP	0.3402	0.0269	**0.0002**	0.0144	0.6114	0.0320	0.0194	0.0102	0.6619	0.0385	0.4079	0.0300	0.4183	0.0281
	Betaine	0.6897	0.0398	**0.0000**	0.0091	**0.0000**	0.0013	0.7783	0.0441	0.0349	0.0052	0.4405	0.0313	0.5025	0.0316
	CDP-ethanolamine	**0.0000**	0.0026	**0.0001**	0.0123	0.8423	0.0429	0.0889	0.0175	0.0750	0.0089	0.5103	0.0340	0.7414	0.0414
	Citraconic acid	0.0104	0.0091	0.0576	0.0326	0.3673	0.0204	**0.0000**	0.0020	0.1370	0.0138	0.0072	0.0023	0.4579	0.0297
	Citrate	0.1830	0.0203	**0.0001**	0.0122	0.0317	0.0054	0.1726	0.0226	0.8251	0.0443	0.7279	0.0425	0.5124	0.0320
	Citrate/isocitrate	0.1830	0.0203	**0.0001**	0.0122	0.0317	0.0054	0.1726	0.0226	0.8251	0.0443	0.7279	0.0425	0.5124	0.0321
	Citrulline	**0.0000**	0.0026	**0.0000**	0.0046	0.0548	0.0067	0.6214	0.0394	0.7766	0.0426	0.5762	0.0368	0.3294	0.0240
	CMP	**0.0020**	0.0064	**0.0002**	0.0145	0.7914	0.0405	0.9060	0.0474	0.4214	0.0285	0.2040	0.0195	0.4693	0.0302

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Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Creatine	0.0183	0.0102	**0.0000**	0.0032	0.4930	0.0267	**0.0036**	0.0061	0.1330	0.0135	0.2149	0.0201	0.5382	0.0333
	CTP	0.3956	0.0291	**0.0185**	0.0274	0.0670	0.0074	0.0258	0.0112	0.8240	0.0442	0.0369	0.0065	0.4616	0.0299
	Cyclic-AMP	**0.0019**	0.0063	**0.0000**	0.0021	0.0597	0.0070	0.1891	0.0235	0.1252	0.0129	0.0087	0.0025	0.0191	0.0042
	Cystathionine	0.6501	0.0383	**0.0000**	0.0052	0.6061	0.0318	**0.0028**	0.0058	0.7844	0.0429	0.0016	0.0010	0.0703	0.0088
	Cysteine	0.6737	0.0391	**0.0017**	0.0192	0.3291	0.0186	0.1648	0.0222	0.4562	0.0301	0.1050	0.0123	0.6201	0.0366
	Cytidine	**0.0000**	0.0024	**0.0000**	0.0091	0.0443	0.0061	**0.0000**	0.0006	0.7640	0.0421	0.4208	0.0305	0.0392	0.0062
	Deoxyribose-phosphate	0.3048	0.0255	**0.0000**	0.0029	0.8472	0.0432	**0.0001**	0.0029	0.1793	0.0164	0.1327	0.0146	0.4333	0.0288
	Deoxyuridine	0.1808	0.0201	**0.0000**	0.0000	0.0071	0.0037	**0.0011**	0.0046	0.7049	0.0399	0.1715	0.0172	0.3997	0.0274
	dGMP	**0.0003**	0.0044	**0.0000**	0.0111	0.3490	0.0195	0.4544	0.0343	0.2681	0.0214	0.6529	0.0397	0.2143	0.0181
	Dihydroxy-acetone-phosphate	0.1376	0.0181	**0.0000**	0.0120	0.0979	0.0089	0.1059	0.0186	0.0808	0.0094	0.0636	0.0090	0.0251	0.0048
	Erythrose-4-phosphate	**0.0000**	0.0015	**0.0000**	0.0025	0.2011	0.0134	0.1512	0.0215	0.0021	0.0009	**0.0001**	0.0004	0.0415	0.0064
	FAD	0.6770	0.0393	**0.0001**	0.0136	0.0626	0.0071	0.5587	0.0377	0.4911	0.0315	0.1791	0.0178	0.0065	0.0027
	Fructose-1,6-bisphosphate	0.4267	0.0303	**0.0000**	0.0029	0.6733	0.0349	**0.0012**	0.0046	0.5929	0.0357	0.2987	0.0246	0.3343	0.0243
	Fumarate	0.9473	0.0483	**0.0007**	0.0172	0.8793	0.0448	0.1891	0.0234	0.7636	0.0421	0.3980	0.0294	0.0710	0.0089
	GDP	0.2692	0.0240	**0.0012**	0.0184	0.3293	0.0187	0.5723	0.0381	0.5431	0.0336	0.4584	0.0320	0.0805	0.0096
	Gluconate	**0.0024**	0.0067	0.1874	0.0389	0.9951	0.0498	0.0110	0.0084	0.7986	0.0434	0.8783	0.0471	0.2835	0.0216
	Glucono-lactone-6-phosphate	**0.0000**	0.0020	**0.0000**	0.0031	0.1396	0.0109	0.0200	0.0103	0.0843	0.0097	0.4512	0.0317	0.0139	0.0036
	Glucosamine-1/6-phosphate	0.1590	0.0192	0.3067	0.0418	0.4988	0.0269	**0.0032**	0.0059	0.2132	0.0183	0.0221	0.0045	0.0362	0.0060
	Glucose-1/6-phosphate	**0.0000**	0.0011	**0.0000**	0.0022	0.4646	0.0252	**0.0073**	0.0074	0.0370	0.0055	0.0016	0.0010	0.0056	0.0026
	Glutamate	0.0916	0.0154	**0.0024**	0.0203	**0.0008**	0.0023	0.3884	0.0318	0.0544	0.0071	0.0059	0.0019	0.1080	0.0117
	Glutamine	**0.0003**	0.0043	**0.0110**	0.0252	0.1537	0.0115	0.2881	0.0280	0.0732	0.0088	0.4706	0.0325	0.2292	0.0189
	Glyceraldehdye-3-phosphate	0.1971	0.0208	**0.0001**	0.0126	0.2033	0.0135	0.0782	0.0167	0.0991	0.0109	0.0870	0.0108	0.0420	0.0065
	Glycerol-3-phosphate	**0.0035**	0.0073	**0.0000**	0.0029	0.1290	0.0105	0.0076	0.0075	0.0008	0.0005	0.5612	0.0361	0.6204	0.0366
	GMP	**0.0009**	0.0053	**0.0000**	0.0031	0.9982	0.0500	0.1827	0.0231	0.2833	0.0221	0.8429	0.0461	0.5142	0.0321

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Histidine	0.0107	0.0092	**0.0000**	0.0014	**0.0002**	0.0019	**0.0021**	0.0053	0.0133	0.0027	0.5881	0.0372	0.1158	0.0122
	Homocysteic acid	**0.0015**	0.0060	**0.0000**	0.0009	**0.0000**	0.0008	0.1069	0.0187	0.3046	0.0233	0.0654	0.0092	**0.0000**	0.0008
	Homocysteine	0.7585	0.0421	**0.0004**	0.0160	**0.0000**	0.0011	0.0116	0.0085	0.5030	0.0320	0.5682	0.0365	0.1078	0.0117
	Homoserine	**0.0032**	0.0072	**0.0035**	0.0214	**0.0000**	0.0003	0.0741	0.0163	0.0320	0.0048	0.7973	0.0447	0.1586	0.0148
	Hydroxyphenylacetic acid	0.0475	0.0127	**0.0008**	0.0174	0.6882	0.0356	**0.0000**	0.0013	0.0534	0.0070	0.5641	0.0363	0.2367	0.0192
	Hydroxyproline	**0.0062**	0.0082	**0.0271**	0.0290	**0.0000**	0.0000	0.9725	0.0493	0.0727	0.0088	0.7487	0.0433	0.3149	0.0232
	IMP	0.0700	0.0140	**0.0000**	0.0066	0.3673	0.0204	0.4483	0.0341	0.5552	0.0341	0.6307	0.0387	0.4130	0.0279
	Inosine	0.0850	0.0149	0.7518	0.0480	0.7809	0.0401	**0.0011**	0.0046	0.5129	0.0324	0.1090	0.0125	0.0216	0.0044
	Isocitrate	0.1830	0.0203	**0.0001**	0.0122	0.0317	0.0054	0.1726	0.0226	0.8251	0.0443	0.7279	0.0425	0.5124	0.0321
	Lactate	0.0607	0.0134	**0.0244**	0.0285	0.3127	0.0180	**0.0033**	0.0060	0.7768	0.0426	0.2374	0.0215	0.5041	0.0317
	Leucine/isoleucine	0.1111	0.0167	**0.0015**	0.0190	**0.0008**	0.0023	0.3168	0.0291	0.1403	0.0141	0.1475	0.0155	0.7102	0.0402
	Lysine	**0.0002**	0.0041	**0.0000**	0.0106	**0.0000**	0.0004	0.0164	0.0096	0.2050	0.0180	0.1185	0.0134	0.1364	0.0133
	Malate	0.6042	0.0367	**0.0002**	0.0143	0.8104	0.0416	0.0771	0.0165	0.6215	0.0369	0.2009	0.0192	0.0174	0.0040
	Methionine	**0.0001**	0.0034	**0.0000**	0.0083	**0.0000**	0.0007	0.1730	0.0226	0.0081	0.0018	0.1177	0.0133	0.2855	0.0218
	Methylcysteine	0.7080	0.0405	**0.0003**	0.0154	**0.0000**	0.0011	0.0102	0.0083	0.4992	0.0318	0.6178	0.0383	0.1196	0.0124
	Methylmalonic acid	0.1990	0.0210	**0.0049**	0.0224	0.7269	0.0375	0.0482	0.0140	0.1769	0.0163	0.2044	0.0195	0.4817	0.0307
	Myo-inositol	0.5719	0.0356	**0.0000**	0.0112	0.2383	0.0148	0.5843	0.0385	0.3961	0.0273	0.5158	0.0343	0.6665	0.0385
	N-acetyl-glucosamine-1/6-phosphate	**0.0025**	0.0068	**0.0000**	0.0050	0.3744	0.0207	0.8535	0.0462	0.0772	0.0091	0.0055	0.0019	0.0871	0.0101
	N-acetyl-glutamate	0.0541	0.0130	**0.0000**	0.0089	0.9853	0.0494	0.2622	0.0268	0.7660	0.0422	0.3365	0.0266	0.7386	0.0413
	N-acetyl-glutamine	**0.0000**	0.0014	**0.0165**	0.0268	0.2282	0.0143	**0.0007**	0.0042	0.1448	0.0144	0.9535	0.0490	0.1669	0.0153
	N-Acetyl-L-alanine	**0.0000**	0.0029	**0.0107**	0.0251	0.8385	0.0427	0.0740	0.0163	0.5533	0.0340	0.3309	0.0263	0.7092	0.0402
	NAD+	0.1876	0.0205	**0.0000**	0.0041	0.3424	0.0191	0.0089	0.0079	0.5314	0.0332	0.3226	0.0259	0.1065	0.0116
	NADH	0.4313	0.0305	**0.0179**	0.0272	0.4605	0.0251	0.3687	0.0311	0.9352	0.0478	0.3559	0.0275	0.3734	0.0263

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	NADP+	0.1318	0.0178	**0.0000**	0.0115	0.8232	0.0421	0.2351	0.0257	0.4457	0.0296	0.7960	0.0446	0.0198	0.0043
	NADPH	0.1244	0.0174	**0.0000**	0.0106	0.4071	0.0224	0.0123	0.0087	0.7213	0.0405	0.0480	0.0075	0.5164	0.0323
	Nicotinamide ribotide	0.3778	0.0284	0.2638	0.0409	0.5009	0.0270	0.1904	0.0235	0.0073	0.0017	0.0069	0.0022	**0.0011**	0.0017
	Ornithine	0.0147	0.0098	**0.0000**	0.0063	0.0043	0.0032	0.0989	0.0182	0.2048	0.0180	0.6521	0.0396	0.7173	0.0404
	Orotate	**0.0000**	0.0009	0.0693	0.0335	0.0912	0.0086	0.0879	0.0174	0.7447	0.0414	0.3943	0.0292	0.1754	0.0157
	Pantothenate	**0.0000**	0.0001	**0.0000**	0.0034	0.1562	0.0116	0.0998	0.0183	0.0067	0.0016	0.2403	0.0217	0.2550	0.0203
	Phosphoenolpyruvate	0.9511	0.0485	**0.0017**	0.0193	0.9133	0.0463	**0.0067**	0.0072	0.9315	0.0477	0.3292	0.0262	0.1161	0.0122
	Proline	0.0996	0.0158	**0.0000**	0.0059	**0.0000**	0.0009	0.0622	0.0153	0.4494	0.0298	0.2988	0.0246	0.0988	0.0110
	Pyrophosphate	0.2282	0.0224	0.0404	0.0309	0.8888	0.0451	**0.0000**	0.0022	0.2598	0.0208	0.0132	0.0032	0.4125	0.0279
	Quinolinate	0.0429	0.0123	**0.0051**	0.0226	0.3700	0.0205	**0.0002**	0.0032	0.0013	0.0007	0.2783	0.0238	0.0262	0.0049
	Ribose-phosphate	0.0118	0.0093	**0.0000**	0.0046	0.2968	0.0174	0.1480	0.0214	0.3928	0.0272	0.8201	0.0456	0.0451	0.0067
	Serine	0.0207	0.0105	**0.0025**	0.0204	**0.0007**	0.0023	0.6352	0.0399	0.3510	0.0255	0.4327	0.0310	0.4561	0.0296
	Shikimate	0.6408	0.0380	**0.0000**	0.0102	0.0065	0.0036	**0.0000**	0.0011	0.9771	0.0493	0.2193	0.0204	0.3566	0.0254
	Succinate	0.1990	0.0210	**0.0049**	0.0224	0.7269	0.0375	0.0482	0.0140	0.1769	0.0163	0.2044	0.0195	0.4817	0.0307
	Taurine	0.1149	0.0168	**0.0118**	0.0255	0.5497	0.0292	0.0324	0.0121	0.0004	0.0003	0.2048	0.0195	0.2178	0.0183
	Taurodeoxycholic acid	0.5504	0.0348	**0.0000**	0.0080	0.2672	0.0161	**0.0007**	0.0042	0.1161	0.0122	**0.0000**	0.0004	0.3763	0.0265
	Threonine	**0.0032**	0.0072	**0.0035**	0.0214	**0.0000**	0.0003	0.0741	0.0163	0.0320	0.0048	0.7973	0.0447	0.1586	0.0148
	Thymidine	0.0709	0.0141	**0.0000**	0.0016	0.2775	0.0164	0.0566	0.0147	0.2483	0.0200	0.4443	0.0314	0.9148	0.0471
	Tryptophan	**0.0000**	0.0023	0.1440	0.0374	**0.0000**	0.0015	**0.0052**	0.0068	0.0002	0.0002	0.0435	0.0071	0.5789	0.0350
	Tyrosine	0.0364	0.0119	**0.0000**	0.0114	**0.0000**	0.0006	0.0097	0.0081	0.0027	0.0010	0.1487	0.0156	0.4456	0.0293
	UDP	**0.0000**	0.0024	**0.0001**	0.0126	0.3289	0.0186	0.2700	0.0272	0.6575	0.0383	0.0683	0.0093	0.0683	0.0086
	UDP-D-glucose	0.8775	0.0460	**0.0000**	0.0023	0.5912	0.0311	0.9917	0.0498	0.1057	0.0115	0.1151	0.0131	**0.0002**	0.0012
	UDP-D-glucuronate	0.2100	0.0215	**0.0000**	0.0039	0.3328	0.0188	0.1449	0.0211	0.1699	0.0159	0.1238	0.0138	0.0021	0.0020
	UDP-N-acetyl-glucosamine	**0.0009**	0.0053	**0.0000**	0.0027	0.8148	0.0417	0.1872	0.0233	0.2415	0.0197	0.0690	0.0094	**0.0001**	0.0008

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	UMP	**0.0014**	0.0059	**0.0000**	0.0062	0.6930	0.0358	0.4694	0.0347	0.2138	0.0184	0.8966	0.0475	0.5068	0.0318
	Uracil	0.2372	0.0227	**0.0008**	0.0174	0.1842	0.0127	0.2904	0.0281	0.3817	0.0267	0.2698	0.0234	0.1845	0.0162
	Uric acid	0.0154	0.0099	**0.0002**	0.0140	0.4695	0.0256	0.7785	0.0441	0.4164	0.0282	0.2189	0.0204	0.0029	0.0022
	Uridine	0.3418	0.0270	**0.0000**	0.0034	**0.0024**	0.0028	**0.0000**	0.0005	0.0393	0.0057	0.0437	0.0071	0.2388	0.0193
	UTP	0.1888	0.0206	**0.0004**	0.0157	0.5637	0.0299	0.0882	0.0175	0.4895	0.0314	0.2541	0.0225	0.7713	0.0423
	Valine	0.6897	0.0398	**0.0000**	0.0092	**0.0000**	0.0013	0.7783	0.0441	0.0349	0.0052	0.4405	0.0313	0.5025	0.0316
	Xanthine	0.1720	0.0197	**0.0102**	0.0249	0.2479	0.0153	0.0246	0.0110	0.7752	0.0425	0.7331	0.0427	0.1210	0.0124
	Xanthosine	0.2670	0.0238	**0.0070**	0.0236	0.3806	0.0211	0.0107	0.0083	0.3209	0.0240	0.1314	0.0146	0.9667	0.0487
Liver	*2-Hydroxy-2-methylbutanedioic acid*	0.3591	0.0348	**0.0000**	0.0099	0.1217	0.0145	0.3291	0.0366	0.3661	0.0224	0.4126	0.0382	0.0966	0.0103
	2-Isopropylmalic acid	**0.0008**	0.0054	**0.0000**	0.0031	0.4955	0.0349	**0.0059**	0.0111	0.6728	0.0371	0.0258	0.0114	0.0311	0.0048
	3-methylphenylacetic acid	**0.0020**	0.0066	**0.0008**	0.0190	**0.0000**	0.0001	**0.0079**	0.0123	0.0844	0.0070	**0.0017**	0.0023	0.0165	0.0033
	3-phospho-serine	0.4091	0.0366	**0.0190**	0.0285	0.7751	0.0442	0.1156	0.0270	0.3121	0.0193	0.1298	0.0241	0.8198	0.0450
	3-phosphoglycerate	0.0897	0.0211	**0.0000**	0.0085	0.8867	0.0471	**0.0026**	0.0086	0.3556	0.0218	0.9809	0.0497	0.2907	0.0223
	6-phospho-D-gluconate	0.9959	0.0499	**0.0032**	0.0225	0.2809	0.0249	0.0674	0.0233	0.0859	0.0071	0.1413	0.0252	0.2221	0.0182
	a-ketoglutarate	0.6638	0.0429	**0.0000**	0.0070	0.2158	0.0210	**0.0001**	0.0027	0.0279	0.0028	0.0080	0.0058	0.0028	0.0013
	Acetyllysine	**0.0028**	0.0071	**0.0016**	0.0209	0.2101	0.0208	0.5183	0.0412	0.3074	0.0190	0.0708	0.0185	0.5246	0.0341
	Adenosine	**0.0010**	0.0057	**0.0000**	0.0077	0.0282	0.0052	0.1796	0.0308	0.2317	0.0151	0.0091	0.0063	**0.0003**	0.0006
	ADP	0.6995	0.0437	**0.0056**	0.0241	0.1167	0.0140	0.4641	0.0402	0.5657	0.0319	0.0181	0.0095	0.0028	0.0013
	ADP-D-glucose	0.1450	0.0254	**0.0002**	0.0159	0.0439	0.0070	0.4617	0.0402	0.2425	0.0156	0.3631	0.0365	0.4644	0.0315
	Allantoin	0.3076	0.0329	**0.0000**	0.0056	0.0578	0.0083	**0.0087**	0.0127	0.0143	0.0016	0.3419	0.0357	0.1513	0.0141
	Aminoadipic acid	**0.0042**	0.0079	**0.0000**	0.0025	0.3708	0.0295	0.5421	0.0418	0.2721	0.0170	0.2925	0.0338	0.9317	0.0482
	AMP	0.5093	0.0392	0.4817	0.0461	0.7868	0.0446	**0.0001**	0.0030	0.0613	0.0052	0.1157	0.0229	0.1117	0.0115

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Arginine	0.0455	0.0160	**0.0096**	0.0260	0.1004	0.0126	0.5090	0.0410	0.3477	0.0213	0.1708	0.0272	0.0939	0.0101
	Ascorbic acid	0.7015	0.0437	**0.0002**	0.0156	0.2869	0.0253	0.6770	0.0445	0.8770	0.0458	0.5495	0.0420	0.0821	0.0094
	Asparagine	**0.0002**	0.0043	**0.0051**	0.0237	0.8771	0.0468	0.0277	0.0179	0.4290	0.0257	0.3392	0.0357	0.0361	0.0055
	Aspartate	**0.0002**	0.0041	**0.0018**	0.0212	0.6974	0.0419	**0.0004**	0.0048	0.0600	0.0051	0.0706	0.0184	0.0008	0.0008
	ATP	0.3083	0.0329	**0.0014**	0.0204	0.0083	0.0030	**0.0043**	0.0103	0.2961	0.0184	0.0945	0.0209	0.0649	0.0081
	CDP-ethanolamine	**0.0000**	0.0012	0.3567	0.0444	0.0264	0.0050	**0.0003**	0.0045	0.8530	0.0448	0.0508	0.0159	0.3961	0.0277
	Cellobiose	0.3255	0.0336	**0.0014**	0.0205	0.5036	0.0352	0.2615	0.0343	0.1263	0.0095	0.0176	0.0094	0.1025	0.0108
	Citrate	0.2100	0.0288	**0.0001**	0.0129	0.9097	0.0477	0.6394	0.0437	0.6814	0.0375	0.2521	0.0320	0.4895	0.0326
	Citrate/isocitrate	0.2100	0.0288	**0.0001**	0.0129	0.9097	0.0477	0.6394	0.0437	0.6814	0.0375	0.2521	0.0320	0.4895	0.0326
	Citrulline	**0.0000**	0.0005	**0.0000**	0.0111	**0.0001**	0.0008	0.0560	0.0221	0.0979	0.0079	0.4249	0.0386	0.0407	0.0059
	CMP	0.0260	0.0132	**0.0001**	0.0132	0.8155	0.0452	0.2727	0.0347	0.5221	0.0300	0.6501	0.0442	0.2638	0.0207
	Creatine	**0.0036**	0.0076	**0.0000**	0.0072	0.0643	0.0090	0.1939	0.0316	0.1919	0.0131	0.1291	0.0240	0.1968	0.0167
	Creatinine	**0.0002**	0.0040	0.0790	0.0355	0.0041	0.0022	0.0956	0.0256	0.3227	0.0199	0.2703	0.0328	0.0825	0.0095
	Cysteine	0.1367	0.0249	**0.0000**	0.0119	0.6311	0.0396	**0.0047**	0.0105	0.0088	0.0010	0.0874	0.0202	0.4040	0.0281
	Cytidine	0.8365	0.0468	**0.0005**	0.0177	0.2141	0.0210	**0.0011**	0.0065	0.7204	0.0394	0.0069	0.0053	0.2997	0.0227
	Deoxyuridine	0.0415	0.0155	**0.0008**	0.0190	0.1249	0.0149	0.1496	0.0294	0.6008	0.0336	0.1677	0.0270	0.1211	0.0121
	dGMP	0.5093	0.0392	0.4817	0.0462	0.7868	0.0446	**0.0001**	0.0031	0.0613	0.0053	0.1157	0.0229	0.1117	0.0115
	Dihydroorotate	**0.0007**	0.0052	0.1802	0.0401	0.0893	0.0116	0.3803	0.0380	0.1285	0.0097	0.1051	0.0218	0.2312	0.0187
	Dihydroxy-acetone-phosphate	0.8518	0.0471	**0.0000**	0.0054	0.1744	0.0182	**0.0064**	0.0114	0.0074	0.0008	0.3033	0.0343	0.1937	0.0166
	dUMP	0.0137	0.0110	**0.0021**	0.0215	0.2003	0.0201	0.0373	0.0197	0.3893	0.0235	0.1069	0.0220	0.6019	0.0372
	FAD	0.3383	0.0341	**0.0079**	0.0253	0.6128	0.0391	0.3780	0.0380	0.6759	0.0372	0.0220	0.0106	0.2740	0.0213
	Fructose-1,6-bisphosphate	0.1885	0.0278	**0.0001**	0.0150	0.6551	0.0405	**0.0020**	0.0080	0.0168	0.0018	0.9126	0.0486	0.6821	0.0405
	Fumarate	0.0596	0.0177	**0.0000**	0.0044	0.7627	0.0438	0.0277	0.0179	0.4617	0.0273	0.0177	0.0094	0.7425	0.0425
	Glucarate	0.0994	0.0219	**0.0076**	0.0251	0.4996	0.0350	0.1150	0.0270	0.4859	0.0285	0.3637	0.0365	0.4262	0.0292

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Gluconate	0.0567	0.0174	**0.0270**	0.0300	0.4462	0.0330	0.0971	0.0257	0.7679	0.0414	0.2675	0.0327	0.3989	0.0278
	Glucosamine-1/6-phosphate	**0.0017**	0.0063	0.0776	0.0354	0.1192	0.0143	0.0243	0.0171	0.3067	0.0190	0.0353	0.0134	0.0340	0.0052
	Glucose-1/6-phosphate	**0.0009**	0.0055	0.3695	0.0447	0.1709	0.0180	0.1226	0.0276	0.8050	0.0430	0.0818	0.0197	0.0984	0.0105
	Glutamine	**0.0026**	0.0070	**0.0000**	0.0024	0.3708	0.0295	0.1311	0.0282	0.2747	0.0172	0.1280	0.0239	0.0106	0.0026
	Glyceraldehdye-3-phosphate	0.8518	0.0471	**0.0000**	0.0054	0.1744	0.0182	**0.0064**	0.0114	0.0074	0.0008	0.3033	0.0343	0.1937	0.0165
	Glycerate	0.2129	0.0289	**0.0027**	0.0220	0.1744	0.0182	0.6039	0.0431	0.3480	0.0214	0.0859	0.0201	0.0980	0.0105
	Glycerol-3-phosphate	0.1424	0.0252	**0.0006**	0.0182	0.1862	0.0190	**0.0129**	0.0141	0.0057	0.0007	0.1882	0.0284	0.0706	0.0085
	GMP	0.2375	0.0300	0.3990	0.0451	0.7902	0.0447	**0.0000**	0.0014	0.0479	0.0042	0.2583	0.0322	0.0761	0.0089
	Guanidoacetic acid	0.1300	0.0243	**0.0000**	0.0097	0.0029	0.0020	0.1765	0.0307	0.9387	0.0479	0.2534	0.0321	0.8941	0.0472
	Guanine	0.0743	0.0196	**0.0000**	0.0095	0.5686	0.0375	**0.0050**	0.0106	0.0611	0.0052	0.0393	0.0141	0.2483	0.0198
	Guanosine	**0.0018**	0.0064	**0.0000**	0.0096	0.1760	0.0183	**0.0036**	0.0098	0.3098	0.0192	0.0751	0.0189	0.1187	0.0119
	Histidine	0.2594	0.0310	**0.0003**	0.0164	0.0344	0.0059	0.9710	0.0496	0.8927	0.0463	0.4148	0.0383	0.0275	0.0045
	Homocysteic acid	0.0356	0.0146	**0.0051**	0.0238	0.7000	0.0419	0.0366	0.0196	0.4872	0.0285	0.3268	0.0353	0.4485	0.0306
	Homocysteine	0.8352	0.0467	**0.0000**	0.0085	0.2918	0.0256	**0.0006**	0.0055	0.8867	0.0461	0.0159	0.0089	0.0384	0.0056
	Homoserine	**0.0001**	0.0038	0.0355	0.0312	0.0606	0.0086	0.0979	0.0258	0.1036	0.0082	0.0973	0.0211	0.0021	0.0011
	Hydroxyisocaproic acid	**0.0024**	0.0068	**0.0000**	0.0015	0.0714	0.0098	0.8148	0.0472	0.5287	0.0303	0.0067	0.0052	0.3967	0.0277
	Hydroxyphenylacetic acid	0.5740	0.0409	0.3127	0.0436	0.7670	0.0440	**0.0053**	0.0108	0.8383	0.0443	0.3317	0.0354	0.3669	0.0263
	Hydroxyphenylpyruvate	0.3703	0.0352	0.0920	0.0364	0.0222	0.0047	**0.0004**	0.0048	0.0372	0.0036	0.0537	0.0163	0.1650	0.0150
	Hydroxyproline	**0.0037**	0.0076	**0.0196**	0.0287	**0.0000**	0.0000	0.2407	0.0336	0.0013	0.0002	0.0037	0.0036	0.3762	0.0267
	Hypoxanthine	0.8138	0.0461	**0.0028**	0.0221	0.2980	0.0259	0.0937	0.0254	0.9118	0.0469	0.0261	0.0114	0.0630	0.0080
	IMP	0.2155	0.0291	**0.0001**	0.0137	0.0062	0.0026	0.1961	0.0318	0.0632	0.0054	0.1536	0.0261	0.0309	0.0048
	Isocitrate	0.2100	0.0288	**0.0001**	0.0130	0.9097	0.0477	0.6394	0.0437	0.6814	0.0375	0.2521	0.0320	0.4895	0.0326
	Lysine	**0.0072**	0.0091	**0.0111**	0.0267	0.3281	0.0274	0.0473	0.0212	0.6954	0.0382	0.1763	0.0275	0.1571	0.0144
	Malate	**0.0029**	0.0072	0.0483	0.0327	0.5864	0.0382	0.1010	0.0261	0.1454	0.0106	0.1144	0.0228	0.1039	0.0110

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Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Methionine	**0.0000**	0.0019	**0.0024**	0.0217	0.4843	0.0345	**0.0049**	0.0105	0.3216	0.0198	0.1236	0.0235	0.0106	0.0026
	Methionine sulfoxide	**0.0000**	0.0015	**0.0000**	0.0070	0.0302	0.0055	0.0612	0.0227	0.5116	0.0296	0.1168	0.0230	0.0090	0.0024
	Methylcysteine	0.9653	0.0494	**0.0000**	0.0108	0.2295	0.0219	**0.0018**	0.0078	0.8053	0.0430	0.0383	0.0138	0.1131	0.0115
	Myo-inositol	**0.0012**	0.0059	**0.0001**	0.0141	0.0163	0.0041	**0.0093**	0.0129	0.5403	0.0308	**0.0011**	0.0018	0.5926	0.0369
	N-acetyl-glucosamine-1/6-phosphate	0.0393	0.0152	**0.0000**	0.0066	0.4362	0.0325	0.0318	0.0187	0.2356	0.0153	0.9045	0.0485	0.2177	0.0179
	N-acetyl-glutamate	**0.0000**	0.0008	0.1612	0.0394	0.0020	0.0018	**0.0002**	0.0042	0.2158	0.0144	0.0258	0.0114	0.4491	0.0306
	N-Acetyl-L-alanine	0.7315	0.0444	**0.0007**	0.0188	0.5160	0.0356	0.2915	0.0354	0.3389	0.0208	0.0699	0.0183	0.4504	0.0307
	N-acetyl-L-ornithine	**0.0062**	0.0088	**0.0300**	0.0304	0.0157	0.0040	0.6739	0.0445	0.0348	0.0034	0.0091	0.0063	0.0134	0.0029
	NAD+	**0.0015**	0.0061	**0.0238**	0.0294	**0.0007**	0.0013	0.0311	0.0185	0.5742	0.0323	**0.0002**	0.0007	0.9326	0.0482
	NADH	**0.0007**	0.0052	**0.0264**	0.0299	0.9249	0.0481	0.9120	0.0487	0.8172	0.0435	0.1247	0.0236	0.8984	0.0473
	NADP+	0.0347	0.0144	0.3693	0.0446	0.1921	0.0195	**0.0016**	0.0075	0.2297	0.0150	0.2037	0.0295	0.6443	0.0390
	NADPH	**0.0024**	0.0069	**0.0074**	0.0250	0.9815	0.0496	0.8686	0.0481	0.9283	0.0475	0.2488	0.0318	0.8663	0.0462
	Octulose 8/1P	**0.0096**	0.0098	0.9317	0.0496	0.0411	0.0067	0.0955	0.0256	0.0286	0.0029	0.0130	0.0078	0.0674	0.0082
	Ornithine	**0.0000**	0.0023	0.4251	0.0454	0.0875	0.0114	0.1179	0.0272	0.3136	0.0194	0.4429	0.0391	0.0033	0.0014
	Orotate	0.0637	0.0183	**0.0055**	0.0240	0.9405	0.0485	0.9810	0.0497	0.4629	0.0274	0.1058	0.0219	0.6614	0.0396
	Pantothenate	**0.0000**	0.0002	**0.0000**	0.0055	0.0084	0.0030	**0.0000**	0.0004	0.0235	0.0024	**0.0002**	0.0007	0.0516	0.0069
	Proline	**0.0001**	0.0031	**0.0023**	0.0217	0.0434	0.0069	0.0216	0.0167	0.5678	0.0321	0.0531	0.0162	0.0125	0.0029
	Pyrophosphate	0.4032	0.0364	**0.0231**	0.0293	0.0278	0.0052	0.7105	0.0453	0.8977	0.0464	0.4674	0.0398	0.1186	0.0119
	Quinolinate	0.1750	0.0271	**0.0000**	0.0042	0.0249	0.0049	0.1320	0.0282	0.8226	0.0437	0.0114	0.0073	0.1389	0.0133
	Ribose-phosphate	0.0318	0.0141	**0.0000**	0.0048	0.2692	0.0243	**0.0008**	0.0060	0.9509	0.0483	0.6383	0.0439	0.9442	0.0486
	S-adenosyl-L-homoCysteine	**0.0000**	0.0003	**0.0000**	0.0044	0.2848	0.0251	**0.0001**	0.0033	0.4586	0.0271	0.0288	0.0120	0.0766	0.0089
	Sedoheptulose-1/7-phosphate	0.1324	0.0246	**0.0000**	0.0061	0.5624	0.0372	0.3638	0.0376	0.0130	0.0014	0.2936	0.0339	0.2532	0.0201
	Serine	**0.0006**	0.0050	**0.0153**	0.0277	0.1951	0.0198	0.0212	0.0166	0.2766	0.0173	0.0272	0.0118	0.0048	0.0017

Table E continued

Tissue	Metabolite	Sex	(i/m)q	Strain	(i/m)q	Diet	(i/m)q	Sex-by-Strain	(i/m)q	Sex-by-Diet	(i/m)q	Strain-by-Diet	(i/m)q	Sex-by-Strain-by-Diet	(i/m)q
	Shikimate	0.2509	0.0306	**0.0013**	0.0203	0.9423	0.0486	**0.0021**	0.0081	0.0372	0.0036	0.1042	0.0218	0.1387	0.0132
	Shikimate-3-phosphate	0.7714	0.0452	**0.0003**	0.0167	0.5578	0.0370	0.8653	0.0480	0.2207	0.0146	0.2984	0.0341	0.3113	0.0235
	Taurine	0.0957	0.0217	**0.0000**	0.0033	0.0194	0.0044	0.1541	0.0298	0.5894	0.0331	0.1035	0.0217	0.0428	0.0062
	Taurodeoxycholic acid	0.0410	0.0154	**0.0010**	0.0195	0.0343	0.0059	0.0665	0.0232	0.4358	0.0260	**0.0013**	0.0019	0.0192	0.0036
	Threonine	**0.0001**	0.0038	0.0355	0.0312	0.0606	0.0086	0.0979	0.0258	0.1036	0.0083	0.0973	0.0211	0.0021	0.0011
	Thymidine	0.0333	0.0143	**0.0017**	0.0210	0.2038	0.0203	0.0755	0.0239	0.2522	0.0160	0.3049	0.0344	0.1822	0.0158
	Thymine	**0.0045**	0.0081	0.1007	0.0368	0.7023	0.0420	0.1651	0.0302	0.2758	0.0172	0.3011	0.0343	0.4404	0.0301
	Trehalose/sucrose	0.4650	0.0380	**0.0000**	0.0101	0.2901	0.0255	0.2803	0.0350	0.3366	0.0207	0.0302	0.0125	0.0580	0.0075
	Tryptophan	**0.0004**	0.0046	0.3154	0.0437	0.5306	0.0361	0.0270	0.0177	0.6649	0.0367	0.1508	0.0259	0.1654	0.0150
	Tyrosine	**0.0001**	0.0033	**0.0038**	0.0230	0.2289	0.0218	0.0206	0.0165	0.5620	0.0318	0.2551	0.0321	0.0117	0.0028
	UDP	0.1094	0.0227	0.2388	0.0419	0.9686	0.0493	**0.0066**	0.0117	0.7293	0.0398	0.1261	0.0238	0.3177	0.0238
	UDP-D-glucuronate	0.4915	0.0388	0.4478	0.0457	0.6484	0.0403	**0.0031**	0.0092	0.9333	0.0477	0.2235	0.0306	0.0826	0.0095
	UMP	0.4726	0.0382	0.4387	0.0455	0.5778	0.0379	**0.0002**	0.0040	0.1982	0.0134	0.1140	0.0228	0.0796	0.0092
	Uracil	**0.0053**	0.0085	0.6503	0.0478	0.5844	0.0382	**0.0002**	0.0040	0.6371	0.0354	0.0340	0.0132	0.1709	0.0154
	Uric acid	**0.0070**	0.0091	0.6690	0.0480	0.5637	0.0373	**0.0005**	0.0052	0.1857	0.0128	0.3580	0.0363	0.3276	0.0243
	Uridine	**0.0035**	0.0076	0.0592	0.0339	0.2255	0.0217	**0.0025**	0.0085	0.1593	0.0116	0.0625	0.0175	0.1262	0.0124
	Valine	**0.0010**	0.0056	**0.0000**	0.0086	0.2494	0.0231	0.0327	0.0188	0.7115	0.0390	0.0316	0.0127	0.0039	0.0015
	Xanthine	0.0360	0.0147	0.6862	0.0481	0.4075	0.0313	**0.0026**	0.0087	0.3041	0.0188	0.0570	0.0167	0.1272	0.0124

All known metabolites considered significant in the ANOVA were identified in liver, skeletal muscle, and adipose tissue. Bold values indicate that the metabolite is statistically significant. Bold metabolites are not tissue specific. Bold and italicized metabolites are shared between all three tissues. Benjamini-Hochberg procedure was performed to determine significance.

APPENDIX F

Table F.6: Number of Edges for Each Node in Correlation Network Analysis Across Strains and Diets

Metabolite	Tissue	Total Degree (+/-)	Degree (Adipose) (+/-)	Degree (Muscle) (+/-)	Degree (Liver) (+/-)	Degree (Phenotype) (+/-)
3-methylphenylacetic acid	Adipose	13 (9/4)	6 (6/0)	2 (0/2)	1 (1/0)	4 (2/2)
4-aminobutyrate	Adipose	5 (3/2)	2 (2/0)	0	1 (0/1)	2 (1/1)
AMP	Adipose	15 (12/3)	12 (10/2)	0	1 (1/0)	2 (1/1)
CDP-ethanolamine	Adipose	18 (15/3)	13 (12/1)	1 (1/0)	1 (0/1)	3 (2/1)
GMP	Adipose	15 (12/3)	12 (10/2)	0	0	3 (2/1)
IMP	Adipose	14 (10/4)	12 (10/2)	0	0	2 (0/2)
N-acetyl-glutamate	Adipose	21 (15/6)	12 (11/1)	2 (1/1)	2 (0/2)	5 (3/2)
NAD+	Adipose	13 (12/1)	12 (11/1)	0	0	1 (0/1)
S-adenosyl-L-homoCysteine	Adipose	18 (9/9)	6 (6/0)	4 (0/4)	4 (1/3)	4 (2/2)
UMP	Adipose	13 (10/3)	12 (10/2)	0	0	1 (0/1)
a-ketoglutarate	Adipose	9 (7/2)	2 (2/0)	3 (2/1)	2 (2/0)	2 (1/1)
Cysteine	Adipose	16 (13/3)	10 (9/1)	3 (2/1)	2 (1/1)	1 (1/0)
dGMP	Adipose	15 (12/3)	12 (10/2)	0	1 (1/0)	2 (1/1)
Inosine	Adipose	6 (3/3)	4 (3/1)	1 (0/1)	0	1 (0/1)
Pantothenate	Adipose	28 (13/15)	12 (11/1)	6 (0/6)	6 (0/6)	4 (2/2)
Quinolinate	Adipose	13 (3/10)	0	5 (1/4)	7 (2/5)	1 (0/1)
Glycerol-3-phosphate	Adipose	11 (10/1)	8 (8/0)	1 (1/0)	1 (1/0)	1 (0/1)
Trehalose/sucrose	Adipose	12 (5/7)	7 (1/6)	1 (1/0)	3 (2/1)	1 (1/0)
Uracil	Adipose	11 (7/4)	6 (6/0)	1 (0/1)	2 (0/2)	2 (1/1)
2-Hydroxy-2-methylbutanedioic acid	Liver	20 (19/1)	1 (1/0)	6 (5/1)	11 (11/0)	2 (2/0)
4-Pyridoxic acid	Liver	9 (5/4)	2 (2/0)	4 (1/3)	2 (2/0)	1 (0/1)
Cellobiose	Liver	12 (11/1)	0	0	10 (10/0)	2 (1/1)
Glucarate	Liver	15 (6/9)	5 (0/5)	1 (0/1)	5 (4/1)	4 (2/2)
FMN	Liver	24 (9/15)	5 (3/2)	7 (1/6)	8 (2/6)	4 (3/1)
IMP	Liver	19 (14/5)	3 (0/3)	1 (0/1)	13 (13/0)	2 (1/1)
N-Acetyl-L-alanine	Liver	22 (19/3)	3 (2/1)	4 (4/0)	13 (12/1)	2 (1/1)
N-acetyl-glutamine	Liver	23 (16/7)	3 (0/3)	5 (5/0)	12 (10/2)	3 (1/2)
Adenine	Liver	10 (8/2)	0	0	7 (7/0)	3 (1/2)
Adenosine	Liver	11 (7/4)	2 (1/1)	2 (1/1)	5 (4/1)	2 (1/1)

Table F continued

Metabolite	Tissue	Total Degree (+/-)	Degree (Adipose) (+/-)	Degree (Muscle) (+/-)	Degree (Liver) (+/-)	Degree (Phenotype) (+/-)
Citrulline	Liver	5 (2/3)	1 (0/1)	0	1 (1/0)	3 (1/2)
Cystathionine	Liver	12 (7/5)	1 (1/0)	2 (1/1)	7 (4/3)	2 (1/1)
dAMP	Liver	4 (2/2)	0	0	1 (0/1)	3 (2/1)
Deoxyinosine	Liver	21 (17/4)	2 (0/2)	4 (4/0)	12 (12/0)	3 (1/2)
Deoxyuridine	Liver	22 (19/3)	2 (1/1)	6 (5/1)	13 (12/1)	1 (1/0)
Guanosine	Liver	16 (15/1)	0	2 (2/0)	12 (12/0)	2 (1/1)
Homocysteic acid	Liver	10 (9/1)	1 (1/0)	4 (3/1)	4 (4/0)	1 (1/0)
Inosine	Liver	19 (14/5)	1 (0/1)	2 (2/0)	13 (11/2)	3 (1/2)
Quinolinate	Liver	4 (3/1)	0	0	3 (2/1)	1 (1/0)
Ribose-phosphate	Liver	22 (16/6)	2 (0/2)	3 (2/1)	14 (13/1)	3 (1/2)
Trehalose/sucrose	Liver	12 (11/1)	0	0	10 (10/0)	2 (1/1)
1-Methyl-Histidine	Muscle	18 (13/5)	2 (0/2)	4 (4/0)	9 (8/1)	3 (1/2)
2-Hydroxy-2-methylbutanedioic acid	Muscle	20 (17/3)	2 (2/0)	6 (5/1)	11 (9/2)	1 (1/0)
2-Isopropylmalic acid	Muscle	14 (2/12)	2 (0/2)	6 (0/6)	5 (2/3)	1 (0/1)
2-dehydro-D-gluconate	Muscle	13 (9/4))	3 (1/2)	8 (6/2)	1 (1/0)	1 (1/0)
6-phospho-D-gluconate	Muscle	12	2 (0/2)	5 (4/1)	4 (3/1)	1 (1/0)
N-Acetyl-L-alanine	Muscle	11 (7/4)	3 (0/3)	5 (5/0)	1 (1/0)	2 (1/1)
N-acetyl-glutamine	Muscle	15 (8/7)	3 (0/3)	6 (5/1)	3 (2/1)	3 (1/2)
NADPH	Muscle	6 (4/2)	1 (0/1)	3 (3/0)	1 (0/1)	1 (1/0)
a-ketoglutarate	Muscle	18 (13/5)	2 (1/1)	8 (6/2)	7 (5/2)	1 (1/0)
Asparagine	Muscle	14 (11/3)	2 (1/1)	8 (7/1)	3 (2/1)	1 (1/0)
Lysine	Muscle	10 (5/5)	3 (3/0)	0	5 (1/4)	2 (1/1)
Orotate	Muscle	15 (8/7)	4 (0/4)	5 (5/0)	3 (2/1)	3 (1/2)
Xanthosine	Muscle	3 (1/2)	1 (0/1)	1 (0/1)	0	1 (1/0)
RER	Phenotype	4 (2/2)	2 (1/1)	0	2 (1/1)	0
VO2	Phenotype	7 (1/6)	5 (0/5)	0	2 (1/1)	0
Weight	Phenotype	28 (11/17)	10 (9/1)	4 (0/4)	14 (2/12)	0
Activity	Phenotype	6 (6/0)	3 (3/0)	1 (1/0)	2 (2/0)	0
Adiposity	Phenotype	53 (30/23)	19 (4/15)	13 (10/3)	21 (16/5)	0
Heat Output	Phenotype	14 (6/8)	3 (3/0)	3 (0/3)	8 (3/5)	0

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APPENDIX G

D12052701 - 06

OpenStandard Diet with tBHQ and Same With Modifications

Product #	D12052701	D12052702	D12052703	D12052704	D12052705	D12052706
	OpenStandard Diet (Modified)	*Mediterranean Diet*	*Japanese*	*Hunter-Gatherer*	*Western*	*Ketogenic*
Ingredient	**gm**	**gm**	**gm**	**gm**	**gm**	**gm**
Casein	200	44	0	0	38.5	160
Soy Protein Isolate, Supro 661	0	0	16	0	0	0
Fish Protein Isolate	0	27	27	152	8.5	0
Egg White	0	9	6	48	55	0
Beef, Cooked	0	61.9	7	126.5	76.9	0
L-Cystine	3	3	3	3	3	3
Corn Starch	641	0	0	0	30	0
Wheat Starch	0	365	0	0	195	0
Rice Starch	0	0	763.5	0	0	0
Potato Starch	0	0	0	333	30	0
Sucrose	0	61	70	60	205	0
Fructose	0	19	6	29	22	0
Cellulose, BW200	75	40.7	10	75	18.2	37.5
Inulin	25	13.5	38	25	6	12.5
Soybean Oil	70	0	10.5	0	0	0
Corn Oil	0	0	0	0	34.4	8
Menhaden Oil (200 ppm tBHQ)	0	13.2	5	24.6	1	8
Trisun Sunflower Oil	0	0	0	0	0	0
Palm Kernal Oil	0	9.9	0	14.7	0	0
Butter, Anhydrous	0	7.4	4.2	0	54.1	161
Lard	0	0	0	0	0	161
Safflower Oil	0	0	0	11.8	0	0
Flaxseed Oil	0	6.1	0	34.4	1	0
Olive Oil	0	117.7	11	0	27.5	0
t-BHQ	0.0049	0.0023	0.0039	0	0.0047	0.0033
Mineral Mix S10026	10	10	10	10	10	10
Dicalcium Phosphate	13	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H2O	16.5	16.5	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10	10	10
Biotin (1%)	0	0.014	0.014	0.1	0.1	0
Choline Bitartrate	2	2	2	2	2	2
Cholesterol	0	0	0.19	0.27	1.5	1.5
Red Wine Extract	0	0.045	0	0	0	0
Green Tea Extract	0	0	0.215	0	0	0
FD&C Red Dye #5	0	0.05	0	0.025	0	0.025
FD&C Yellow Dye #40	0.05	0	0.025	0.025	0	0
FD&C Blue Dye #1	0	0	0.025	0	0.05	0.025
Total	**1071.0549**	**855.5113**	**1034.6729**	**994.42**	**864.7547**	**609.5533**

D12052701 - 06

OpenStandard Diet with tBHQ and Same With Modifications

	D12052701	D12052702	D12052703	D12052704	D12052705	D12052706
g	OpenStandard Diet	Mediterranean Diet	Japanese	Hunter-Gatherer	Western	Ketogenic
Protein	177.0	115.7	120.8	271.1	140.7	142.2
Carbohydrate	570.3	406.7	689.1	362.1	455.3	3.1
Fat	72.4	172.3	44.6	123.2	139.7	339.9
Cholesterol	0.0	0.21	0.23	0.62	1.81	2.09
Fiber	93.8	50.8	50.0	93.8	22.7	46.9
g%						
Protein	16.5	13.5	11.7	27.3	16.3	23.3
Carbohydrate	53.2	47.5	66.6	36.4	52.6	0.5
Fat	6.8	20.1	4.3	12.4	16.2	55.8
Cholesterol	0.0	0.024	0.023	0.063	0.210	0.342
Fiber	8.8	5.9	4.8	9.4	2.6	7.7
kcal						
Protein	708	463	483	1084	563	569
Carbohydrate	2281	1627	2757	1448	1821	13
Fat	652	1551	401	1109	1257	3059
Total	3641	3641	3641	3641	3641	3641
kcal%						
Protein	19	12.71	13	30	15	16
Carbohydrate	63	44.69	76	40	50	0
Fat	18	42.60	11	30	35	84
g% Contribution to Protein sources						
Casein	98.3	33.1	0.0	0.0	23.8	97.9
Soy Protein Isolate, Supro 661	0.0	0.0	11.5	0.0	0.0	0.0
Fish Protein Isolate	0.0	22.2	21.2	53.3	5.7	0.0
Egg White	0.0	6.3	4.0	14.4	31.7	0.0
Beef, Cooked	0.0	35.8	3.9	31.3	36.6	0.0
Rice Starch	0.0	0.0	56.9	0.0	0.0	0.0
L-Cystine	1.7	2.6	2.5	1.1	2.1	2.1
g% Contribution to Carb sources						
Corn Starch	100.0	0.0	0.0	0.0	5.9	0.0
Wheat Starch	0.0	80.1	0.0	0.0	38.4	0.0
Rice Starch	0.0	0.0	88.8	0.0	0.0	0.0
Potato Starch	0.0	0.0	0.0	74.7	5.3	0.0
Sucrose	0.0	15.1	10.3	17.0	45.6	0.0
Fructose	0.0	4.7	0.9	8.2	4.9	0.0
Cellulose, BW200 (Insoluble) (g/kg)	70.0	47.6	9.7	75.4	21.0	61.5
Inulin (Soluble) (g/kg)	23.3	15.8	36.7	25.1	6.9	20.5
Total Fiber (g/kg) (includes starch source)	87.5	59.4	48.3	94.3	26.3	76.9
Cholesterol (mg/kg)	37.3	239.8	225.3	627.8	2096.2	3422.9
Red Wine Extract (mg/kg)	0	52.6	0	0	0	0
Green Tea Extract (mg/kg)	0	0	207.8	0	0	0
tBHQ (mg/3641 kcals)	4.9	4.9	4.9	4.9	4.9	4.9
Biotin (mg/3641 kcals)	0.20	0.34	0.34	1.20	1.20	0.20

APPENDIX H

Typical Fatty Acid Profiles of D12052701 - 06 Based on
Data from the Manufacturers of the Fats Listed

	D12052701	D12052702	D12052703	D12052704	D12052705	D12052706
	OSD	Mediterranean	Japanese	Hunter-Gatherer	Western	Ketogenic
Ingredient (g)		gm	gm	gm	gm	gm
Corn Oil					34.4	8
Menhaden Oil, ARBP-F		13.2	5	24.6	1	8
Beef, Cooked		61.9	7	126.5	76.9	
Butter, Anhydrous		7.4	4.2		54.1	161
Lard						161
Palm Kernal Oil		9.9		14.7		
Olive Oil		117.7	11		27.5	
Safflower Oil, USP				11.8		
Flaxseed Oil		6.1		34.4	1	
Soybean Oil, USP	70		10.5			
Total	70	216.2	37.7	212	194.9	338
C2, Acetic	0.0	0.0	0.0	0.0	0.0	0.0
C4, Butyric	0.0	0.2	0.1	0.0	1.7	5.2
C6, Caproic	0.0	0.1	0.1	0.0	1.0	3.1
C8, Caprylic	0.0	0.6	0.0	0.8	0.6	1.8
C10, Capric	0.0	0.6	0.1	0.6	1.4	4.1
C12, Lauric	0.0	4.7	0.1	6.7	1.5	4.7
C14, Myristic	0.0	3.6	0.8	4.9	6.1	18.5
C14:1, Myristoleic	0.0	0.3	0.1	0.3	1.0	2.4
C15:0	0.0	0.1	0.0	0.3	0.1	0.2
C16, Palmitic	7.3	22.1	4.6	14.5	25.9	75.3
C16:1, Palmitoleic	0.0	3.5	0.8	3.6	2.4	6.7
C16:2	0.0	0.2	0.1	0.4	0.0	0.1
C16:3	0.0	0.2	0.1	0.4	0.0	0.1
C16:4	0.0	0.2	0.1	0.4	0.0	0.1
C17:0	0.0	0.2	0.0	0.5	0.2	0.6
C17:1, n-9	0.0	0.2	0.0	0.3	0.2	0.0
C18, Stearic	2.7	6.2	1.5	6.2	10.2	36.9
C18:1, Oleic, n-9	17.0	95.5	12.6	26.2	50.5	96.1
C18:1, Vaccenic, n-7	0.0	0.7	0.1	1.5	0.9	0.0
C18:2, Linoleic, n-6	37.5	17.6	7.3	16.9	26.5	47.9
C18:3, gamma-Linolenic, n-	0.0	0.0	0.0	0.0	0.0	0.0
C18:3, alpha-Linolenic, n-3	5.5	4.4	1.0	19.4	2.0	4.6
C18:4, Stearidonic, n-3	0.0	0.4	0.2	0.8	0.0	0.2
C20, Arachidic	0.0	0.6	0.1	0.1	0.7	1.8
C20:1, n-9	0.0	0.5	0.1	0.5	0.2	1.1
C20:2, Eicosadienoic, n-6	0.0	0.0	0.0	0.1	0.0	1.3
C20:3, n-6	0.0	0.1	0.0	0.2	0.0	0.2
C20:3, n-3	0.0	0.0	0.0	0.0	0.0	0.0
C20:4, Arachidonic, n-6	0.0	0.4	0.1	0.7	0.1	0.6
C20:4, n-3	0.0	0.0	0.0	0.0	0.0	0.0
C20:5, Eicosapentaenoic, n	0.0	1.9	0.7	3.5	0.1	1.1
C21:0	0.0	0.0	0.0	0.0	0.0	0.0
C21:5, n-3	0.0	0.1	0.0	0.2	0.0	0.1
C22, Behenic	0.0	0.0	0.0	0.0	0.0	0.0
C22:1, Erucic	0.0	0.0	0.0	0.1	0.0	0.0
C22:4, Clupanodonic, n-6	0.0	0.0	0.0	0.1	0.0	0.0
C22:5, n-3	0.0	0.4	0.1	0.7	0.0	0.4
C22:5, n-6	0.0	0.0	0.0	0.0	0.0	0.0
C22:6, Docosahexaenoic, n	0.0	1.4	0.5	2.5	0.1	0.8
C24, Lignoceric	0.0	0.1	0.0	0.2	0.0	0.0
C24:1	0.0	0.0	0.0	0.1	0.0	0.0
Total	69.9	167.4	31.7	113.5	133.7	316.3

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**Typical Fatty Acid Profiles of D12052701 - 06 Based on
Data from the Manufacturers of the Fats Listed**

	OSD	Mediterranean	Japanese	Hunter-Gatherer	Western	Ketogenic
Saturated (g)	9.9	39.0	7.6	33.9	49.1	151.3
Monounsaturated (g)	17.0	100.6	13.6	31.9	54.1	106.4
Polyunsaturated (g)	42.9	26.5	10.0	44.8	29.0	57.3
Saturated (%)	14.2	23.3	23.9	29.9	36.7	47.9
Monounsaturated (%)	24.3	60.1	43.0	28.2	40.5	33.6
Polyunsaturated (%)	61.4	15.9	31.6	39.5	21.7	18.1
Total Omega-6 (g)	37.5	18.1	7.5	17.9	26.7	50.1
Total Omega-6 (%)	53.6	10.8	23.6	15.8	20.0	15.9
Total Omega-3 (g)	5.5	8.5	2.6	27.1	2.4	7.2
Total Omega-3 (%)	7.8	5.1	8.1	23.9	1.8	2.3
n6 : n3 Ratio	6.9	2.1	2.9	0.66	11.3	6.9

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APPENDIX I

Uncertainty Analysis Addendum For Chapter 5

kNN Imputation

Values may be missing from a dataset because they are not measured in a sample or because they are below the limit of detection. Imputation methods may be used to replace missing values. Missing values in metabolomics data are often replaced with some value since it is often accepted that a metabolite may be missing because it is below the detection limit of the mass spectrometer. Furthermore, missing values in metabolomics data are not well tolerated when performing multivariate analyses, such as PLSDA and PCA. The data analyzed in this dissertation used the kNN imputation method.

The original kNN imputation was performed using data that was missing 70% or less of metabolite values across samples and averaged 10 neighbors (see chapter 5 for results). This follow-up analysis has reduced the number of missing values to 10% or less and reduced the number of neighbors from 10 to 4. This resulted in a reduction of initial metabolites in the dataset by 33.1%, 26%, and 35.1% for adipose, skeletal muscle, and liver tissue with 13.7%, 14%, and 14% of the metabolites for each tissue is attributable to knowns (Table I.1).

Table I.7: Number of Metabolites Present in Each Raw File Before Imputation

Tissue	Total Metabolites	Knowns	Unknowns	Total
Adipose	70% Missing Values	139	4864	5003
	10% Missing Values	120	3228	3348
	Percent Difference	13.7%	33.6%	33.1%
Muscle	70% Missing Values	143	4877	5020
	10% Missing Values	123	3591	3714
	Percent Difference	14.0%	26.4%	26.0%
Liver	70% Missing Values	165	6292	6457
	10% Missing Values	142	4051	4193
	Percent Difference	14.0%	35.6%	35.1%

Comparison of ANOVA Analyses

ANOVA was used to assess the significance of each metabolite for sex, strain, and strain-by-sex interaction. The previous analysis determined that there were 85, 10, and 2 significant metabolites for strain, sex, and sex-by-strain interaction (Fig. 5.2), while the new analysis found 66, four, and one significant metabolites for each factor (Fig. I.1). Several metabolites were specific to one analysis for each factor (Fig. I.2). Since there were fewer metabolites in the second analysis due to the more stringent requirements, some of the metabolites in the first analysis are specific because they are no longer present in the second analysis (Table I.2). In adipose tissue six metabolites that were significant for strain were removed from the second analysis and in liver tissue 11 metabolites were removed from the second analysis for strain.

Comparison of Pathway Analyses

Functional pathway analysis was performed to map significant metabolites onto KEGG pathways. Several pathways were found in both analyses; however, some pathways were unique to each analysis (Table I.3, and Table 5.2). In the first analysis there were no significantly altered pathways for sex-by-strain interaction; however, in the second analysis sex-by-strain interaction altered pyrimidine metabolism in liver tissue. In addition, the number of hits for each pathway that were found in both analyses was altered. Purine metabolism, in liver, which was altered by strain, had 20 metabolites mapped onto this pathway but in the new analysis 17 metabolites mapped onto this pathway.
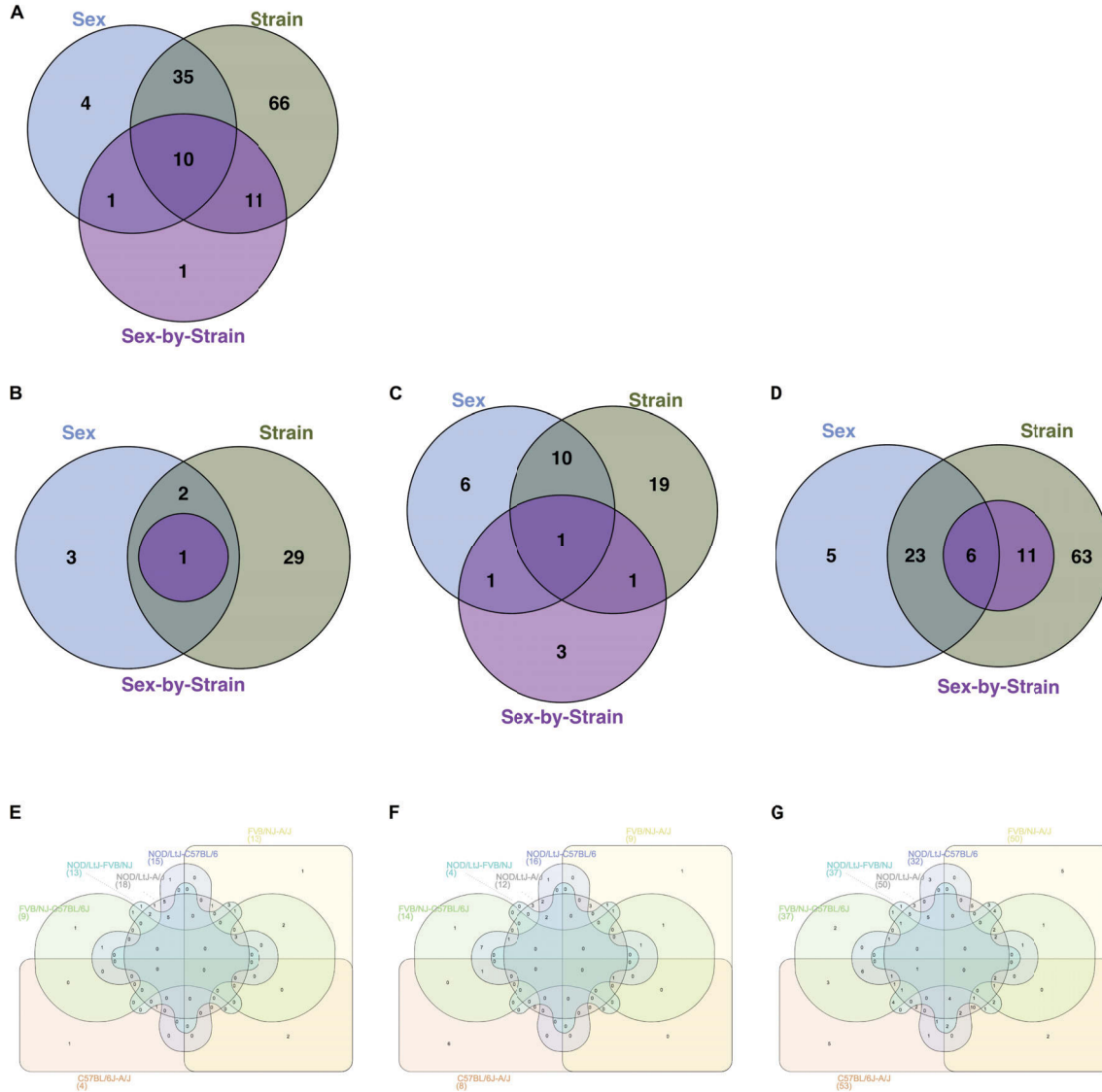
Figure I.1: Shared and unique effects of strain on tissue metabolomes. The sets of metabolites that differed significantly, based on ANOVA (FDR < 0.05) by strain across tissues, (A), and by sex, strain, and sex-by-strain interaction in liver (B), adipose (C), and muscle (D) were visualized for shared and factor-specific effects using Venn diagrams. Metabolites that differed significantly for strain in adipose (E), muscle (F), and liver (G) were assessed for pairwise comparisons using Tukey's HSD and visualized for shared and specific effects using Venn diagrams.

Figure I.2: Venn diagrams depicting overlap of significant metabolites from ANOVA that began with up to 70% of the values missing from each metabolite and ANOVA that began with up to 10% of the values missing from each metabolite. Comparison of each data analysis was performed for sex (A), strain (B), and sex-by-strain (C) for all tissues combined; sex (D), strain (E), and sex-by-strain (F) for adipose tissue; sex (G), strain (H), sex-by-strain (I) for muscle; and sex (J), strain (K), and sex-by-strain (L) for liver tissue.
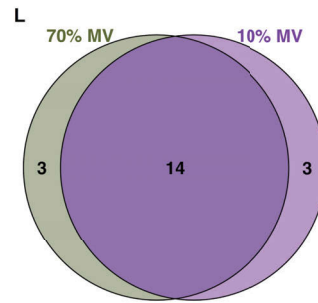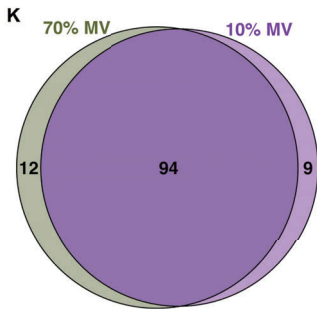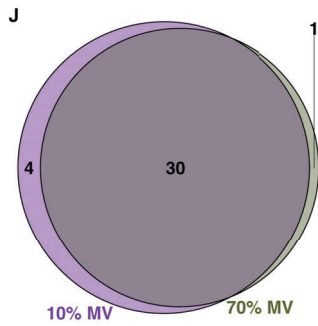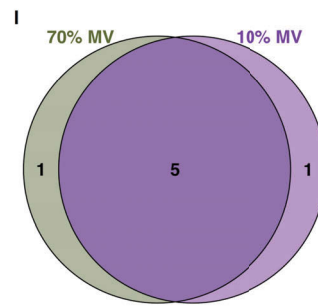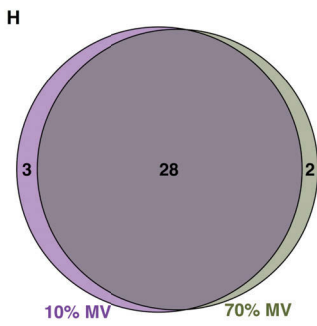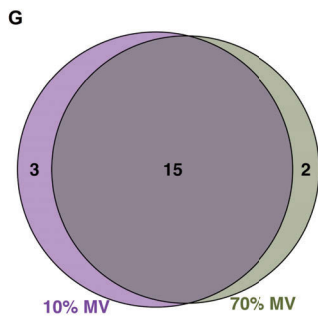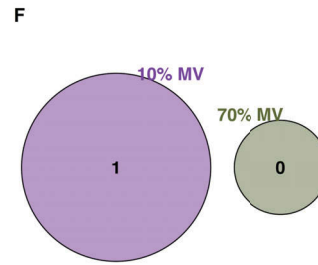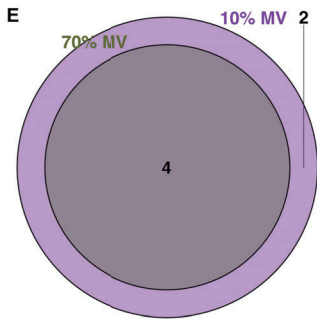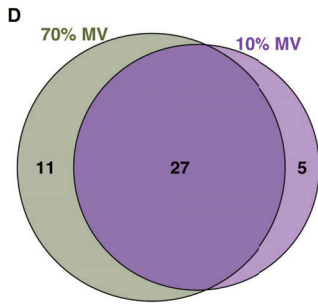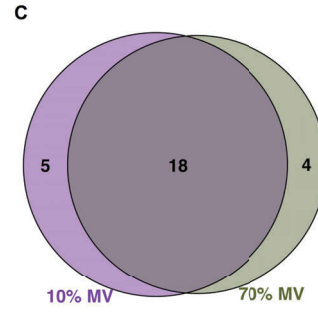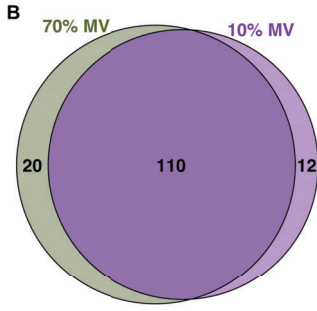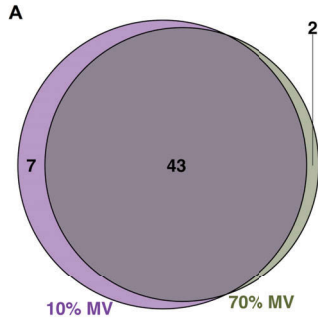
Table I.8: Comparison of Significant Metabolites from ANOVA

Tissue	Significant Metabolites	Sex	Strain	Sex-by-Strain
Adipose	70% KNN (removed from 10% KNN)	0	12 (6)	0
	Both	4	27	0
	10% KNN (Present but Not Significant in 70%)	2 (2)	5 (5)	1 (1)
Muscle	70% KNN (removed from 10% KNN)	1 (0)	1 (0)	1 (0)
	Both	17	30	5
	10% KNN (Present but Not Significant in 70%)	1 (1)	1 (1)	1 (1)
Liver	70% KNN (removed from 10% KNN)	4 (0)	11 (11)	3 (1)
	Both	29	96	14
	10% KNN (Present but Not Significant in 70%)	1 (1)	7 (7)	3 (3)

Table I.9: KEGG Pathway Enrichment of Metabolites Affected by Sex, Strain, and Sex-by-Strain

| Tissue	Factor	Pathway	Hits	P-value	(i/m)q
All Tissues	**Sex**	**Purine metabolism**	9	<0.001	0.001
	Strain	**Purine metabolism**	20	<0.001	0.008
		Pyrimidine metabolism	15	<0.001	0.008
		Alanine, aspartate and glutamate metabolism	8	<0.001	0.008
		Ascorbate and aldarate metabolism	5	<0.001	0.008
		Citrate cycle (TCA cycle)	6	0.002	0.008
		Pantothenate and CoA biosynthesis	5	0.003	0.008
		Aminoacyl-tRNA biosynthesis	12	0.003	0.008
		D-Glutamine and D-glutamate metabolism	3	0.003	0.008
Adipose	**Sex**	**Cysteine and methionine metabolism**	2	0.005	0.010
	Strain	**Purine metabolism**	8	<0.001	0.007
		Pyrimidine metabolism	5	0.001	0.007
		Arginine and proline metabolism	5	0.002	0.007
		Aminoacyl-tRNA biosynthesis	6	0.002	0.007
Muscle	**Sex**	**Aminoacyl-tRNA biosynthesis**	5	0.001	0.002
	Strain	**Aminoacyl-tRNA biosynthesis**	6	0.001	0.004
		Lysine biosynthesis	2	0.002	0.004
Liver	**Sex**	**Purine metabolism**	9	<0.001	0.003
		Riboflavin metabolism	3	0.001	0.003
	Strain	**Purine metabolism**	17	<0.001	0.003
		Pyrimidine metabolism	13	<0.001	0.003
		Pantothenate and CoA biosynthesis	5	0.002	0.003
	Sex-by-Strain	Pyrimidine metabolism	4	0.001	0.002

Pathways in bold represent pathways also found in chapter 5.

Comparison of Correlation Analyses

Assessing correlations between metabolite profiles in each tissue and systems level energy balance traits revealed that 32, 11, and 51 metabolites were associated with one or more phenotypes for adipose, muscle, and liver tissue in the previous analysis (Fig. 5.5). There were 24, 11, and 55 metabolites in adipose, muscle, and liver tissue associated with one or more phenotypes in the new analysis (Fig. I.3). Many of the metabolites that were associated with one or more phenotypes were in both analyses; however there were a few metabolites that were specific to each analysis. In adipose tissue, nine metabolites were specific to the previous analysis, while one metabolite was specific to the new analysis. In muscle, two metabolites were specific to the previous analysis and two were specific to the new analysis. In liver, two metabolites were specific to the previous analysis and three metabolites were specific to the new analysis.

When comparing correlations that were assessed between and across tissues the new analysis had a greater number of metabolites correlated with phenotypes and one or more tissues. The previous analysis showed that eight, 15, and 24 metabolites were associated with one or more traits and one or more metabolites within or across tissues in adipose muscle, and liver tissue. The new analysis showed that 11, 19, and 30 metabolites were now significantly correlated for adipose, muscle, and liver tissue (Fig. I.4).
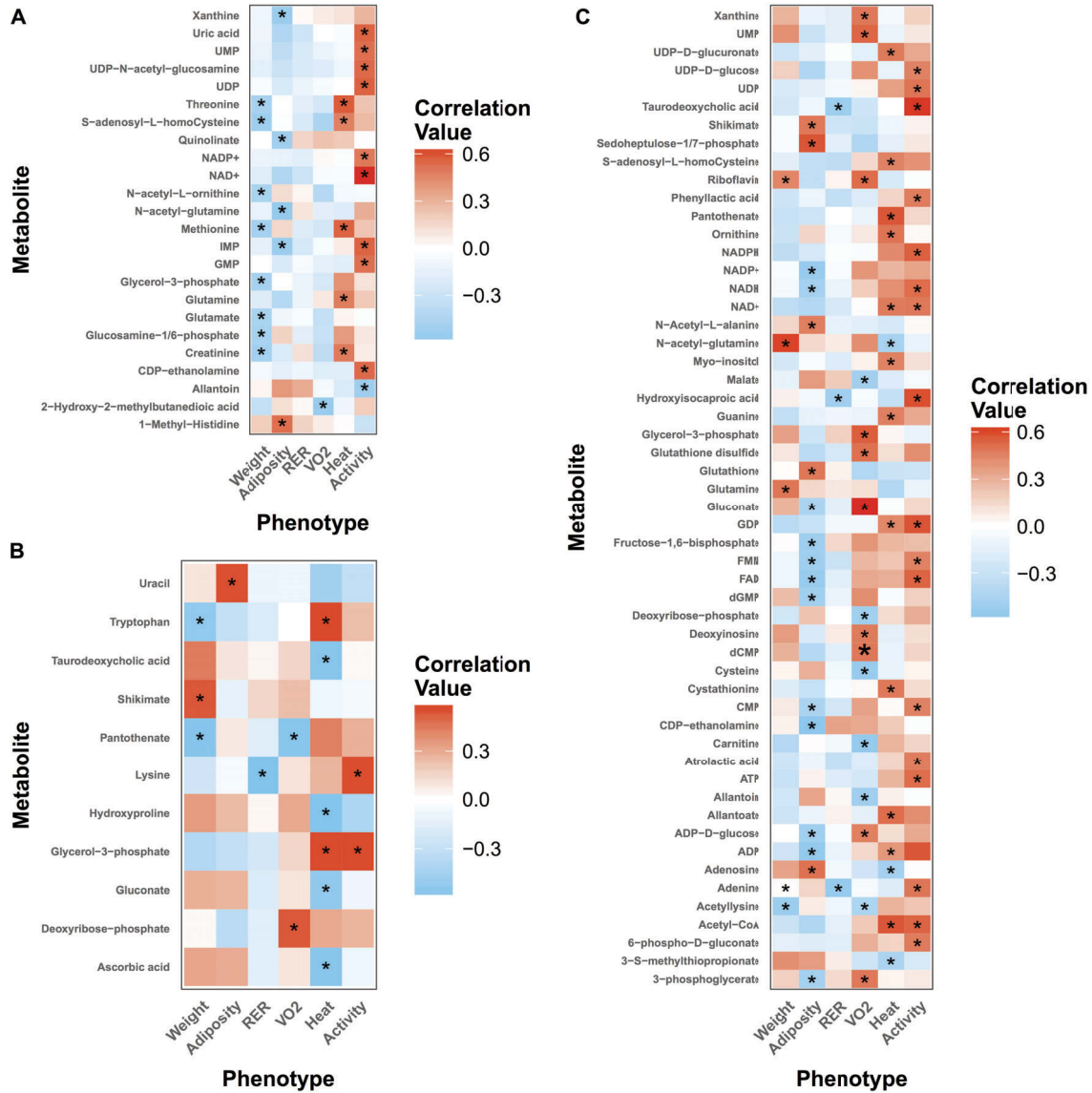
Figure I.3: Correlations between metabolites and system phenotypes. Pearson correlation was used to associate metabolites in adipose (A), muscle (B), and liver (C) with weight, adiposity, and metabolic phenotypes. Significant correlations (FDR < 0.05) are represented by an asterisk (*).
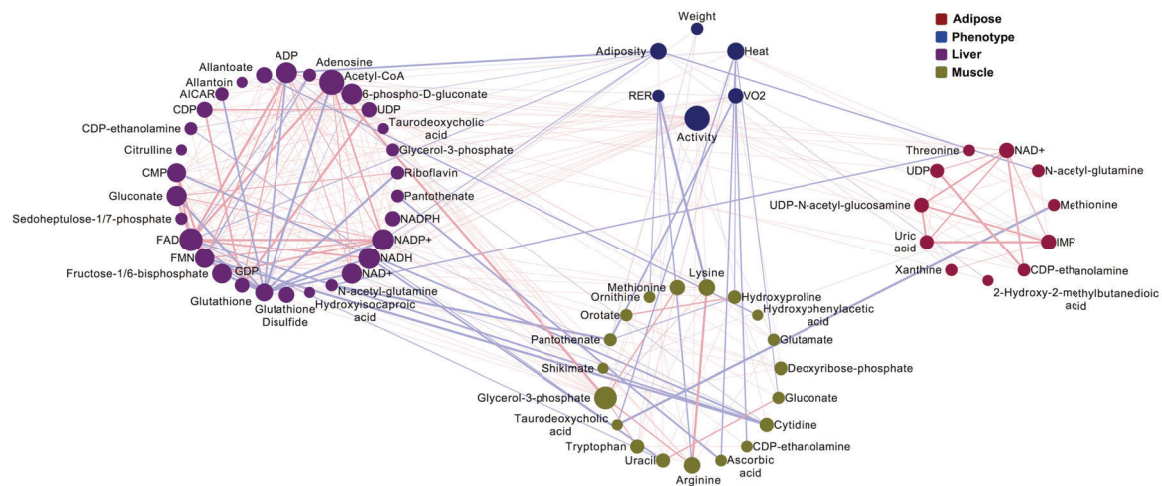
Figure I.4: Cross-tissue metabolite and phenotype network. Pearson correlation was used to associate metabolites within and across each tissue with weight, adiposity, and metabolic phenotypes. Only significant correlations (FDR < 0.05) are shown. To assess relationships within and across tissues and phenotypes, metabolites are clustered by their respective tissue or phenotype. The size of each node represents the number of edges connected to the node. A red line represents positively correlated metabolites, while a blue line represents negatively correlated metabolites.

Summary

Comparing the results from two analyses showed that the level of stringency applied to the KNN imputation might result in alteration of the results. The first analysis used 10 nearest neighbors and allowed for up to 70% of the samples to be missing from each metabolite. The second analysis used 4 nearest neighbors and allowed for up to 10% of the samples to be missing from each metabolite. Reducing the number of neighbors increased the local effects and reduced the chance of averaging values across treatments. This may have produced values that were more specific to the treatment and less general to the entire population. Caution should be taken when determining the number of missing values and the number of neighbors to perform KNN imputation as it can impact the results of the data.

VITA

Ann E. Wells was born in Melrose, MA. She received her Bachelor's degree of Science in Microbiology at the University of Tennessee-Knoxville. She began her Ph.D. at the University of Tennessee-Knoxville in the UT-ORNL Graduate School of Genome Science and Technology in August 2012. She expected to receive her Ph.D. in December 2017.