THE EVOLUTION & REGULATION OF PHYSICAL ACTIVITY LEVELS

A Dissertation

by

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ABSTRACT

Physical activity is a well-established preventative for many diseases, yet the average United States citizen does not engage in regular exercise. Thus, there is a need to understand the biological and environmental mechanisms that regulate physical activity adherence. Altered caloric intake has been shown to be a mediator of physical activity levels, with undereating leading to increased activity and overeating leading to decreased activity. The exact mechanisms through which *diet al*ters activity are not yet known, but literature suggests the dopaminergic system as a primary central integration center where physical activity can be reinforced in times of need to allow the organism to acquire needed nutrients. As such, the purpose of this dissertation was to determine the following: 1) what single nucleotide polymorphisms are associated with physical activity regulation in humans and when in history did they mutate; 2) are the effects of diet on wheel running sustained through the lifespan; 3) how does a high fat/high sugar diet alter the production of relevant metabolites in the gut; and 4) are microbiota essential in mediating the relationship between diet and physical activity? Methods consisted of genetic mutation predictions, wheel running, diet interventions, metabolomics, and microbial rRNA sequencing.

There was a total of 104 single nucleotide polymorphisms associated with physical activity in humans. The range of emergence was between 200-800 thousand of years ago. Caloric restriction proved to maintain wheel running throughout the lifespan, while overeating lead to progressive reductions. The gut metabolome was substantially

altered between a chow diet and a high fat/high sugar diet in mice including metabolites associated with sex hormones, neurotransmitters, and inflammation. Microbial transplants from high active animals in combination with improving diet increased wheel running quicker than an improved diet alone. Fecal transplantation without altering diet modestly increased microbe diversity of fecal pellets but did not change wheel running. A single genera, Lachnospiraceae, was correlated with improved physical activity during the treatment. However, the microbial and metabolite composition was primarily determined by diet with no clear distinction of treatment benefit. These results support the primary importance of diet and a supportive role of the microbiome on influencing levels of physical activity throughout the lifespan.

DEDICATION

To my parents, Cash Letsinger and Kathryn Gose. How could I be so lucky?

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1. INTRODUCTION AND LITERATURE REVIEW

Physical activity is known to prevent and reverse many chronic conditions such as obesity (153, 235), type 2 diabetes (110, 141, 201, 259), non-alcoholic fatty liver disease (94), cardiovascular diseases (127, 142, 190, 271), brain dysfunction (66, 146, 184, 272, 279), osteoporosis (74, 277), accelerated biological aging/premature death (81, 87, 194, 198) and multiple types of cancer (57, 186, 189). Despite the overwhelming upside to participating in physical activity, the majority of humans are not active enough to avoid hypokinetic related disease (29). A study of 6,329 participants monitored using accelerometers in the United States revealed only ~3.5% of adults met the American College of Sports Medicine's guidelines for physical activity of just 30 minutes per day of moderate intensity activity (8). Coupled with poor diet, the low level of physical activity in the United States has been considered the second leading cause of actual death in the United States (5) and has been estimated to cost $67 billion a year in health care (3). Given the amount of literature and public health initiatives promoting physical activity, United States citizens should be aware of the benefits but are not being physically active.

Pre-technology humans were required to move to carry out tasks for survival such as foraging, hunting, escaping prey, finding shelter, reproduction, and trade/communication. Many modern day societies have nearly eliminated the need for high levels of physical activity to perform those same functions in humans; motorized vehicles are the primary mode of transportation, automated machines carry out

physically demanding labor such as washing clothes, instant connection with the world and entertainment via the internet and television, and temperature-controlled homes are much more comfortable than life outside (89). Further, physically demanding jobs have diminished over the decades (44, 57), the walkability of many cities is poor (4), and total energy expenditure of household maintenance has dropped by half (7). Since there is little need for survival based physical activity in the typical United States citizen's day to day life, determining ways to get humans more active is of the utmost importance.

To date, there are an abundance of studies focusing on improving built environments for increasing physical activity such as sidewalks, gymnasiums, and/or city-wide interventions (258). However, improving the built environment only fixes a fraction of the inactivity. In a nationally representative cohort, individuals who live in the highest developed regions for physical activity facilities (i.e. gyms, parks, basketball courts) were only 26% more likely to meet physical activity guidelines (283). It seems most humans still avoid physical activity if not required. For example, a field study recorded less than 10% of individuals choose to take the stairs if an escalator is present (183). In a systematic review and meta-analysis on school interventions, we found that children be forced to be more physically active (10 minute mandatory movement during class) or persuaded by novelty (new games or playground equipment), but adherence drops in the majority of children as soon as requirements or novelty wears off (accepted for publication in Journal of Sport and Health Science). However, physical activity does not drop for all students as it seems some children are more likely to enjoy exercise than others. When analyzing activity levels within and between families and twins, there is a

large portion of physical activity adherence that is not driven by the environment (164). Since human traits are compartmentalized into genetic, environmental, or gene-by-environmental influences, the remainder of variability in physical activity in these studies not from the environment is genetic (95). A study of 37,051 twin pairs from seven countries concluded shared common environment was not a determinant of exercise participation (except for Norwegian males) while the broad sense heritability ranged from 48% to 71% (247). Additional studies have repeatedly confirmed these results in both humans and rodents (82, 126, 247, 251, 252, 260, 131, 151, 156, 165, 167, 168, 202, 246). Despite these results, the amount of literature devoted to defining the genetic and gene-by-environmental determinants of physical activity level is minimal in comparison to studies investigating psychosocial and built environments. Thus, our laboratory has focused on the biological factors that may contribute to physical activity levels. Previous studies from our laboratory have focused on the interactions of sex hormone levels (33–35, 161), exposure to environmental toxicants (227), protein expression (78, 79), miRNA expression (63, 64), inhibition of various proteins (33, 34, 80, 137), and diet (265). During our laboratory's pursuit to find the biological determinants of physical activity, Vellers *et al*. carried out a study in which mice fed a high fat/high sugar diet drastically decreased voluntary wheel running by 57 ± 26% in females and 70 ± 28% in males (265). The current studies and focus of this dissertation takes Veller's research an additional step to delineate the mechanism(s) that connect diet and physical activity regulation. The following sections review existing literature that led us to this pursuit.

1.1 The Obesity Epidemic

The United States' population has experienced radical alterations in body composition since the industrialization of agriculture has led to the ability to produce greater amounts and easier access to food. According to the most recent National Health and Nutrition Examination Survey (NHANES), the estimated percentage of obese US citizens increased from 14.5% in 1971-74 to 39.8% in 2015-16 (83, 104). Further, obesity in children and adolescents has more than tripled since the 1970s, from ~5% to 18.5% in 2016 (83, 104). The exact cause of this increased adiposity is a close race between overeating and physical inactivity. Finding a decisive answer to what is driving the current obesity epidemic is difficult due to challenges associated with obtaining accurate caloric intake data. Self-reported caloric intake surveys are by far the most commonly used in national surveys, but these surveys can often be erroneous. A review of 24 self-reported dietary intake studies revealed individuals typically under-report total caloric intake by ~20% (112). The same review noted obese individuals tended to under-report total caloric intake by ~40%, double the average participant. While food surveys may be inaccurate, epidemiologists have used data from increased sales of food (207), larger proportions per meals (218, 282), and the emergence of high fructose corn syrup (39) to determine that total caloric intake has increased. Even though individuals underreport caloric intake, NHANES data from 1971 to 2010 reveal a significant 12% increase in adjusted mean energy intakes of adults older than 20 from 1955 kcal/day to 2195 kcal/day (104). However, increased caloric intake may not be the worst of the US health issue as the composition of food is also suffering.

Comparing and interpreting trends in diet composition is also difficult due to the lack of a true standard for what humans should be eating. This is evident in the ever-changing macronutrient recommendations given by the government. In the 1970s, there was a large push to lower total fat intake as a few studies like the Minnesota Coronary Study (86), which suffers severe flaws (211), linked dietary fat with early mortality. Regardless of the debate whether fats are unhealthy or not, low-fat became the new trend in food sales. The decrease in fats led to a replacement with more carbohydrates and sweeteners (206). A review in macronutrient intake comparing 1971 with 2006 revealed an increase in carbohydrate intake from 44.0% to 48.7% (10). While a 4.7% increase may not seem to be an issue, the calories tend to consist of minimal nutrients and fiber (104). When high fructose corn syrup was introduced into the marketplace in the 1970s, the per capita consumption of high fructose corn syrup in the 2000s increased over 100-fold, from 0.6 lb per person a year to 73.5 lb (207). In 2008, fructose accounted for 10% of the total calories of the average American, with the highest intake among adolescents at 12.1% (267). Fructose is interesting for multiple reasons: 1) While glucose is typically metabolized after passage through the liver, fructose is primarily metabolized within the liver (121). For this reason, high levels of fructose in the diet can lead to *de novo* lipogenesis and deposits of fat in the liver, typically coinciding with non-alcoholic fatty liver disease (121); and 2) fructose does not stimulate insulin secretion or enhance leptin production, which would otherwise suppress hunger (73).

While fructose is found in many fruits, the fiber that is also contained in fruit decreases the ability of fructose to readily absorb into the small intestines and is

associated with decreased rates of type II diabetes (103). The lack of fiber intake may be the largest nutrient missing in the current human diet (147). Compared to the paleolithic diet, the best guess of anthropologists is that fiber intake has dropped by from ~100 g/day to ~16 g/day (143, 178). Early hunter-gatherer diets relied heavily on a variety of plants, particularly fibrous vegetables (69), while ~85% of grains eaten today (the source of most calories) are refined grains that have the fiber removed to improve shelf life (60). As described in Section 1.4.2, the disappearance of fiber in the diet has enormous implications on the gut microbiome.

1.2 Diet Influences Physical Activity

The first documented account of caloric intake altering physical activity levels dates back to a 1898 study by Stewart and Toronto (244). In this study on rats, a calorie dense diet consisting of beef, cheese, sugar, chocolate, and bread led to a decrease in wheel running and a plain but "apparently sufficient" diet (as animals had no clear health problems) consisting of bread increased running. While this study did not create a revolution, the implications of dietary intake as a driver of low physical activity in the United States needs more attention. Since physical activity is associated with diminishing total energy availability, it appears logical for an individual with low caloric intake to have decrease physical activity to conserve stored energy. Likewise, an increase in physical activity is typically followed by an increase in caloric intake after temporary suppression in humans (70) and mice (128). However, the following sections summarize literature that suggest caloric intake and physical activity are inversely related.

1.2.1 Undereating

The bulk of literature on undereating has focused on murine models due to the ease of controlling and measuring total caloric intake and physical activity. Wheel running as well as total cage activity have both been utilized. The difference between the two methods will be discussed in detail in Section 1.5.2, but for now, both methods are typically in agreement. In a study by Lusseau *et al.*, mice exposed to caloric restriction were hypothesized to show suppressed body temperature via decreased cage ambulation and more bouts of torpor, a state of mental inactivity (172). However, Lusseau's results matched much of previous research; physical activity increases as caloric intake decreases (62, 203, 237, 245, 270). While creating a murine model for anorexia nervosa, Pierce and Epling demonstrated a 10-fold increase in wheel running when rats were allowed a single 60 or 90 min meal per day resulting in an approximate 70% decrease in calories (62). The authors were surprised as a decrease in energy intake was thought to lead to fatigue and energy saving phenotypes. However, this adaptive response may be evolutionarily appropriate since a hungry rodent may increase locomotion to find more food. The unusual part is that when the diet restricted rats from Pierce and Epling's study were returned to an *ad libitum* diet, they continued to run at higher than baseline levels with caloric intake not returning to baseline levels. Thus, it appears rodents can experience a sort of addiction to the wheel running. This same phenomenon is seen where rodents will press levers in order to receive access to a wheel for running (140). Further, short-term and long-term caloric restriction studies (30% less intake than *ad libitum*) in rhesus monkeys yield increases in cage ambulation (270, 280).

These findings have led to basic science research suggesting physical activity elicits a reward-like response, regardless of species, likely being centralized in the dopaminergic system located in basal ganglia structures: the dorsal striatum (caudate nucleus and putamen), ventral striatum (nucleus accumbens and olfactory tubercle), globus pallidus, ventral pallidum, substantia nigra, and subthalamic nucleus. (140). Section 1.3.1 will dive deeper into this "reward" based paradigm.

Caloric restriction in humans has not yielded consistent results as in murine and non-human primate models, but this may be due to the difficulty in controlling for a wide range of factors. Research in humans is much harder to come by as intake and physical activity are not always straightforward. For example, a set of studies done by Martin *et al*. calorically restricted participants by 25% for six months; there was a significant decrease in activity energy expenditure compared to baseline, but accelerometer data was not different (176). Thus, it is difficult to separate out what was activity-based heat (the authors concluded there must have been less activity from fidgeting in the restriction group) or from other organ systems suppressing metabolism to make up for a loss in calories (197). Another potential confounding variable is that caged animals are not aware of where and when their next meal is coming from, while humans in a clinal setting understand their intake schedule. Still, there is evidence for increased activity in humans. Shuval *et al*. using accelerometer data of 4,910 US adults from the NHANES found an association between meeting physical activity guidelines and eating 37 fewer empty calories (24). However, these types of studies inevitably end with a chicken or the egg conundrum. The most delineated examples of caloric

restriction increasing physical activity in humans were summarized by Casper in 2006 in relation to anorexia nervosa (47). Casper determined the increase in "the drive for activity" associated with anorexia nervosa is not a consequence of the preoccupation to lose weight as prepubertal children demonstrated the same activity phenotype as older individuals. Rather, there is likely to be increased reward associated with physical activity in the restricted state of individuals with anorexia nervosa.

1.2.2 Overeating

The result of overfeeding, defined as taking in more calories than required for bodily function resulting in an increase in body fat, on physical activity is much more convoluted in literature. The few murine studies that attempt to overfeed mice do so by altering caloric density of food, primarily by increasing total fat composition. Due to the many approaches to simulate overfeeding, studies reveal decreases in activity (264), no differences in physical activity (43), or even an increase in activity of high activity bred mice (181). There is evidence to show rodents will adjust their total volume of food of various compositions to match caloric intake (160). Thus, changing composition does not necessarily alter total caloric intake, just like eating a nutrient poor diet does not require an individual to also be overconsuming calories. To simulate true overfeeding of total calories, our laboratory has added additional calories through fructose dissolved in drinking water. In this case, mice are not able to regulate total caloric intake, rapidly gain weight, and voluntary wheel running drops drastically by 50-70% (265). While added calories in drinking water should be considered overfeeding, rodents allowed food *ad libitum* regardless of composition should also be considered a degree of overfeeding.

Mice in the wild typically cannot eat until total satiation and spend a large majority of time foraging (234). However, mice in the wild still intake more food than strain matched laboratory mice (determined via doubly labeled water) likely because of their increased need for calories (foraging, mating, temperature control, etc.), though wild mice do not become obesogenic (234). Overeating should not be labelled to any diet unless calories exceed need resulting in increased adiposity and glucose intolerance compared to strain matched wild mice.

The longest human overfeeding study was done by Levine *et al*. who overfed 10 lean and 12 obese men and women by 1000 kcal above baseline values for 8 weeks resulting in a drop of 1.5 km of daily walking in both groups (158). A shorter study done by Schmidt *et al*. overfed obesity resistant and prone individuals 40% more than their basal metabolic rate for three days (226). While there were no differences in "spontaneous physical activity", obesity prone individuals had decreased walking bouts while obesity resistant individuals maintained walking distance. Studies that attempt to compare caloric intake surveys and accelerometer data are largely irrelevant to the primary question as it is impossible to determine if those who are health-conscious and monitor caloric intake are also more motivated to be active (233).

There are multiple studies reporting total energy expenditure increases during overfeeding. Most consider a larger production of energy created by the thermic effect of food (digestion and associated metabolism). However, calculations of thermic effect of activity have been disputed and misunderstood. The paradigm many scientists take is that when the body is overfed, energy-wasting activity will take place, such as fidgeting,

to burn excess energy and keep the body closer to a preferred weight/composition. While it may seem to make sense in a modern world where food is in excess, the physiological purpose of this does not seem to be rooted in any logical evolutionary perspectives. Humans are remarkably efficient at storing excess calories as fat for survival. A mechanism that functions to burn extra calories outside of thermogenesis would not fit any evolutionary mechanism for increased survival. A study by Apolzan *et al*. refers to this difficulty of total energy expenditure and increased physical activity (5). In this study, 25 men and women were kept in an inpatient clinic and overfed by 40% above their basal metabolic rate with 5, 15, or 25% of extra calories coming from protein for eight weeks. Accelerometer counts and total energy expenditure increased as body weight increased, though total energy expenditure was significantly different from baseline until total body weight was controlled for. The increase in accelerometer counts may be more likely to do with being confined in an inpatient clinic and barred from doing any deliberate physical activity. This study is a prime example of how complicated of a phenotype physical activity is in humans to study. It may be entirely possible for deliberate exercise/ambulation to decrease while fidgeting-like movements increase. Given the present literature, physical activity is a complex phenotype that has a strong connection to caloric intake.

1.3 Mechanisms of Action

The mechanisms through which diet alters the drive to be physically active are not yet delineated, but multiple central and peripheral modulators are recognized. While early research focused on energy availability as the sole contributor, the bulk of

literature suggests the dopaminergic system of the brain is the primary central integrating center and sex hormones are the most potent peripheral modulators. The following sections will cover the dopaminergic system and sex hormones' dominant roles, touch on the influence of malnutrition, and the lack of correlation with body weight.

1.3.1 Dopamine

The dopaminergic system is considered the "reward center" of the brain. The same system is responsible for basic motor function production; as first realized in Parkinson's victims, when dopamine secreting neurons in the substantia nigra of the midbrain become deteriorated, sporadic/uncontrollable movements occur (22). The critical role of dopamine as the integrating center of physical activity and caloric intake was demonstrated in a study by Zhu and Palmiter, in which mice with knocked out tyrosine hydroxylase, an enzyme needed to synthesize L-3,4-dihydroxyphenylalanine (L-DOPA), completely stopped all physical activity and food intake after weaning (286). Physical activity and eating was transiently recovered when the brain was supplied L-DOPA, (253, 254). Knab *et al*. determined dopaminergic function controlling physical activity also has a genetic basis. High active mice (C57L/J) showed under expression of dopamine receptor 1 and tyrosine hydroxylase RNA (136) and responded differently to dopaminergic antagonists/agonists (138) compared to low active mice (C3H/HeJ).

The question becomes, how do physical activity and eating interact with each other through the dopaminergic system? Generally, activation of dopamine receptors sets off a cascade of events associated with "satisfaction" or "reward" like behaviors

(14). I refrain from explicitly saying an increase in dopamine implies increased reward as the dopaminergic system is complex and dependent on stimuli from multiple other neural and chemical signals. For this reason, all dopaminergic cascades that result in a reinforcing sensation will be referred to as "reward". Eating (26, 200, 230) and physical activity (27, 49) both have an abundance of literature revealing a strong interaction with reward. The general paradigm our lab and others have proposed is when reward from food is gained, additional reward gained from wheel running is blunted. In the previous section, the calorically restricted mice and humans having an addiction-like connection with physical activity fits the dopaminergic reward system model: if an organism is not receiving adequate reward from nutrient intake, the reward from physical activity could be greater. This exact balance can also be seen when bouts of exercise increases reward and temporarily suppresses food intake (177); this suppression is followed by an increased caloric intake.

It is important to consider if studies that force participants to overeat may be blunting the typical reward response that would be gained if the overeating was *ad libitum*, thus explaining why physical activity is unchanged or even increased. For this reason, studies aiming to determine food's impact on physical activity in humans and mice should carefully consider the palatability of diet. A study by Hall *et al*., observed inpatient participants consumed an average of 500 kcals more when offered an *ad libitum* ultra-processed diet compared to an *ad libitum* unprocessed diet (105). Energy expenditure was not different between the groups when placed in a metabolic chamber. However, another study which compared isocaloric processed vs. unprocessed menus

revealed subjects on the processed diet expended about half the energy as the unprocessed (12). In studies that provide added sucrose in drinking water, wheel running did not decline like they did in fructose (53). It appears there are distinct differences in brain activity between fructose and sucrose: sucrose is preferred by murine species, but fructose is registered as sweeter (99). Thus, the exact diet composition, total caloric intake, and palatability all are likely to make an impact.

1.3.2 Sex Hormones

Many mechanisms have been recognized as peripheral components influencing physical activity levels for a potential role in influencing the neural drive to be physically active. An example that affects activity, but not through neural drive, would be the limited substrate availability in McArdle's disease. Those burdened with McArdle's disease lack myophosphorylase which prevents the breakdown of glycogen in muscle, a needed source of energy for exercise. Another example is difficulty breathing during even light bouts of activity in chronic obstructive pulmonary disease (COPD). In both diseases, the individual may have a neural drive to be more physically active but performing the activity is difficult. While many peripheral mechanisms have been studied for a potential role in regulating activity levels, circulating sex hormones and androgen receptors have proven to exhibit a compelling relationship (119, 161).

First recognized in 1927 by Richter (214), there is a tight relationship between wheel running and the menstrual cycle in female mice. The 4 to 5-day menstrual cycle creates a distinguishable "saw tooth" pattern in wheel running with the highest bouts of activity occurring during estrus or ovulation. The evolutionary implication of this

relationship is intuitive as animals who are more mobile during peak reproductive periods are more likely to find a mate. The removal of sex organs, an ovariectomy or orchidectomy, results in near total abrogation (~90%) of physical activity as seen in our lab (34) and others (119). The same study from our lab discovered sex hormone replacement recovers 35-110% of baseline wheel running (34). The connection with neural drive was not clear until a well performed set of studies using orchidectomies, testosterone replacement, and dopamine agonistic amphetamines by Jardi *et al*. determined the loss of sex hormones worked independently of muscular dysfunction and interacted with dopamine receptors in the brain (120).

While there is sufficient evidence to conclude sex hormones play an important role in the control of physical activity in murine species, human research is entirely inconclusive. Sex hormone replacement studies are scarce in humans, and those that have been done typically do not have physical activity as the main outcome of the study (241). A pilot study did show that intramuscular injections of gonadotropin-releasing hormone agonist in post-menopausal women trended toward improvement in physical activity levels. There is also evidence that sex hormones are depleted during chronic overfeeding and weight gain (31). Though, the correlation is an observation that has yet to be mechanistically connected with physical activity level in humans. Improvements in technology and methodologies will likely allow studies to directly target these potential links.

1.3.3 Malnutrition

It is critical to distinguish between different circumstances of low caloric intake and how they may fit in the current paradigm. Nearly one third of the United States is at risk of deficiency in at least one vitamin due to poor diet that lacks vegetables and fruits (24). It is possible nutrient deficiencies could cause any number of cell signaling issues that could be integrated through locomotor centers such as seen in iron, vitamin B6, or vitamin D (13, 150, 212). It is difficult to determine if individual caloric restriction studies do so without inducing nutrient deficiencies, but those that result in increased activity clearly do not have this issue. The connection between diet composition and neural drive is a complex interaction that is worth investigation. The potential for diet composition to send direct signals to the brain will be further discussed in Section 1.4.2.

1.3.4 Body Weight

During chronic overfeeding, an increase in total body weight and fat will occur. It has been hypothesized this increase in body weight (in humans and mice) is primarily responsible for the decrease in activity. The association is difficult to determine in humans as those who are overweight may have gained weight in the first place due to physical inactivity rather than the weight being the cause. Among wheel running studies performed in our laboratory, total body weight did not correlate with wheel running indices in 14 inbred mouse strains (167), 41 inbred mouse strains (166), and the same strain fed different diets (265). A study by Friend *et al*. determined wheel running was reduced by diet-induced obesity, as well as reductions in dopamine receptor 2 binding compared to controls (91). Genetic removal of the dopamine receptors in lean mice

lowered activity and restored G_i signaling in the obese mice returned activity to lean levels (91). While these types of studies cannot be performed in humans, it is interesting to note individuals who lose weight through bariatric surgery do not have increased activity levels (20).

1.4 Microbiota & the Gut-Brain Axis

If the paradigm is accepted that caloric intake can alter physical activity motivation in the brain, there must be a connection between the two. One of the most promising regulators is gut microbiota. The presence of gut microbiota was first discovered in 1665 by Antoni van Leeuwenhoek, but the body-wide influence of the trillions of bacterial cells residing in our intestines has not been appreciated until recently. The creation of the germ-free mouse model has provided invaluable information on the critical homeostatic role of gut microbiota. Germ-free mice are riddled with physiological dysfunction such as cardiovascular disease, irritable bowel syndrome, obesity, type 2 diabetes, and CNS diseases (171). This realization has sparked much interest in deciphering which bacterial phyla are playing a role in which specific dysfunction and if supporting or killing those phyla can mitigate or even prevent dysfunction. There are 300 to 2200 different species of gut microbiota that fall into 12 phyla (255). The four dominant, and most studied, bacterial phyla are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. The most dominant species, *Bacteroides*, represents about 30% of the microbiome. The bulk of research has been dedicated to analyzing the changes in phyla ratios, using rRNA sequencing, caused by interactions with the environment. Due to the ever-complex data sets that result,

generalizations between the relationship of phyla ratios and phenotypes are difficult. Ley *et al*. were some of the first scientists to find a strong correlation between a high ratio of *Firmicutes* to *Bacteroidetes* (F/B ratio) and obesity (255). Since then, microbiota research has focused heavily on the F/B ratio as a marker for poor gut health typically labeled a "dysbiosis". However, recent reports conclude that F/B is much too simple of a method to determine gut health. The search for specific "good" or "bad" bugs with large effects on physiological function is likely to be rare. Of course, there are occasions such as *H. pylori* or *C. difficile* causing severe distress of the gut and colon, respectively. However, these moments are likely as rare as a gene variant being solely responsible for a phenotype.

The gut microbiome is an intricate system with many interactions. With the creation of more complex analyses, a clearer picture of what is happening in the gut and how it responds to the environment has emerged. Gut function may be as complicated as the epistatic steps from gene to protein production as gut metabolite productions depends on the interaction of microbial presence and function with nutrient influx. Different species have unique DNA coding that can generate enzymes that break down the nutrients ingested by the host and either allow greater nutrient absorption (65) or create metabolites that interact with host physiology (240). For this reason, it has been assumed that a large variety of microbe species, deemed "microbial diversity", is a sign of a healthy gut.

Metabolite production from the microbiome interact with the body through two main methods: 1) diffusing directly into the splanchnic veins and/or 2) interacting with

the vagus nerve to send signals to the brain. Metabolite absorption into bloodstream via the blood-gut barrier of the splanchnic veins is no different compared to the rest of substances that are absorbed. Interactions of the metabolites with the body were thought to be limited to peripheral organs only, as most neurotransmitters cannot cross the blood-brain barrier. However, studies that cut the vagus nerve of the enteric nervous system discovered many metabolites were able to interact directly with the nerve (58). While it has been known the brain can communicate and control gut function, a study by Perez-Burgos *et al*. determined the bi-direction signal is 90% in the gut to brain direction (58).

1.4.1 Gut & Brain

The germ-free mouse model has allowed researchers to observe a myriad of physiological abnormalities, but many physical activity related alterations were not expected. Germ free mice were noted to have altered states of anxiety, depression, metabolic syndrome, and even autism (18, 117, 123, 130, 285). While these studies reveal clear behavioral traits, a study of *Tocoplasma gondii* gut infection in rats reveals the ability to alter desire of the host organism. While *T. gondii* can infect nearly all warm-blooded animals, it can only reproduce in the gut of felines. Rats typically avoid the smell of feline urine, but when infected with *T. gondii*, feline urine becomes an attractant causing rats to move toward the smell (19). The altered phenotypes observed do not end with altered behavior, but the changes are likely rooted in physical characteristics of the brain. For example, microglial cells in germ-free mice, which are responsible for immune defense, are defective (58). In another study, the prefrontal cortex had hypermyelinated axons, which is associated with shorter a lifespan (58).

Heijtz *et al*. determined germ-free mice also have alterations in the production and atrophy of synapses between neurons (109). These microbiota-based changes in behavior and brain architecture are potentially caused by the altered levels of brain neurotransmitters and proteins such as dopamine (6, 72, 223), serotonin (175, 275), brain-derived neurotrophic factor (BDNF) (18, 109, 222, 249), and gamma-Aminobutyric acid (GABA) (38) produced by microbiota metabolism. Asano *et al*. determined gut microbes are necessary for the production of half the body's dopamine (9), and about 90% of the bodies total serotonin (5-HT) (109). As mentioned in Section 1.3.1, the neural drive to be physically active is likely integrated within the dopaminergic axis, so the implications of gut microbiota producing and altering dopaminergic related neurotransmitters and proteins is worthy of investigation. In the following sections, literature is described for two relevant influencers on gut microbiota: nutrition and exercise.

1.4.2 Gut & Diet

The gut microbiome has high variability from person to person depending on genetic and environmental factors. Of the many factors, medication and diet are the two primary environmental factors responsible for microbial composition (77, 284). Falony *et al*. in 2016 determined medication intake explained 10% of the microbial variation, with antibiotics having the largest effect (77). Interestingly enough, a study in healthy adults noted microbe species that were unintentionally altered by antibiotics returned to baseline levels 35 days after the final administration (170). Thus, the microbiome seems to have a "default" state that responds to acute exposures but is not permanently altered.

This may be a ray of light in terms of reversing a potential dysbiosis. However, there is also evidence that early life exposures when microbiota colonies are establishing in the gut, antibiotics and food choices can make a life-long impact (21). While medications have the largest effect, dietary habits are more chronic and consistent. Citizens of farming societies exhibit a greater variety of gut microbiota than the average city dwelling United States citizen (281). The lack of vegetable consumption and associated fiber in the United States could be the primary difference between the two demographics (278). Interestingly, hunter-gatherer population have a very high diversity of gut microbes, followed by high diversity in traditional farmers and fishing populations, and low diversity in Westernized populations (102). Not only are United States citizens potentially creating a dysbiosis with low fiber and vegetable intake, but the high fat/ high sugar composition causes further disorder (192, 229).

1.4.3 Gut & Physical Activity

Few studies have begun to investigate the interrelationship between physical activity and the gut microbiome. A review of literature concluded that while exercise alters the microbiome, the alterations in phyla diversity and proportion are not consistent likely due to the high level of confounding variables (174). In the only study to control for diet, Estaki *et al*. found 13 adults with high aerobic fitness had increased gut microbial diversity compared to dietary intake matched individuals with lower cardiorespiratory fitness (76). Studies in mice also indicate wheel running leads to increased diversity (50). No study to date has determined if the microbiome effects activity levels.

1.5 From Mice to Humans: Translation is Key

1.5.1 Wheel Running

The ability to translate neural drive to be active using wheel running in mice to the same drive responsible for physical activity levels in humans is paramount. Spontaneous physical activity can be measured via running wheel or general cage activity (ambulation and/or rearing). While the two have decent agreement among the studies relevant for this dissertation's purpose, wheel running has been selected as the ideal model for assessing reward and physical activity (71, 232). Mice express a highly repeatable wheel running phenotype (139) which is tightly regulated by strain and gender (165) allowing for the comparison of results across studies and aims. As seen in previous cited studies, mice will engage in wheel running without any external reward, even in the wild, hinting the activity is enjoyable (182). Further, when mice are allowed access to a wheel, general cage activity is decreased (46). This is hypothesized to be similar in humans engaging in more sedentary behavior after exercise (216).

This wheel running model is beneficial for our study as it can be used as a dependent variable as diet and other interventions can directly impact levels of wheel running (265). Wheel running can also be used an independent variable that directly impacts phenotypes such as body weight, glucose tolerance, neurological changes, and heart rate similarly as running does in humans (36).

1.5.2 Fecal Microbial Transplants

Fecal microbial transplants (FMT) first appeared in the fourth century in China by the medical doctor, Ge Hong, as an orally ingested therapy to cure digestive

dysfunction. It is no surprise Ge Hong's "Yellow Soup" did not catch on as a popular home remedy even though it was effective. FMTs have made a resurgence recently as the direct benefits become better understood. The only current medical application in humans is alleviating a *Clostridium difficile* infection (the same primary target of Yellow Soup), in which a direct duodenal infusion from a "healthy" donor has been proven to be more effective than the standard vancomycin regimen (195). Evidence is growing for a much wider benefit for FMTs in human populations (219). In an interesting case of FMT, a lean donor developed obesity after receiving an FMT from her overweight 16-year old daughter (3). This case study led to much interest in the role of gut microbiota in altering physiological status of the host. Two studies in this dissertation focus on the role of gut microbiota in a mouse model. While the mouse and human microbiomes have distinct differences such as a large variance in relative abundances of microbial genera, there are highly functional similarities between the two as both species share a representative from 89% of all genera (148). To emphasize the translatability of mouse microbiome studies to humans, a study by Ridaura *et al*. performed fecal transplants from the gut of obese or lean humans into the guts of lean mice (215). The mice receiving the FMT from obese humans became obese and those receiving the FMT from lean humans did not have a change in adiposity (215). Not only does this study show the surprising role gut microbiota play in certain phenotypes, it shows the microbes may play a similar enough functional roll between humans and mice to be a valid experimental model.

Since FMTs are a relatively new experimental method, there is no gold standard when it comes to preparation, dosage, and frequency. A review of literature involving effective transplants that altered body composition or other physiological function suggests a dosage of 100-200 µl every day for three weeks is sufficient for physiological changes to be observable, but the lasting effects are variable (133, 159, 179, 208, 220). The repeated doses allow for the new colony of microbiota to have a larger probability of engrafting and maintaining a presence in the gut. Binary fission of a microbe typically occurs in 20 minutes and will continue to grow at an exponential rate if in a perfect environment. The primary determinate of growth/success rate however is substrate availability. Shepherd *et al*. tested this hypothesis by performing a microbial transplant of a rare strain of *Bacteroides ovatu* (can digest fructans and marine polysaccharides) with or without their substrate of choice, red algae poryphyran (108). With increasing levels of poryphyran, the microbes had a higher chance of engraftment (108). Of course, filling the stomach of a mouse with a cocktail of fresh fecal microbes is difficult to translate to humans as adding the same volume of microbes to a human stomach is highly unlikely, but intraduodenal infusions and fecal capsules are currently being performed.

1.6 Summary & Purpose

It seems humans have evolved to engage in daily physical activity as disease onset follows long periods of inactivity, which is not shared among other primates. However, the average United States citizen does not engage in physical activity. In order to improve activity levels, understanding the biological regulation of physical

activity is necessary. A summary of research points to a strong interaction of diet, the microbiome, and physical activity. Thus, the purpose of this dissertation was to answer the following primary questions: 1) what single nucleotide polymorphisms are associated with physical activity regulation in humans and when in history did they mutate; 2) are the effects of diet on wheel running sustained through the lifespan; 3) how does a high fat/high sugar diet alter the production of relevant metabolites in the gut; and 4) are microbiota essential in mediating the relationship between diet and physical activity?

2. MUTATION AGE OF SNPS ASSOCIATED WITH PHYSICAL ACTIVITY LEVELS[1]

2.1 Synopsis

The purpose of this study was to determine the estimated mutation age and conservation of single-nucleotide polymorphisms (SNPs) associated with physical activity (PA) in humans. All human SNPs found to be significantly associated with PA levels in the literature were cross-referenced with the National Heart, Lung, and Blood Institute's Grand Opportunity Exome Sequencing Project to find estimated African-American (AA) and European-American (EA) mutation age. As a secondary measure of mutation age, SNPs were searched for in Hawk's mutation age prediction database which utilizes linkage equilibrium. To determine conservation among hominids, all SNPs were searched in the University of California, Santa Cruz Genome Browser, which contains Neanderthal and chimpanzee reference genomes. Six of the 104 SNPs associated with PA regulation were exon-located missense variants found in *IFNAR2, PPARGC1A, PML, CTBP2, IL5RA,* and *APOE* genes. The remaining 98 SNPs were located in non-protein coding regions. Average AA and EA estimated mutation age of the exon-located SNPs were 478.4 ± 327.5 kya and 542.1 ± 369.4 kya, respectively. There were four selective sweeps (suggestive of strong positive selection) of SNPs in humans when compared to Neanderthal or chimpanzee genomes. Exon-located PA

[1] Reprinted from (157)

candidate SNPs are older than the hypothesized emergence of anatomically modern humans. However, 95% of PA associated SNPs are found in intron and intergenic location. Across all SNPs, there seems to be a high level of conservation of alleles between humans, Neanderthals, and chimpanzees. However, the presence of four selective sweeps suggests there were selection pressures or drift unique to *Homo sapiens* that influenced the development of mutations associated with PA regulation

2.2 Introduction

While the benefits of physical activity (PA) are well known [1,2], a study of 7176 participants utilizing accelerometers [3] reported less than 3.5% of adults met the United States daily PA guideline of at least 30 minutes of daily moderate PA [4]. This level of physical inactivity among most humans has been shown to cause and exacerbate many diseases and increase rates of early mortality [5,6]. However, our closest primate relatives manage the same PA level as the most inactive humans, but seemingly do not suffer similar health consequences (263). It seems as there is a physiological need for humans to engage in PA that is not shared with close relatives. Thus, understanding the biological factors responsible for increasing the likelihood of participating in PA is critical to avoid associated hypokinetic diseases and their impact on human health. In this article, PA refers to the conscious engagement of locomotion via muscle contraction. Thus, PA regulation refers to the biological factors that result in the engagement or disengagement of PA.

Substantial evidence indicates the regulation of PA levels in adult humans is heritable, as shown by over 45 twin and population studies (163). In spite of the

numerous studies in this area, there is still minimal agreement regarding which genetic mechanisms are involved due to the inherent complexity of PA regulation (132, 163) as well as the many environmental factors (258) that may have genetic interactions. To date, there are few genome-wide association studies (GWAS) in humans that link the time spent engaged in PA with genomic variants. The available PA GWAS have generated a number of significant single-nucleotide polymorphisms (SNPs) at multiple genomic locations associated with PA, yet there is no particular region that is considered to be responsible for the majority of the variance in PA (40, 132, 163). Due to this ambiguity, parsing out which of these regions are important and how/if they regulate PA requires novel approaches.

Predicting the emergence and spread of genetic regulators of PA in specific time eras could help identify potential environmental factors that would have served as selection pressures. For example, approximately 11,000 years ago (11 kya; (1)) agriculture rapidly spread, requiring a fivefold increase in duration of PA for farmers on a daily basis (162). A farmer who did not meet the needs for increased PA could not produce enough food to support fecundity and subsequent population growth. This agricultural-induced increase in daily PA could have induced genetic alterations favoring increased PA adherence in some population by altering preferred metabolic pathways (162). However, once farming methods of a region could produce larger yields, the greater percentage of associated humans no longer needed to engage in the same amount of PA. While this example is limited in its explanatory value due to variation in agriculture progress between regions of the world, estimating the age of the identified

PA-related genomic variants could provide initial information of when and how the current state of human PA regulation could have been established. Alternatively, potential mechanisms regulating PA could be relatively recent products of genetic drift. Thus, the primary purpose of this study was to determine the mutation age and conservation of all currently identified human SNPs associated with PA regulation. The outcomes of this study could then be used to initially determine: 1) if significant genomic variants associated with PA were a part of an evolutionarily conserved gene set shared among other species; and 2) if variants emerged at any point in human history that would have required altered engagement in PA due to survival strategies (e.g. hunting/gathering transition to farming)

2.3 Methods

2.3.1 Overview

The goal of our approach was to combine all published SNPs associated with PA regulation in humans, determine their conservation between humans and closely related species (chimpanzees and Neanderthals), determine conservation among placental mammals, estimate mutation age using two existing prediction databases, and compare current population allele frequencies in Africa, Eastern Asia, and Europe.

2.3.2 Literature Search

To identify the currently known genomic variants associated with PA regulation in humans, the PubMed database was searched on April 20th 2018 with the following combined keywords: (Genome-wide[Title/Abstract] OR SNP[Title/Abstract] OR allele[Title/Abstract]) AND (Physical Activity[Title/Abstract] OR leisure[Title/Abstract]

OR sedentary[Title/Abstract] OR exercise participation[Title/Abstract]). Resulting manuscripts were downloaded and placed into a reference management software (Mendeley, INC., New York, NY). Final inclusion was based on a three-step screening process through abstract text and full text when necessary. The inclusion criteria applied were: 1) the study was completed in human populations; 2) PA levels were the primary dependent variable; and 3) the study was not a review of literature. A list of all PA-related SNPs were compiled and searched in the National Center for Biotechnology Information's SNP Database (https://www.ncbi.nlm.nih.gov/projects/SNP/) to confirm function, location, and allele frequencies.

2.3.3 Conservation of Alleles

To compare conservation of PA-related SNPs with Neanderthal and Chimpanzee genomes, the SNP reference numbers (rs#) were entered into the University of California Santa Cruz Genome Browser (https://genome.ucsc.edu/Neandertal/) which contains data generated by Green *et al*. (100). SNPs from five human genomes of diverse ancestries (San, Yoruba, Han, Papuan, and French) and three Neanderthal genomes were labeled as ancestral (A) or derived (D) characterized as such by comparison with the chimpanzee reference genome. Comparisons of genomes were limited to 30% of substitutions and 14% of indels in the human lineage due to incomplete sequencing coverage, disproportionately higher mutation at CpG sites, and a low sample size (n=3) in the Neanderthal genome. The final database contained 3,202,190 substitutions and 69,029 indels. Additionally, all SNPs were cross-checked with a database created by Bejerano *et al*. (16) containing 13,736 ultra-conserved elements which were sequences over 100

base pairs that are identical between at least three of five placental mammals. The comparison with the Bejerano database allowed determination of whether the PA-related SNPs emerged in early mammals before human evolution.

2.3.4 Mutation Age Prediction

To determine mutation ages, each PA-related SNP was cross-referenced with the National Heart, Lung, and Blood Institute's Grand Opportunity Exome Sequencing Project (NHLBI GO ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/) as well as Hawk's Linkage Disequilibrium Database (106). The Exome Variant Server contains SNP data from 6,515 unrelated Americans compiled from 20 different cohorts. Mutation origin predictions were produced by Fu *et al*. (92) using Griffiths and Tavares's (101) age of mutations in a coalescent tree formula which indicates the origination of gene variants with a common ancestor. This coalescent tree formula was derived from Kimura and Ohta's (134) formula for calculating the expected age of neutral mutations, variations that are not selected for or against, in a stable population. All simulations were based on the Out-of-Africa Model theorized by Schaffner *et al* (221) which characterized a bottleneck of non-African populations approximately 51 kya, a second bottleneck for European populations 23 kya, and an accelerated world population growth occurring 5.1 kya. The Linkage Disequilibrium Database contains estimated years since the mutation of 6,509 selected SNPs from the HapMap Project (Northern and Western European ancestry only), which were calculated using rates of linkage disequilibrium decay. This linkage disequilibrium decay indicates the rate at which sections of DNA depart from predictable reshuffling (106). The use of these

techniques produced an estimation of when each PA-related SNP emerged in the human genome as indicated by years before current day.

2.3.5 Population Allele Frequency Comparisons

To determine the similarity of PA-related SNPs among difference populations, population allele frequencies of Africa, Eastern Asia, and Europe were searched within the 1000 Genomes Project Database using the GRCh37 reference assembly (http://grch37.ensembl.org/Homo_sapiens/Info/Index). Chi-square statistics were used to determine if allele frequencies were significantly different between populations. The alpha level was set at 0.00048 per Bonferroni's correction for multiple tests.

2.4 Results

2.4.1 Literature Search

The PubMed search yielded 902 results, of which 12 studies met the three-inclusion criterion. Two papers (45, 135) were added due to appearance in the literature after the initial search. Of the 14 studies, seven were GWAS and seven were candidate gene studies. Of the seven GWAS, four used SNPs, while all pre-2009 studies utilized micro-satellite technology to estimate Quantitative Trait Loci (QTL). The pre-2009 studies that used micro-satellite technology did not provide specific locations for conservation or age predictions and as a result, were dropped from the analysis. After compiling all results, the eight included studies included reported a total of 104 unique SNPs significantly associated with PA (45, 59, 97, 135, 169, 173, 187, 188, 276).

Of the 104 unique SNPs associated with various measurements of PA, six were located in exons, 49 were located in introns, one was in a 3' UTR region, two were

considered upstream of a gene (potential promoter/enhancer), three were considered downstream of a gene (potential transcription unit/terminator), and 43 were intergenic.

2.4.2 Conservation of Alleles

Twenty-nine of the significant PA-related SNPs had chimpanzee/Neanderthal derivations in the University of California Santa Cruz Genome Browser. Of these 29 SNPs, effect alleles were provided in the original articles for 16 SNPs (Table 2.1). Of those 16 SNPs, the allele associated with a more active phenotype was the most common allele for 10 of 16 alleles in humans, 9 of 16 alleles Neanderthals, and 8 of 16 alleles in chimpanzees. A *post-hoc* analysis using chi-square statistics revealed no significant differences between frequency of "higher PA" associated alleles in humans, Neanderthals, or chimpanzees. However, four PA-related SNPs were predicted to have strong selective sweeps (evidence of strong selection pressure in favor of these alleles) in *Homo sapiens* since their divergence from *Homo neanderthalensis*: rs1051393 exon in *IFNAR2*, rs2267668 intron in *PPARD*, rs1638525 intron of *AKAP10*, and rs10145335 intergenic region near *C14ord177*. There were no matches between PA-related SNPs and the Bejerano *et al*. database of 13,736 ultra-conserved elements.

2.4.3 Mutation Age Prediction

Exon-located SNPs found in *IFNAR2, PPARGC1A, PML, CTBP2, IL5RA,* and *APOE* were matched in the NHLBI GO ESP's Exome Variant Server. *APOE* age was not estimated in the reference database for unknown reasons. For the remaining PA-related SNPs (n=5), average AA estimated mutation age was 478.4 ± 327.5 kya and average EA mutation age was 542.1 ± 369.4 kya (Table 2.2). These age ranges can be

compared to the average age across all 6,515 SNPs in the Exome Variant Server database of 47.6 ± 1.5 kya in AA and 34.2 ± 0.9 kya in EA. Amongst the non-exonic SNPs, only a SNP located in the intron for *DNAJC1*, rs7910002, was found in the linkage disequilibrium database. The estimated age of selection of this SNP was predicted to be 7.8 kya (Table 2.2).

2.4.4 Population Allele Frequency Comparisons

Of the 104 chi-square tests ran, 38% of the PA-related SNPs had at least one population, African, Eastern Asian, or European, with significantly different allele frequencies. A list of all the tests can be found in S1 Supporting Information. Of the six SNPs within exons and four SNPs who are strong candidates of selective sweeps, there was only a significant difference between population frequencies in the *PPARGC1A* gene (Figure 2.1).

2.5 Discussion

The present study is the first to attempt dating the currently known human-related genomic sequence variations associated with PA in an attempt to further understand the origin of PA regulation and its conservation among species. In general, our results estimate PA-related SNPs in protein-coding genomic areas are as old or older than the hypothesized emergence of anatomically modern humans (~200-350 kya) (51, 118, 236). This estimated SNP age range is considerably older than the majority of exon-located mutations given that Fu *et al*. predicted 73.2% of modern day exonal SNPs arose less than 5 kya (92). A mutation age range from 210 to 785 kya suggests the genetic control of PA in humans, through these particular exon-located SNPs, may have

emerged during periods of *Homo* energy expenditure alterations such as the dramatic increase in *Homo*'s brain size, body size, and/or gathering range (51, 236). A proposed timeline equating the emergence of PA associated mutations with possible selection pressures is in Figure 2.2. Given the evolutionary divergence of humans and primates has been approximated to be 4-12 mya (17, 107), our species may not share the exact intragenic-based modulators of PA present in primates. Further, while many PA genetic studies are completed in mice models, the evolutionary split between humans and mice is estimated to have occurred approximately 96 mya similarly suggesting intragenic-based modulators controlling activity in humans may not be similar to those controlling activity in mice (17, 96, 107). However, we urge caution in interpreting our limited dataset to mean there are no similarities among human PA genetic regulation and PA regulation in other species given that the direct causal link between the available SNPs and PA is yet to be determined.

Further supporting our estimated mutation age of the PA-related SNPs, there were no matches between PA-related SNPs and the database of 13,736 ultra-conserved elements (16). Thus, there is little to no known conservation of any PA-related SNPs in humans among other earlier mammalian placental species, suggesting that the PA-related SNPs emerged after the split of *Homo erectus* from other mammals (Figure 2.2). Comparison of the 16 SNPs where Neanderthal and chimpanzee genome data were available revealed the effect allele for "more physical activity" was possessed by the majority of humans 63% of the time and 56% of the time for sequenced Neanderthals. The chimpanzee reference genome had the effect allele as the primary allele 50% of the

time. Statistical analysis determined these differences could be accredited to chance alone, indicating no clear genetic basis for any species as "more active". Thus, this genetic data does not explain why chimpanzee daily PA is 3-6 times lower than modern day hunter-gatherers (204, 205, 238).

The four selective sweeps in the *Homo sapiens* lineage reveals at least a partial divergence from *Homo neanderthalensis* with increased fitness at these specific locations. Mutations that altered the amount of PA could have improved fitness by increasing the motivation for movement. For example, there is evidence for increased ranging size in early *Homo* relatives around 2 mya that laziness would have directly decreased fitness (154). However, the split between *Homo sapiens and Homo neanderthalensis* is estimated to have occurred much later (~200-350 kya). It is possible the altered regulation of PA could have been a secondary phenotype as gene functions of the four selective sweeps have no clear connection with PA regulation. The protein coded by *IFNAR2* forms a part of membrane receptor for interferons eventually leading to the phosphorylation of many proteins typically associated with infection prevention. Knockouts of the *PPARD* gene suggest a role in myelination of the corpus callosum as well as lipid metabolism. *AKAP10* is known to confine regulatory subunits of protein kinase A to discrete regions of mitochondria. Polymorphisms within *AKAP10* have been associated with increased risk of arrythmias and sudden cardiac death. rs1014533 is found intergenically located closest to *C14orf177* (243). It is unknown if this SNP is connected to this particular open reading frame but would likely suggest a role in gene expression. The suggestions that genetic factors regulate PA as a secondary phenotype

is supported by the fact other PA-related SNPs that were not included in this aging analysis due to location outside of coding areas or lack of age-estimation data have been related to various other phenotypes such as obesity, sensation seeking, depression, blood flow, mitochondrial function, inflammation, Alzheimer's, blood lipid levels, metabolic syndrome, and height.

Of the 104 PA-related SNPs, only 38% of the SNPs had at least one population (African, Eastern Asian, or European) with a significantly unique allele frequency. The largest disparity was found between African and European populations in rs1993246, an intron of *KCCATT33*, where the C allele is 60% more common in Europeans. Due to the large disparity, this allele is likely to have emerged after the splitting of these two populations. Other than rs1993246 and rs8192678, an exon in the *PPARGC1A* gene (Fig. 2.1), the generally low disparity in the other allele frequencies between the populations may indicate at least some conservation within human populations since initial mutation development and growth.

Predictions of the emergence and conservation of PA-related SNPs are complicated by assuming these mutations improved fitness as mutations aged 400-500 kya would likely be fixed in current day, but the data suggest these mutations are not currently fixed. It is possible genetic mutations altering rates of PA were dependent on the environment and were not always advantageous throughout time or between groups. For example, methods of obtaining food are different depending on region, climate, and innovations. While selection may have occurred during periods of time 400-500 kya, genetic drift may have played the strongest effect on allele frequencies in recent years.

A limitation of this paper is only six of 104 unique human PA-related SNPs found to date are located in protein-coding exons. The large number of PA-related non-exon located SNPs raises the question whether these SNPs have any PA regulation function or if they are significant due to random chance alone. Previous papers in rodent models have observed a similar percentage of PA-related SNPs in intergenic areas (166) as well as sets of micro-RNAs associated with high- and low-activity mice (63). The available databases only enable the age estimation of one of the human PA-related intron SNPs with the age estimated for this intron SNP (7,796 kya) being much younger than those we observed from the exon SNPs. The age of this intron SNP (from the *DNAJC1* gene), is close to the beginning of the onset of farming in humans (1). While we will make no speculation of how the *DNAJC1* mutation may have improved fitness and rose in frequency, it is possible that with further dating of the other activity-associated SNPs a more comprehensive timeline of the evolution of the genetic mechanisms regulating PA can be developed. Further limiting our predictions, SNPs with larger minor allele frequencies are more likely to be discovered via GWAS. Since the method by Fu *et al*. uses allele frequency as the heaviest weighted factor to determine mutation age, GWAS discovered SNPs are expected to be predicted as older than the average SNP.

In summary, our results show that where the age-estimation data is available, exon-located SNPs that are associated with the regulation of PA in humans arose between 210 and 785 kya while intergenic SNPs may be much younger. The estimations at this point represent just a small fraction of the known PA-related SNPs but provide an initial framework for better understanding the origins of PA genetic regulation. Further

resolution of this evolutionary timeline will require additional studies and an understanding of the role genomic factors located outside of the protein-coding sequences play in the regulation of PA.

3. WHEEL RUNNING LEVELS ARE MAINTAINED THROUGHOUT LIFESPAN WITH MILD CALORIC RESTRICTION COMPARED TO *AD LIBITUM* INTAKE WITHIN A TUMORIGENESIS MOUSE MODEL

3.1 Synopsis

Breast cancer susceptibility can be altered by poor diet, physical inactivity, as well as *in utero* exposures. The purpose of this study was to determine if physical activity could protect female SENCAR mice from developing mammary tumors after being exposed to a carcinogen (dimethyl-benz[a]anthracene (DMBA)) and a high fat-high sugar (HFHS) or mild caloric restriction (DR) diet during various stages of early life development. Female pups born from dams fed either a HFHS or DR diet during gestation and lactation were randomized post-weaning to either a HFHS or DR diet with or without access to running wheels. Starting at seven weeks of age, mice were orally gavaged via DMBA or corn oil for six weeks. Mice were analyzed for body composition, glucose and insulin tolerance, mammary tumor incidence and latency, and wheel running activity for one year. Mice fed post-weaning *ad libitum* diets had a greater risk of tumor incidence and shortened latency compared to the mice who were fed the post-weaning DR diet. There were significant improvements in body composition and glucose tolerance when given access to running wheels. Access to running wheels did not protect mice from mammary tumorigenesis even though glucose tolerance and body composition were improved. Post-weaning DR diet mice born from dams fed *ad libitum* with previously high resistance to tumorigenesis had significant

increases in mammary tumor incidence and shortened latency when given access to running wheels. Physical activity is not adequate to prevent diet-altered risk for mammary tumorigenesis in the SENCAR model and can exhibit pro-tumorigenic responses in DR mice who would otherwise have high resistance to tumorigenesis.

3.2 Introduction

Women in the United States have approximately a one in eight risk of being diagnosed with breast cancer resulting in an estimated 40,920 deaths in 2018 (196). Glucose dysregulation and the associated chronic inflammation is a commonly targeted mechanism associated with the incidence of breast cancer (23). While genetic predisposition to glucose dysregulation has been demonstrated (287), it is primarily a result of increased adipose tissue, poor nutrition, and physical inactivity (115). The most recent National Health and Nutrition Examination Survey (NHANES) estimated 41.5% of United States adult females are obese (104), and a nationwide accelerometer based study in 2008 revealed only 3.5% of adult women were meeting the daily recommended physical activity guidelines (257). Regardless of whether glucose dysregulation is primarily caused by poor body composition and/or physical inactivity, these lifestyle choices are major contributors to breast cancer risk (8).

Although an individual's current lifestyle impacts breast cancer incidence, data from various species show that early life programming (e.g. *in utero*, nursing, growth) affects the propensity for cancer later in life (268). For example, female offspring of parents that endured the Dutch famine of 1944-1945 had increased rates of cardiovascular disease, diabetes, and breast cancer incidence (2). These effects of a mother's nutritional and metabolic status during gestation on offspring health, deemed the fetal origins hypothesis, has been extensively studied in animal models (15, 111, 114, 149, 155, 242). We previously demonstrated in mice born from mothers who were mildly diet restricted, then post-weaning fed a high fat/high sugar diet, had

significantly increased glucose dysregulation and mammary tumor incidence (149). As such, preventing fetal origins of disease is vital in order to reduce rates of cancer and metabolic diseases (11).

It is well recognized that physical activity is essential for establishing proper glucose regulation and improving body composition (36, 153). Further, the World Health Organization's International Agency for Research on Cancer estimates that 25-30% of cancer (primarily breast, colon, and lung) could be mitigated by avoiding weight gain and engaging in physical activity (261). While animal models have been used to study the effects of physical activity on tumorigenesis, the majority use forced treadmill running or wheel running with one mouse and one wheel per cage. Mixed results from these studies may be due to the associated stress from forced treadmill running or social isolation (125, 129). For example, one study with singly housed mice concluded physical activity via treadmill or wheel running were pro-tumorigenic (56). Additionally, no studies have determined if wheel running can reverse the effects (primarily glucose dysregulation) of pro-tumorigenic diet exposure *in utero* and across the lifespan. Therefore, the purpose of this study was to examine the efficacy of co-housed wheel running activity to prevent mammary tumor susceptibility when induced by carcinogen in mice who were fed either a mild diet restricted or high fat/high sugar diet in early development periods (gestation, lactation, and post-weaning) using a combination of our previously established tumorigenesis model (149) and a mouse wheel-running model (165).

3.3 Methods

3.3.1 Study Approval

All experimental procedures were approved and performed in accordance with the guidelines set by the Institutional Animal Care and Use Committee (Texas A&M IACUC 2013-0132).

3.3.2 Mice and Diets

Outbred SENCAR breeder mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in our AAALAC-accredited facility under temperature and light controlled conditions (24° C and 12-hour light and dark cycles). Mice were randomized onto their respective diets as described previously (12). Briefly, at four weeks of age, mothers were randomly placed on either an *ad libitum* high fat/high sugar Western-like diet (HF), *ad libitum* chow-like diet (C), or a mild 12% calorie restricted chow-like diet (DR). All diets (Table 3.1) were purchased from Research Diets, Inc. (New Brunswick, NJ). After two weeks of diet acclimation, HF-fed breeder mice were provided with a 10% fructose drinking water solution to simulate consumption of high-fructose corn syrup sweetened beverages (266, 267). The DR diet group mimicked a mild portion control diet to prevent excess weight gain in the sedentary *ad libitum* mice without resulting in insufficient macro- or micronutrients.

3.3.3 Breeding and Offspring Experimental Groups

At 15 weeks of age, female mice from the HF and DR diets were paired with male SENCAR mice previously fed a C diet creating "gestation" exposure groups (Figure 3.1). Male pups of the resulting litters were culled immediately, and female pups were fostered within 24 hours of birth to female SENCAR mice with the HF or DR diet to create "lactation" exposure groups. At three weeks of age, pups were then co-caged and provided a HF or DR diet to create "post-weaning" exposure groups for the remainder of the study. Pups from a C-fed mother remained with C-fed mothers during lactation and were given a C diet at weaning to provide a C control group in all three phases (gestation, lactation, and post-weaning). Group names used were based on diet exposures created in our previous study (149) to maintain continuity. Body weights and total food consumed were recorded weekly using an electronic scale (Mettler Toledo, Columbus, OH) until 20 weeks of age when weight reached a plateau.

3.3.4 Voluntary Wheel Running

To develop a group-housing model of wheel running, four-week-old SENCAR mice were housed one to a cage with one wheel (1v1), two to a cage with one wheel (2v1), or two to a cage with two wheels (2v2). The running wheel setup used was similar to our previously validated model (165). Briefly, plastic running wheels with a 410 mm circumference solid running surface (Kaytee, Chilton, WI) were secured to the top of the cage and wheel rotations were individually measured using an odometer (BC8.12, Sigma Sport, Batavia, IL) with a magnet glued to the wheel's outer rim. Unrestricted access to the wheel was provided throughout the day and night cycle. Daily distance (km/day) and time (min/day) were recorded each day between the 12:00-14:00. Average daily speed (m/min) was calculated by dividing distance run (km) by duration of activity (mins). Wheel functionality was assessed by manually spinning each wheel until a change of 0.01 km was registered on the odometer. Wheel calibration was determined by matching the number of spins to reach 0.01 km to the theoretical number of spins based on wheel circumference. If the number did not match, the magnet and odometer alignment was adjusted, spins were recounted, and data for that day were removed from the final analysis. Given the dual-wheel activity set-up was deemed to result in adequate activity levels for the involved mice (see results), the 2v2 wheel set up was used during the tumorigenesis phase of this study such that at four weeks of age, half of the mice from each diet group were randomly selected to receive two running wheels or no wheels.

3.3.5 Glucose and Insulin Tolerance Tests

A subset of animals from each diet and activity group were randomly assigned to receive glucose tolerance tests (GTT) and insulin tolerance tests (ITT). Tests were performed at 10 and 12 weeks of age, respectively. Animals were fasted for 6 hours starting at 6AM, then baseline blood glucose levels were taken by drawing blood from the submandibular vein using a lancet

(Goldenrod Animal Lancet, MEDIpoint Inc., Mineola, NY). Glucose levels were measured using the AlphaTrak animal glucometer (Zoetis, Inc., Parsippany, NJ) at 0, 30, 60, and 120 minutes post-intraperitoneal injection of 2 g/kg glucose (GTT) or 0.75 U/kg insulin (ITT).

3.3.6 Mammary Carcinogenesis

Starting at seven to nine weeks of age, a subset of animals received 20 µg/mouse/day of 7,12-dimethylybenz[a]anthracene (DMBA) or corn oil (vehicle-control) by oral gavage for five days per week for six weeks, as described previously (149, 209). Mice were weighed weekly and monitored daily for health status and tumors. Mice were sacrificed upon tumor detection or when they reached 1 year of age. After euthanasia via CO_2, lean mass, fat mass, and bone mineral density were measured using a dual energy x-ray absorptiometry scan (DXA, Lunar PIXImus Densitometer, GE Medical Systems, Chicago, IL).

3.3.7 Statistical Analysis

To determine if housing density and wheel availability affected physical activity levels, average daily distance, duration, and speed differences between housing/wheel conditions were compared using separate 1-way ANOVAs with housing density/wheel condition as the factor. Given we could not determine how much activity each mouse completed in the double-wheel cages, the data was analyzed by wheel; i.e. cages with two wheels in them had activity values averaged within cage that resulted in two equivalent values. In all instances, the alpha value was set *a priori* at 0.05. Paired student t-tests were performed between weight and body composition of mice within each cage as an indirect confirmation of whether animals that were co-caged were eating and exercising similarly.

Individual mouse wheel running metrics (distance, duration, and speed) were analyzed relative to the total lifespan of the individual mouse, resulting in wheel running metrics at specific percentages of lifespan (5, 25, 50, 75, and 100% of lifespan). Analyzing the data by

absolute lifespans (as opposed to using relative percentage of lifespan) resulted in the later age groups being skewed by 'healthy' animals. The resulting data were compared using a two-way ANOVA with diet group and relative percent of lifespan as factors. A Tukey's *post hoc* test was employed in the case of significant effects.

Body weight at 20 weeks was examined by comparing all diet groups using a Welch's t-test. Glucose and insulin tolerance test responses were quantified via a trapezoidal area under the curve analysis and subsequently compared using a Wilcoxon rank sum test. Survival analysis was carried out using Kaplan–Meier statistics with all diet groups compared using the Mantel–Haenszel test, and final tumor incidence proportions compared using Pearson χ^2 test statistic. *Tukey's post hoc* tests between relevant groups were performed in the case of significant effects. If groups with similar post-weaning diets were not significantly different, this was interpreted as undetectable influence from mother's diet. All statistical analyses were completed using JMP statistical software (SAS Inc., Cary, NC). All graphs were created using GraphPad Software (La Jolla, CA).

3.4 Results

3.4.1 Wheel Running Setup

When singly-housed (1 mouse and 1 wheel, 1v1), the SENCAR mice ran an average of 10.03 km/day, which places them among the highest active mice compared to the 41 strains we have previously tested (165). There was a significant effect of housing density and wheel availability on wheel running levels (Figure 3.2). Mice housed in 2v1 (2 mice and 1 wheel) and 2v2 (2 mice and 2 wheels) arrangements ran 6.19 and 6.27 km/day respectively, which is significantly less ($p = 0.0001$) than those in 1v1 cages (10.03 km/day). While wheel running decreased in a group caged setting, total distance was still among the top 50% of previously tested strains. Given the overall purpose of this study was to determine if wheel running would

offset diet dependent risk of DMBA induced tumorigeneses, it was determined the observed level of wheel running in the 2v2 setup would be a sufficient model for the remaining phases of the study.

3.4.2 Wheel Running Outcomes

Baseline wheel running (5% of lifespan) was not different between any diet group (daily distance: 5.62 ± 2.64 km, $p = 0.2543$; Figure 3.3). Due to the outbred nature of the colony, there was larger variation in wheel running activity within groups than typically seen in our previous studies using inbred mouse strains (165). Post-weaning DR diet groups maintained their baseline wheel running distance throughout their lifespan but displayed a gradual decrease in running duration that did not become significantly lower than baseline until 100% of their lifespan (-33.7%) with a similar gradual increase in running speed at 100% of their lifespan compared to baseline (+10.9%). Group AL (C/C/C) and post-weaning HF diet groups D (DR/HF/HF) and G (HF/DR/HF) had significant decreases in wheel running distance, duration, and speed across the lifespan compared to the post-weaning DR diet groups A (DR/DR/DR), F (HF/HF/DR), and H (HF/DR/DR) ($p < 0.01$). By 100% of animal lifetime, post-weaning C and HF diet groups ran on average 2.36 less km/day, 87.24 less minutes/day, and 6.1 m/min slower than post-weaning DR diet groups. While the post-weaning HF diet animals were on average 11 g heavier than the post-weaning C diet animals, there were no differences between these groups in running distance, duration, or speed at any point across the lifespan.

To determine if wheel running levels were related to body weight, *post hoc* linear regression correlations were performed comparing body weight with distance, duration, and speed at 20 weeks of age. Twenty weeks of age was the final week of body weight measurement for all animals ((DR = 37.0 ± 4.1 g; C = 41.2 ± 3.7 g; HF = 50.9 ± 10.3 g). Body weight did not correlate with distance, duration, or speed in the post-weaning C or HF diet mice. However,

there was a significant negative correlation between duration and body weight in the post-weaning DR diet mice ($R^2 = 0.43$, $p < 0.0001$; Figure 3.3).

3.4.3 Body Composition

Mice within the same post-weaning diet had similar body weights throughout the study via repeated ANOVA analysis suggesting no significant influence of gestation or lactation diet (Figure 3.4). The post-weaning HF and C diet mice were on average 57% and 23% heavier than the post-weaning DR diet mice by 20 weeks of age ($p < 0.0001$; Figure 3.4). The differences in body composition between the groups were solely due to greater body fat with no differences in lean body mass between groups ($p = 0.0879$, Figure 3.4). Even though the post-weaning C diet mice consumed approximately the same calories from solid food as the post-weaning HF diet mice (average of 2-0.5 kcal/day less), post-weaning C diet mice weighed on average 11.0 g less (20.7%) than the post-weaning HF diet mice at 20 weeks of age ($p < 0.0001$, Figure 3.4). Whereas fluid intake was not measured during the study, the increased weight gain in the HF mice is likely attributed to the additional calories as well as fructose decreasing fat oxidation leading to greater fat storage (213). Access to a running wheel resulted in significantly lower body weight in all post-weaning *ad libitum* diet groups ($p < 0.0001$; Figure 3.4). While all post-weaning diet restricted groups with access to a running wheel had consistently lower average body weights across the entire study compared to their non-wheel diet groups, only diet group A (DR/DR/DR) reached consistent statistical significance (Figure 3.4). As such, the extent of body weight gain prevention via wheel running access was larger in post-weaning HF (group D = 35%; group G = 17%) and C diet groups (group AL = 21%) compared to the post-weaning DR diet groups (group F = 11%; group A = 16%; group H = 9%) diet mice.

3.4.4 Glucose and Insulin Tolerance Tests

Glucose tolerance tests (GTT) showed the mice fed the post-weaning HF diet, group D (DR/HF/HF) and group G (HF/DR/HF), were significantly more glucose intolerant compared to the mice fed the post-weaning DR diet, group H (HF/DR/DR) and F (HF/HF/DR), similar in our previous study (149). Wheel running significantly improved glucose tolerance in all groups except group F (HF/HF/DR, $p = 0.089$) and group AL (C/C/C, $p = 0.384$; Figure 3.5). The lack of a significance difference in group F (HF/HF/DR) may have been due to the group having the best glucose tolerance of all groups even without access to running wheels, so there was little room for activity-induced improvement. Insulin tolerance tests (ITT) revealed groups D (DR/HF/HF) and G (HF/DR/HF) had impaired ability to clear glucose from the blood stream. Animals with access to running wheels in groups D (DR/HF/HF) and A (DR/DR/DR) had superior insulin sensitivity compared to their non-wheel diet group ($p = 0.015$; Figure 3.5). However, no ITT differences were seen in any other group despite the clear metabolic changes seen in the GTT.

3.4.5 Mammary Carcinogenesis

Kaplan-Meier survival curves revealed a significant difference in mammary tumorigenesis (latency) between diet groups without wheels ($p = 0.0005$; Figure 3.5). *Post hoc* analysis revealed post-weaning *ad libitum* diet groups (D (DR/HF/HF), G (HF/DR/HF), and AL (C/C/C)) had significantly shorter mammary tumor latency compared to the DR diet groups (A (DR/DR/DR), F (HF/HF/DR), and H (HF/DR/DR); Table 3.2). Tumor incidence rates between groups were similar to tumor latency (Table 3.2). Groups with the same post-weaning diet did not have significantly different tumor latency or incidence. When analyzing the effect of post-weaning diet alone, only 14.5% of mice who were fed the DR diet had a tumor halfway between the end of dosing and the end of one year compared to 61% with tumors in the post-weaning HF diet groups. This result corresponds to a combined hazard ratio of 3.391 in groups G

(HF/DR/HF) and D (DR/HF/HF) compared to a combined hazard ratio of 0.2949 in groups F (HF/HF/DR), H (HF/DR/DR), and A (DR/DR/DR). A *post hoc* analysis to determine if tumor growth rates were significantly higher in any diet group did not meet significance ($p = 0.6311$).

For groups with access to running wheels, group F (HF/HF/DR) and group H (HF/DR/DR) had significanctly shorter tumor latencies (Figure 3.6) and 63% and 68% significantly greater tumor incidence at one year, repsectively (Table 3.2) compared to the non-wheel group F and H. All other diet groups had non-significant differences in tumor latency or incidence regardless of running wheel exposure and improved body weight and glucose tolerance.

3.5 Discussion

The overall purpose of this study was to determine the efficacy of co-housed wheel running activity to prevent mammary tumor susceptibility when induced by carcinogen in mice who were fed either a mild calorie restricted diet or high fat/high sugar diet in early development periods (gestation, lactation, and post-weaning). While we observed that the dual wheel running protocol was sufficient to prevent excess body fat gain and glucose intolerance, mammary tumorigenesis was not mitigated in any diet group that had access to running wheels compared to their corresponding diet groups without wheels. Further, mice who were calorically restricted and birthed from mothers who were fed high fat diets had significantly shortened latency and increases in tumor incidence with the addition of wheels. The major findings of this work suggest co-caged wheel running cannot overcome the effects of a high fat/high sugar diet and becomes pro-tumorigenic in mild calorie restricted female SENCAR mice.

We hypothesized physical activity would improve glucose tolerance and protect mice from mammary tumorigenesis. Previous studies have shown that caloric restriction can protect mice from developing mammary tumors (52). Attempts at mimicking the anti-tumorigenic

effect of a negative energy balance via treadmill running or wheel running has yielded opposite results (56), which supports our observations of a trend towards an increase in tumorigenesis in those animals that were active. To date, however, no other study has investigated the tumorigenesis outcome of wheel running in DR or HF diet animals from mothers that were fed DR or HF diets during gestations and lactation. As such, our observations of a significant increase in tumorigenesis when given access to a running wheel in post-weaning DR diet mice that were born from HF diet fed mothers (63% and 68% increase in groups F (HF/HF/DR) and H (HF/DR/DR), respectively) are novel. No other diet group experienced a change in tumorigenesis with access to a wheel, including post-weaning DR diet mice from DR diet mothers. We would suggest that these groups did not see an increase in tumorigenesis with added wheel running because diet groups without wheels [besides groups F (HF/HF/DR) and H (HF/DR/DR)] may have been at a physiological ceiling with tumor incidence already ranging from 68% to 79% (Table 3.2). Conversely, we would suggest that the observed very high levels of wheel running across the entire lifespan observed in both group F (HF/HF/DR) and group H (HF/DR/DR) (Figure 2) could have augmented the growth of tumors due to an increased oxidative stress (122) and/or lactate production fueling tumor growth via the Warburg effect (116) increasing tumorigenesis. Additionally, while literature suggests adding a cage mate would reduce social stress, it is possible there was added competition between mice in DR situation leading to stress and increased activity. Wheel running in underfed mice can become excessive and has been shown to lead to dramatic weight loss and even death (62). Since the level of running of the post-weaning DR diet mice would place them among the highest runners in terms of distance, duration, and speed of any of the 41 strains of mice we have tested before (165), it is possible that increased competition increased stress and potentially tumorigenesis (217).

Parallel with the Dutch famine studies and the fetal origins hypothesis (2), the mouse mothers in this study who were calorically restricted gave birth to offspring with greater disease risk and mammary tumorigenesis when exposed to a carcinogen. Similar to our previous study (149), post-weaning HF diet mice born from DR diet mothers had the greatest incidence of mammary tumorigenesis regardless of activity. DR diet mice born from DR diet mothers also had slightly elevated tumorigenesis compared to DR diet mice born from mothers on a HF diet. All other maternal diet combinations did not have a detectable influence on tumorigenesis. Further, it was not clear that the unique diet differences in mothers alone did not play a role in altering body weight, glucose, or insulin tolerance. It is possible the post-weaning diet had such a large magnitude of effect on future tumorigenesis, that any potential epigenetic effect on body composition or glucose tolerance from gestational or lactation dietary influences was masked.

The dietary effect on the amount of wheel running performed in each group was not an original hypothesis in the design; however, other studies have recently shown significant decreases in daily activity in mice chronically fed high fat / high sugar diets (213, 265). We observed that animals fed either of the post-weaning *ad libitum* diets (groups F and H) with either chow or high-fat diet, had significantly lower daily distance, duration, and average speed throughout the lifespan which was evident as early as four weeks of age (Figure 3.3). Mice who were on the post-weaning DR diet (groups A, F, and H) did not have a decrease in distance or speed throughout the lifespan and did not have a significant drop in duration until the final weeks of the lifespan (Figure 3.3). Higher activity levels in animals fed the post-weaning DR diets compared to the post-weaning C diet is in agreement with previous literature (203). The cause of this increase is thought to be a coping mechanism to manage the lack of reward obtained from eating by increasing the reward obtained from activity (203). The mechanisms regulating physical activity via alterations in dietary food composition and quantity are unclear at this point,

but are active areas of investigation (213, 265). Supporting a potential independent dietary regulation of physical activity, we observed (Figure 3.3) that wheel running in the post-weaning C and HF diet groups was largely independent from body weight, which we and others have previously observed (90, 213, 265). Conversely, post-weaning DR diet mice showed a significant correlation between lower body weight and increased running duration which may be explained by the well documented increased drive for movement in proportion to the magnitude of diminished weight below body weight during *ad libitum* intake.

3.5.1 Limitations

While the wheel running protocol was successful in preventing weight gain and glucose intolerance, the running intensity and duration were lower in post-weaning C and HF diet groups. It is possible these groups did not reach a level of physical activity that could prevent tumorigenesis; however, it is unknown what level of physical activity is needed to prevent tumorigenesis. Compared to past activity studies in mice (165), the mice in these studies would have been considered moderately active. Moderate activity in humans has been shown to lead to prevention of several forms of cancer (42), so until there is further data regarding necessary levels of activity for tumorigenesis reduction/prevention, whether our mice do enough activity is an open question. Additionally, since the mice in this study were co-caged, it was not possible to distinguish the caloric intake and wheel running of each individual mouse. The purpose of adding cage mates was to prevent social isolation factors to better model human populations since social isolation is not generally present in human populations. Although the evidence for avoiding social isolation is sound (125, 129), SENCAR mice may require additional numbers per cage for interaction or the co-caging may have introduced unknown factors that are not usually evident in human models.

3.5.2 Conclusions

The purpose of the current study was to determine if wheel running would offset the increased mammary tumorigenesis from certain dietary regimens we previously observed when exposing mice to DMBA (19). Complicating our results, is the understanding – including our own observations – that diet independently has its own effects on physical activity (265) which may also influence tumorigenesis. Our observations of an increase in tumorigenesis with activity – especially in the diet restricted groups – suggest it is possible that in some of the animals, the increased physical activity, despite a normalization of body weight and glucose tolerance, increased tumorigenesis due to the high volume of activity completed. In this instance, we conclude improved glucose tolerance and body composition from vigorous exercise is not in itself protective from tumorigenesis as there may be further complexities and interactions arising from the high intensity and duration of activity.

4. A HIGH FAT/HIGH SUGAR DIET ALTERS THE GASTROINTESTINAL METABOLOME OF MICE IN A SEX-DEPENDENT MANNER

4.1 Synopsis

We characterized the alterations in the gut metabolome of male and female C57BL/6J mice fed a high fat (45%), high sugar (20% fructose drinking solution) diet. C57BL/6J female and male mice were weaned at 3 weeks of age, individually housed, and randomly assigned to either a standard "chow" diet (CHOW) or a high fat/high sugar diet (HFHS) for nine weeks. Total caloric intake and body composition were measured weekly. Cecal metabolites were extracted and analyzed on the QExactive mass spectrometer coupled to liquid chromatography. Data were analyzed using Progenesis QI, Mummichog and Metaboanalyst. The HFHS mice (female: n=6, male: n=6) consumed significantly more calories per day than CHOW mice (female: n=6, male: n=5; 22.0 and 26.3% kcal) and had significantly higher body fat (12.8 and 26.3%). Significant changes were found in the cecal metabolome of HFHS fed mice in a sex dependent manner. Data analysis reveals 2,443 and 1,669 metabolome features to be significantly altered with a HFHS diet in the females and males respectively. In females, metabolites from eleven pathways were significantly altered with a HFHS diet primarily altering the tryptophan metabolism and the vitamin B9 (folate) metabolism pathways. In males, metabolites from eight pathways were significantly altered with a HFHS diet primarily the lysine metabolism and the vitamin B3 (nicotinate) metabolism pathways. Three pathways were differentially altered with a HFHS diet between the animals, including androgen and estrogen biosynthesis and metabolism. A HFHS diet depletes many beneficial metabolites in the gut, alters the sex hormones, with some metabolomic changes being sex dependent. The distinct

metabolomic features from female and male mice shows that sex plays an important role and should be considered while investigating the effects of diet on the host.

4.2 Introduction

The gut microbiome has been suggested as a critical component of host metabolic homeostasis (14). The microbiota colonizing the intestinal tract affect host physiology by metabolizing ingested nutrients leading to increased molecular diversity (18). These microbial derived metabolites interact with the digestive system just like any other digested nutrient: absorption into the blood stream, interaction with the enteric nervous system, stimulation or inhibition of hormone signaling, interaction with other metabolites, or excretion. As seen in germ-free mice, microbial-derived metabolites are not required for living, but the absence can lead to dysfunction of organ systems such as liver disease (9), immune deficiency (16), and psychological state (10). A gut completely devoid of microbes is not plausible in humans, but the loss of mutualistic microbes (1) and/or the addition of opportunistic pathogens (15) has been linked to poor health (21). For this reason, much research has focused on characterizing microbial diversity of humans and animals to generate connections to various phenotypes.

Microbial diversity is highly susceptible to environmental factors such as, but not limited to, antibiotics (4), diet (3), and geographical location (22). Diet is the second strongest predictor, behind medication usage (5), of which species of microbes thrive in the gut as microbe species that cannot metabolize the nutrients available will die and be replaced with species that can. Given that we have recently observed that a high fat/high

sugar diet significantly decreases physical activity levels in mice (19), we hypothesized this physical activity reduction may be dependent on alterations in the gut microbiome.

Low microbial diversity and/or the abundance of pathogenic microbes has been termed a "dysbiosis". However, what constitutes a "healthy" microbiome has been proven difficult to determine. To create a simple diagnostic marker of gut health, many studies have used the ratio of the two most abundant microbe phyla, the gram-negative *Bacteroidetes* and the gram-positive *Firmicutes*. Early microbiota studies found a strong correlation between obesity and an increase in the abundance of *Bacteroidetes* and a depletion of *Firmicutes* (6), although the correlation has not always been repeatable and varies through weight loss interventions (17). Clearly, alterations in two phyla cannot describe the complexity of microbial changes with diet alterations. Still, in a study by Moeller et al, humans have a substantially lower microbial diversity compared with other primates, and US citizens had even less diversity than the guts of agricultural based communities in Venezuela and Malawi (13). Evidence is available suggesting that the Westernized diet of high fat, processed foods lacking fibrous vegetables may be affecting the microbiome in a harmful manner (6).

The lack of an easy marker for dysbiosis or specific microbe species that influence disease states is likely due to the incredibly high degree of interconnectivity between microbes and their bi-directional interaction with the body. A microbe species may be able to colonize the intestinal lumen but may not have a specific nutrient available to generate various metabolites. Thus, research has focused on measuring changes in known beneficial microbial derived metabolites, such as the short chain fatty

acids derived from indigestible carbohydrates, to determine health outcomes. The short chain fatty acids are known to function in various ways, primarily improving the gut/blood barrier preventing excessive inflammation. Another metabolite, trimethylamine N-oxide (TMAO) generated from choline, betaine, and carnitine, has been related to the occurrence of cardiovascular events. However, ignoring the thousands of other metabolites produced by the microbes could be disregarding critical components of the gut-body connection. Thus, in the present study, we aim to use an untargeted metabolite analysis to generate a repository of altered metabolites from the cecum of C57BL/6J mice on a standard mouse diet containing fiber compared to a high fat (45%), high sugar (20% fructose solution) mimicking a poor Westernized diet. The compiled data will provide a resource for future studies to causally test the connection of specific metabolite production and physiological function.

4.3 Methods

4.3.1 Study Design

The cecal tissues used in this study were taken from a previously published study (265). Briefly, at three weeks of age, C57BL/6J pups were weaned, individually housed, and randomly assigned to an intervention diet. Two *ad libitum* diets were utilized: a standard "chow" diet (CHOW) consisting of 4% fat, 25.2% protein, 39.5% carbohydrate, 3.3% crude fiber, 10% neutral fiber, and 9.9% ash (Harlan Labs, Houston TX) and a high fat/high sugar diet (HFHS) consisting of 45% fat, 20% protein, 35% carbohydrate, 5% fiber, and a 20% fructose solution in place of regular drinking water (product D12451, Research Diets, Inc., New Brunswick, NJ) (Female on CHOW n=7; Female on

HFHS n=5; Male on CHOW n=5; Male on HFHS n=5). Caloric intake was measured weekly using an electronic scale. Body composition was measured weekly using a MRI (EchoMRI, Houston, TX). Feces from the cecum were extracted during animal sacrifice by squeezing content into a cryotube, immediately flash frozen via liquid nitrogen, and stored at -80 °C.

The HFHS diet combined with fructose drinking water is used to mimic a United States citizen with a worse than average diet as according to NHANES's surveys where the United States mean total fat intake is ~34% (7) and mean total calories from fructose is ~10% (20). Due to the increased caloric intake of the *ad libitum* HFHS compared to an *ad libitum* CHOW diet, we termed the HFHS diet intervention "overfeeding" (19). Inbred C57BL/6J were selected as the best model for human translation due to their susceptibility to diet induced obesity (2), slight preference for fructose water over regular water (8), and their consistent and repeatable wheel running phenotype (11, 12).

4.3.3 Metabolite Extraction.

Using tissues from Vellers, et al (265), cecal metabolites were extracted using a solvent-based method (17). Ice-cold methanol/chloroform (2:1, v/v) was added to pre-weighed cecal samples. Samples were homogenized using garnet bead tubes and Precellys 24 homogenizer at 5,000 rpm for 20 seconds. After homogenization, the samples were centrifuged at 3,000 rpm for 10 min at 4 °C and the supernatant was collected. A second sequential extraction was performed to maximize the metabolites extracted and the supernatants were pooled. 600 µl of ice-cold water was then added to the supernatants, vortexed and centrifuged at 5,000 rpm for 5 min at 4 °C to obtain phase

separation. The upper and lower phases were collected. The upper phase was passed through a 0.2 micron filter and lyophilized. The concentrated sample were resuspended in 200 µl of methanol/water (1:1 v/v) for the LC-MS analysis. The samples were stored at -80 °C until the metabolomic analysis was carried out using Q-Exactive Plus orbitrap mass spectrometer (Thermo Scientific) coupled to Dionex 3000 UHPLC system. A C18 Synergi Fusion-RP 4µ 80Å 150 x 2.0 mm column (Phenomenex) was used for chromatographic separation with 0.1% formic acid in water (Solvent A) and with 0.1% formic acid in ethanol (Solvent B). MS1 and MS1-dependent MS2 spectra was collected at a m/z resolution of 37,500. Metabolites were eluted at a flow rate of 0.4 ml/minute. The flow gradient was 40% of solvent B for 5 min, 95% of solvent B for 7 min and 10% solvent B for 8 min. Blanks (methanol and water at 1:1 v/v) were inserted between every sample to prevent any sample carryover. Deuterated indole-3-acetic acid was used as a labeled internal quality control standard. Pure standards of metabolites of interest were used to generate a standard curve for absolute quantification. Data were analyzed using Progenesis QI software (Waters), Human Metabolome Database (HMDB) and the Kegg database for metabolite identification. Raw abundance data were normalized to fecal sample weights and statistical analysis was performed using KaleidaGraph (Synergy).

4.3.4 Statistical approach.

The statistical comparison of caloric intake and body composition data (total mass, fat mass, and lean mass) across the experimental timeline were analyzed and reported previously (265) using independent one-way ANOVAs (diet treatment) with an

alpha level of 0.05 set a priori. The statistical analyses of caloric intake and body composition were performed using JMP statistical software (SAS Inc., Car, NC). All graphs were created using GraphPad Software (La Jolla, CA). For this paper, comparisons of the medians between the metabolite levels of CHOW and HFHS fed mice were performed with the non-parametric two-sided Mann–Whitney U-test with an alpha level of 0.05 set *a priori* then adjusted for multiple comparisons.

4.4 Results

4.4.1 Body Composition, Total Food, and Water Consumption

As analyzed previously (265), mice on the HFHS diet consumed similar levels of total calories from pellets, but significantly greater total calories due to the additional fructose intake (Table 4.1; published previously (265)). Mice on the HFHS diet also had greater body fat and lean mass (Table 4.1; published previously (265)). In a separate analysis, if given a running wheel, the male and female HFHS diet mice ran approximately 70 ± 28% and 57 ± 26% less than the CHOW diet mice, respectively (265). This previous data showed that overfeeding the mice significantly decreased physical activity.

4.4.2 Metabolite Analysis

The PLS-DA plot organizing the untargeted analysis of cecal metabolites revealed significant changes between diet groups in a sex dependent manner (Figure 4.1). The Mann–Whitney U-tests of metabolites yielded 2,443 and 1,669 features significantly altered between diet groups in females and males respectively (Figure 4.2). Significant metabolites identified via similar m/z ratios using PubChem, the Human

Metabolome Database (HMDB), and the Kyoto Encyclopedia of Genes and Genomes database were compiled and analyzed for known metabolic pathways with altered abundance. In females, metabolites from eleven pathways were significantly altered with a HFHS diet including the tryptophan metabolism pathway (Figure 4.3). In males, metabolites from eight pathways were significantly altered with a HFHS diet including the androgen biosynthesis/metabolism pathway (Figure 4.3). Three pathways were differentially altered with a HFHS diet between sexes, including leukotriene metabolism and androgen and estrogen biosynthesis and metabolism (Figure 4.3). Also of interest, 2-Indole Carboxylic Acid, an anti-inflammatory beneficial metabolite, was depleted with HFHS diet in both males and females (Figure 4.4).

4.5 Discussion

4.5.1 Cecal Metabolome

The HFHS diet had profound changes on the gut metabolome in the cecum of both female and male C57Bl/6J mice. While the difference is likely due to nutrient availability between the diets, females and males exposed to the same HFHS diet have unique metabolites expressed. This data suggests female and male mice respond differently to the same diet as early as digestion and may partially explain why male mice experience greater decreases in physical activity with exposure to the HFHS diet compared to female mice (265). A sex-dependent dichotomy is recognized in humans for pharmacokinetics (84); however, more evidence is needed for sex interactions with diets and should be considered in future studies.

4.5.2 Inflammation

One of the primary signs of poor intestine health is the presence of inflammation. If the intestinal epithelial cells become damaged, barrier integrity can be compromised potentially leading to elevated bacterial endotoxins or unwanted metabolites in the bloodstream (224). High fat foods and fructose have both been linked to the presence of intestinal inflammation, increased epithelial permeability, and the onset of non-alcoholic fatty liver disease (88). The addition of indole, a metabolite generated from the breakdown of tryptophan, helped mitigate this inflammation (273). From our current analysis, mice on the HFHS had no detectable levels of indole in the cecum suggesting a lack of protection from inflammation in these mice (Figure 4.4). However, only male mice exhibited overexpression of multiple inflammatory metabolites such as prostaglandins 1-4, arachidonic acid, and thromboxane. Although, these metabolites were detected with a 5 ppm error and cannot be verified.

4.5.3 Androgen Biosynthesis and Metabolism

Androgens are critically important for many physiological functions affecting growth, mood, and vitality. While limited, studies have linked diet with alterations in sex hormones. Bouchard *et al.* overfed male subjects by 1000 kcal/day, six days a week, for 100 days resulting in depletion of circulating androgens (142). There is evidence that endotoxemia via chronic intestinal inflammation is negatively associated with circulating androgens (256). However, increases in androgen levels are associated with overfeeding in females (142) indicating critical sex-dependent considerations should be made for making causal links.

Elevated levels of cortisol were found in mice on the HFHS diet (unconfirmed; Figure 4.4). Although cortisol can be a product of diet, it is primarily secreted into the intestines by the host especially in times of stress (199). Corticosteroids are important modulators of intestinal inflammation which can also be reabsorbed back into circulation (191). Ridlon *et al.* discovered a gut microbe, *Clostridium scindens,* that can convert corticosteroids in the gut into androgens (142). Our untargeted analysis revealed a significant increase in the production of gut androgens in females and males exposed to the HFHS diet (Figure 4.3). The effects of elevated androgens in the gut are not well documented and while our results cannot demonstrate a direct link between diet and circulating androgens or the activated levels of cortisol (hydrocortisone) these data provide an intriguing basis for future studies.

4.5.4 Neurotransmitter Biosynthesis and Metabolism

The production of neurotransmitters in the gut has been a target of many studies attempting to connect mood states with gut function; however, the causal connection to mood states have yet to be determined (124), and generally the production of neurotransmitters is associated with gut motility and irritable bowel syndrome. The HFHS diet resulted in elevated levels of both serotonin (via tryptophan metabolism) and glutamate (via lysine metabolism) associated metabolites (Figure 4.3). Interestingly, neurotransmitter expression was also sex-dependent (Figure 4.3).

4.5.5 Conclusion

The findings of our untargeted analysis have characterized distinct changes in the cecal metabolome that are dependent on diet as well as sex. The results are limited as

the specificity of metabolite identifications were not validated with purified samples. However, there is evidence for alterations in multiple pathways critical for host homeostasis such as inflammation, androgens, and neurotransmitter production. These findings are important as it may be possible to replace depleted metabolites critical for host health by supplementing specific nutrients supporting the formation of indole or removal of nutrients that lead to formation of pro-inflammatory molecules. Additionally, the variety in metabolomic signatures between sexes on the HFHS diet should be considered while investigating the effects of diet in future studies.

5. FECAL MICROBIAL TRANSPLANTS COMBINED WITH DIET IMPROVEMENT REPAIRS LOW PHYSICAL ACTIVITY LEVELS FASTER THAN DIET ALONE IN C57BL/6J MICE

5.1 Synopsis

In this study, we tested the hypothesis that gut microbiota modulate the effect of nutrient intake on physical activity. C57BL/6J male mice (n = 40; 5 weeks of age) were individually housed and divided randomly into four groups: Group 1 (control group) received an *ad libitum* "chow" diet with regular drinking water (CHOW) and Groups 2, 3, and 4 received an *ad libitum* high fat diet with a 20% fructose drinking water solution (HFHS) for a total of 13 weeks. Each group was given a running wheel for physical activity monitoring after three days on the new diet. After 13 weeks, Groups 2 and 3 were switched to a CHOW diet. Fresh fecal pellets from Group 1 (control group) were collected, homogenized, and orally gavaged in Groups 2 and 4 once a week for four weeks. At week 13, Groups 2, 3, and 4 (HFHS diets) ran significantly less distance, duration, and speed than Group 1 (CHOW diet). The HFHS animals ate significantly greater calories, had more body fat, and had less microbial richness and diversity at 13 weeks. After the treatment initiation at the beginning of week 14, Group 2 (switched to CHOW plus a fecal transplant from Group 1) had a significant increase in wheel running indices after one week while Group 3 (switch to CHOW diet plus a vehicle transplant) had no increases until two weeks. Non-metric multidimensional scaling indicated the communities were distinct between the Group 1 and Groups 2-4 at baseline.

Communities microbial diversity were similar between Groups 2 and 3 throughout both weeks. Thus, a HFHS diet decreases wheel running activity, increases body fat, and decreases microbial diversity compared to a CHOW diet in C57BL/6J male mice. Improvements in wheel running, body composition, and microbial diversity was accomplished within 2 weeks by switching mice from a HFHS diet to a CHOW diet. However, switching diets plus receiving a fecal transplant from a desirable gut provided quicker results than diet alone. A fecal transplant from a desirable gut without altering diet did not recover activity levels or body composition even though alpha diversity increased. Our results suggest diet is the primary mediator of physical activity levels while microbes can support the effectiveness of the diet potentially dependent on presence or absence of multiple microbes including *Lachnospiraceage*, *Alistipes*, *Clostridialvadin*, *Lactococcus*, *Faecalibaculum. Erysipelatoclostridium*, *Bifidobacterium, and/or Romboutsia*.

5.2 Introduction

Poor diet and physical inactivity are well recognized as significant contributors to the onset of many avertible diseases and premature death (30). Mokdad *et al*. noted poor diet combined with physical inactivity was the second leading cause of preventable death in the United States only behind tobacco use (185). Delineating which of the two lifestyle choices is the primary driver of physiological dysregulation and subsequent sickness is difficult as overeating and physical inactivity are commonly coexisting in humans (28, 248, 269). On the opposite side of the spectrum, while anorexia nervosa is viewed primarily as an undereating disorder, these individuals also display greater time

spent in physical activity (47). Using a rodent model of anorexia nervosa, deemed activity nervosa, Epling and Pierce concluded caloric restriction leads to vast increases in wheel running (75). A similar increase in locomotion is found in *C. elegans*, *drosophila*, and rhesus monkeys (41, 152, 270). These conclusions led our lab to test if overfeeding via a high fat diet paired with a fructose solution as drinking water (HFHS) led to decreased wheel running. Macronutrient and fructose percentages were selected based on a worse than average US citizen diet (85, 267). After six weeks of exposure to the *ad libitum* HFHS diet, wheel running decreased by 57% in female mice and 70% in male mice compared to *ad libitum* chow controls (CHOW) (265). This may partially explanation low levels of physical activity in the US (257). Comparing our results to other attempts at "overfeeding" reveals high fat diets did not produce similar decreases (181) in wheel running unless a fructose solution was also used (213). Thus, there is a need to determine what potential factor(s) is/are responsible for altering physical activity levels via diet.

The gut microbiome is a likely candidate for altering physical activity level as many studies have uncovered a direct connection between gut function (210), physical activity (174), and psychological state (262). Germ-free rodents and drosophila have distinctive increased locomotion (109, 228). The lack of gut microbiota has been directly linked to alterations of neurotransmitter levels and brain architecture which has the potential to be the causal link in altering physical activity levels. Without gut microbiota, however, nutrient absorption in the gut is severely limited which is likely a similar model to caloric restriction. A study by Schretter *et al*. demonstrated the

colonization of a microbe species, *lactobacillus brevis*, in the gut of germ-free drosophila decreased locomotion to similar levels of drosophila with intact gut microbiomes (228). When the group knocked out the gene coding for xylose isomerase (Xi) in *lactobacillus brevis* responsible for catalyzing the reversible conversion of glucose to fructose, the colonized drosophila did not exhibit decreased locomotion. In this model, it is clear the gut metabolome modulated by microbiota can directly influence physical activity levels.

The latest National Health Interview Survey (NHIS) indicated probiotics are the third most used natural supplement in the United States with use growly rapidly (55). However, there is low support for the efficacy of probiotics (250) and fecal microbial transplants (FMT; (144)). A recent clinical trial regarding the efficacy of FMTs for weight loss indicated that FMTs from lean individuals slightly altered the guts of obese individuals but did not promote weight loss (144). It appears alterations of microbiota presence does not play a significant role in altering host physiology if the new microbes are not supplied with specific nutrients to generate relevant metabolites. Thus, the purpose of this study was to determine if low levels of physical activity induced by exposure to a high fat/high sugar diet could be reversed by improving diet alone, the transplantation of fecal microbiota from high active animals on the better diet alone, or a combination of the two.

5.3 Methods

5.3.1 Animals

All protocols in this study conformed to the standards of animal care approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP 2019-0345). Male C57BL/6J mice (n = 40; Jackson Laboratory, Bar Harbor, ME) were purchased at 5 weeks of age and individually housed in the University Vivarium with 12-h light/dark cycles. Inbred C57BL/6J mice were selected as the best model for translation due to their repeatable wheel running phenotype (139, 166), susceptibility to diet induced reduction in wheel running (265), and a slight preference for fructose solution over regular drinking water (98). Female mice were not used as has previously shown to not induce as large of a wheel running deficit compared to males (265). Additionally, our previous work has shown that there are sex-based differences in microbiome response to diet, suggesting that our responses will only be generalizable to male mice.

5.3.2 Diet

After a week of acclimation, mice were split evenly into four groups (5.1). Group 1 was provided an *ad libitum* "chow" diet (CHOW) consisting of 4% fat, 25.2% protein, 39.5% carbohydrate, 3.3% crude fiber, and 10% neutral fiber (Diet 8604, Harlan Labs, Houston, TX). Groups 2-4 were provided an *ad libitum* high fat/high sugar diet (HFHS) consisting of 45% fat, 20% protein, 35% carbohydrate, 5% fiber, and a 20% fructose solution in place of regular drinking water (Diet D12451, Research Diets, Inc., New Brunswick, NJ). The HFHS diet combined with fructose drinking water was used

to mimic a United States citizen with a worse than average diet as the estimated United States mean total fat intake is ~34% (85) and mean total calories from fructose is ~10% (267). Due to the increased caloric intake and subsequent body fat caused by the *ad libitum* HFHS compared to the *ad libitum* CHOW diet, we termed the HFHS diet intervention "overfeeding" (265). Total caloric intake (kcal/day) was measured by weighing food (g/day) and fluid (ml/day) at the beginning and end of each week using an electronic scale.

5.3.3 Wheel Running

Physical activity levels were measured by means of running wheels with a 410 mm circumference and solid running surface (Kaytee, Chilton, WI). Bicycle computers (BC8.12, Sigma Sport, Batavia, IL) were attached to the top of the animal cage and received signals from a magnet glued to the outside rim of the running wheel. Daily distance (km/day), duration (min/day), and calculated average speed (m/min) were collected every 24 h at the same time of day. Sensor alignment and wheel function were monitored daily and adjusted as needed. If a wheel was determined to be malfunctioning (wobbly, chewed, or stuck) data from the previous 24 hours was removed from the week's average total. We have repeatedly validated this model of activity measurement in mice (139, 166).

5.3.4 Body Composition

Total body mass was measured weekly starting at 5 weeks of age using an electronic scale. Fat mass, lean mass, and hydration were analyzed weekly using an MRI made specifically for rodents (EchoMRI, Houston, TX).

5.3.5 Fecal Collection

During the weekly body composition measurements, mice were placed in empty, sterilized cages and allowed to roam freely. Fresh fecal pellets were collected in sterile cryotubes immediately after passing, flash frozen, and stored at -80 °C.

5.3.6 Diet & Fecal Transplant Intervention

After 13 weeks, Group 2 (indicated as "HF/CH+") were switched to the CHOW diet and were given a fecal transplant from Group 1 (indicated as "CH/CH"), Group 3 (indicated as "HF/CH") were switched to the CHOW diet and given a sham transplant, and Group 4 (indicated as "HF/HF+") remained on the HFHS diet and were given a fecal transplant from Group 1 (Figure 5.1). The fecal transplants were generated by collecting a fresh fecal pellet from all 10 animals in Group 1 (CH/CH) which was mixed in an anerobic tube filled with 500 ml of pre-reduced phosphate buffered saline and cysteine (for additional oxygen scavenging). 150 µl of the fecal solution was orally gavaged into the stomach of mice in Group 2 and Group 4. To control for the additional handling and stomach filling, mice in Group 1 and Group 3 received an oral gavage of 150 µl of pre-reduced phosphate buffered saline and cysteine only. Due to the stress induced reduction of wheel running for 1-3 days immediately following the oral gavage, only one transplant was given to each animal at the start of the week. Transplantations were performed for 4 total weeks.

5.3.7 Tissue Extraction

Animal sacrifices were performed after the 4 weeks of the intervention (17 total weeks of the study timeline, 23 weeks-old) via anesthetization using 3-4% isoflurane,

removal of blood by cardiac puncture, and ensuring death via cervical dislocation. All bodily tissues were extracted, immediately freeze clamped, then stored at -80 °C. Cecums were weighed, then contents were squeezed into a cryotube, immediately flash frozen via liquid nitrogen, and stored at -80 °C.

5.3.8 Microbial Sequencing

DNA was extracted from the fecal pellet samples taken throughout the study (Figure 5.1) using the DNA Powersoil Kit (Qiagen) and the V4 region of 16S rRNA was sequenced using the method previously described by Kozich *et al*. (145) on a MiSeq Illumina platform at Microbial Analysis, Resources, and Services facility (MARS) at University of Connecticut. Data was analyzed using Mothur (225), Phyloseq (180) and Calypso software (283).

5.3.9 Metabolome

Metabolites were extracted from homogenized fecal samples using methanol/chloroform, concentrated using a lyophilizer (Labconco) and re-suspended in methanol/water (1:1 v/v) as previously described (240, 274). Metabolomic analysis was carried out using Q-Exactive Plus orbitrap mass spectrometer (Thermo Scientific) coupled to Dionex 3000 UHPLC system. A C18 Synergi Fusion-RP 4µ 80Å 150 x 2.0 mm column (Phenomenex) was used for chromatographic separation with 0.1% formic acid in water (Solvent A) and with 0.1% formic acid in ethanol (Solvent B). MS1 and MS1-dependent MS2 spectra was collected at a m/z resolution of 37,500. Metabolites were eluted at a flow rate of 0.4 ml/minute. The flow gradient was 40% of solvent B for 5 min, 95% of solvent B for 7 min and 10% solvent B for 8 min. Blanks (methanol and

water at 1:1 v/v) were inserted between every sample to prevent any sample carryover. Deuterated indole-3-acetic acid was used as a labeled internal quality control standard. Pure standards of metabolites of interest were used to generate a standard curve for absolute quantification. Data were analyzed using Progenesis QI software (Waters), Human Metabolome Database (HMDB) and the Kegg database for metabolite identification. Raw abundance data were normalized to fecal sample weights and statistical analysis was performed using KaleidaGraph (Synergy).

5.3.10 Liver Histology

Livers extracted at the time of sacrifice were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc.), frozen with liquid nitrogen, and stored at -80 °C. Livers were sliced into 4 µm sections at -15° C and stained with hematoxylin and eosin. Liver steatoses were quantified at 40x magnification using OsteoMeasure (OsteoMetrics, INC, Decatur, GA) and severity was calculated by the percent area of steatosis over total area. Values were averaged between the exact center of three unique liver slices per mouse.

5.3.11 Statistical Approach

The statistical comparison of wheel running indices (distance, duration, and speed) and body composition data (total mass, fat mass, and lean mass) were analyzed from baseline to week 13 with separate repeated ANOVAs (group) with an alpha level of 0.05 set *a priori*. Tukey's post-hoc analyses were employed in the case of significant main effects. A Tukey's post-hoc analysis was employed in the case of significant effects. Once diets and transplants started at the beginning of week 13, repeated

ANOVAs were rerun comparing changes in physical activity and body composition. The Statistical analyses of caloric intake, body composition, and physical activity indices were performed using JMP statistical software (SAS Inc., Car, NC). All graphs were created using GraphPad Software (La Jolla, CA). Comparisons of the medians between the metabolite levels of CHOW and HFHS fed mice were performed with the non-parametric two-sided Mann– Whitney U-test with an alpha level of 0.05 set *a priori* and then corrected for multiple comparisons.

5.4 Results

5.4.1 Wheel Running Pre-Treatment

Wheel running indices were similar across all groups after week 1 (7.2 ± 2.1 km/day, 235.4 ± 46.5 min/day, 30.6 ± 5.4 m/min; $p > 0.6526$; Figure 5.2). As expected, mice of all groups had increased average speed (+36.9%) by the end of week 2 due to acclimation (32). There was a slight drop in duration by the end of week 2 (-9.5%). The combination of the two led to a peak in distance for all groups across the study timeline (+22.2%). For this reason, the end of week two is used as a baseline for subsequent calculations. By the end of week 13, all wheel running indices had similar reductions in Groups 2-3 (all fed a HFHS diet) compared to baseline (-56.1% distance, $p < 0.0001$; -39.2% duration, $p < 0.0001$; -25.7% speed, $p < 0.0001$; Figure 5.2). Group 1 animals (fed a CHOW diet) had comparatively minor decreases in wheel running indices (-23.4% distance, $p = 0.0215$; -26.3% duration, $p = 0.0094$; -2.2% speed, $p = 0.8051$; Figure 5.2). The animals in Group 1 were running 127.8% further ($p < 0.0001$), 70.8% longer ($p =$

0.0002), and 41.8% faster ($p < 0.0001$) than Groups 2-3 by the end of week 13 ($p < 0.0001$).

5.4.2 Body Composition and Intake Pre-Treatment

Mice in Groups 2-4 had significantly greater body mass by the end of week 13 compared to Group 1 (38.2 vs. 27.0 g, $p < 0.0001$; Figure 5.3). The difference in body mass was due to body fat (12.5 vs. 2.8 g, $p < 0.0001$; Figure 5.3) as lean mass was not consistently different between any group (23.6 ± 1.0 g, $p = 0.0779$; Figure 5.3). At the end of week 13, mice in Groups 2-4 were eating fewer total grams of food per day compared to Group 1 (2.6 ± 0.2 vs. 4.2 ± 0.3 g, $p < 0.0001$; Figure 5.4), but same total kcal from pellets (12.2 ± 1.2 kcal per day) as the HFHS pellets had greater caloric density. The additional kcal from the fructose drinking solution added roughly 6.3 ± 1.6 kcal more per day in Groups 2-4.

5.4.3 Wheel Running Post-Treatment

Week 13 was used as the baseline for changes in wheel running and body composition as diet changes and microbe transplants occurred on the first day of week 14. After the first week of transplants, running distance decreased in Group 1 (CH/CH) by -25.3% ($p = 0.0001$), increased in Group 2 (HF/CH+) by +31.2% ($p = 0.0303$), was unchanged in Group 3 (HF/CH; +5.5%, $p = 0.9784$), and decreased in Group 4 (HF/HF+) by -22.3% ($p = 0.0114$; Figure 5.5). Duration decreased in Group 1 by -21.2% ($p = 0.0001$), increased in Group 2 by +12.3% ($p = 0.02872$), was unchanged in Group 3 (-5.0%, $p = 0.5202$), and was unchanged in Group 4 (-0.1%, $p = 0.0140$; Figure 5.5). Speed decreased in Group 1 by -5.5% ($p = 0.0019$), increased in Group 2 by

+12.0% ($p = 0.0006$), was unchanged in Group 3 by (+6.6%, 0.0537), and decreased in Group 4 by -7.6% ($p = 0.0016$; Figure 5.5). One animal from Group 2 was removed from analysis as wire connecting the wheel running monitor was chewed two days in a row by the mouse resulting in skewed data showing wheel running distance increasing by +354%. After two weeks of treatment, there were no further significant changes in any group except Group 3 which increased distance by +38.8% ($p = 0.0107$), duration by +14.9% ($p = 0.04291$), and speed by +19.8% ($p < 0.0001$). By the end of the final week of treatment (four weeks total), wheel running averages of Groups 1-3 (all CHOW) were similarly greater than Group 4 (HFHS), but due to high variability within groups, only speed was significantly higher than Group 4 ($p < 0.0001$).

5.4.4 Body Composition and Intake

Mice in Groups 2 and 3 had dramatic decreases in body mass (-15.0% and -13.2%) within one week of exposure to the CHOW diet and reached a plateau after an additional drop after two weeks (-4.9% and -4.1%). The decreases in body mass were solely due to loss in body fat mass (-47.5% and -51.8%). However, mice in Groups 2 and 3 remained heavier than Group 1 throughout the remainder of the study (31.0 ± 1.9 vs. 27.7 ± 1.3 g; $p < 0.0001$; Figure 5.3). Groups 1 and 4 did not experience any significant changes in body mass or composition throughout the treatment. Groups 2 and 3 had substantial drops in total caloric intake during the first week on the CHOW diet (-64.3% and -63.1%) which increased to intake levels of Group 1 by the end of the study. Groups 1 and 4 did not experience any significant alterations in caloric intake.

EchoMRIs indicated no differences in hydration between any groups during the transplantation periods.

5.4.5 Microbes

Diet type had a significant effect on the microbial community structure. The 16S rRNA sequencing of the V4 region generated 3,215,089 reads corresponding to 4169 operational taxonomic units (OTU's). Groups 2-4 pre-treatment on the HFHS diet had significant reductions in richness and diversity, as measured by Shannon and Chao1index respectively, compared to Group 1 on the CHOW diet (Figure 5.6). After one week of treatment, diversity remained significantly lower in Groups 2-4 compared to Group 1 (Figure 5.6). However, the diversity of the Group 2 (HF/CH+) increased to the levels of the Group 1 (CH/CH) by two weeks of the treatment while Group 3 (HF/CH) did not (Figure 5.6). Non-metric multidimensional scaling (NMDS) analysis indicated clear separation between Groups 1 and Group 2-4 samples pre-treatment (Figure 5.7). After one week of treatment, the community structure of Group 2 and 3 (both switched to a CHOW diet with or without a fecal transplantation) clustered together and were significantly different than Group 1 and Group 4 (Figure 5.7). After two weeks of treatment, Groups 1-3 clustered together and were significantly different than Group 4 (Figure 5.7). At the phylum level, *Bacteroidetes* were significantly lower in Group 2-4 at baseline, and remained lower in Group 4 throughout the treatment (Figure 5.8). The opposite pattern was apparent for Firmicutes (Figure 5.8). These results are consistent with the literature where a HFHS diet is known to decrease the relative abundance of *Bacteroides* and increase the relative abundance of *Firmicutes* (68, 193). The relative

abundance of *Proteobacteria* had no clear pattern during the treatment between groups (Figure 5.8). After two weeks of treatment, *Verrucomicrobia* were significantly lower in Groups 1-3 (Figure 5.8). .

LefSe, a metagenomic biomarker tool, was used to identify genera taxa that were significantly different between groups after one and two weeks of treatment (Figure 5.9 and 5.10). After one week of treatment, nine genera were significantly higher in Group 1 than all other groups, predominantly *Ruminococcus1*, *Anaeroplasma*, and *Turicibacter*. Seven genera including *Clostridialesvadin* BB60, *Lachnospiraceaege*, and *Alistipes* were higher in Group 2. *Lachnospiraceae UCG001*, *Tyzzerella 3*, and *Acetatifactor* were higher in Group 3. The relative abundance of 18 genera including *Faecalibaculum, Lactococcus*, and *Ruminococcaceaege* were dominate in Group 4.

Relatively high abundance of genera *Lachnospiraceae. Alistipes, and Clostridiales vadin* BB60 were strongly associated with animals fed a CHOW diet at baseline and throughout treatment (Figure 5.10). The relative abundance of these three genera in Group 4 did not increase from the baseline levels even with fecal transplants. This trend was opposite for *Lactococcus, Faecalibaculum, Erysipelatoclostridium, Bifidobacterium, and Romboutsia* genera which were abundance on a HFHS diet and depleted when fed a CHOW diet (Figure 5.11). *Lactococcus* genera is known to be colonized in obese people (48). These results indicate that diet plays the primary role in regulating the bacterial community structure and a single fecal transplantation a week is not effective at substantially altering the gut community. However, this does not mean transplants did not alter the community as diversity variation increased within the

HFHS+ group (Figure 5.6). However, it is clear significant engraftment could not be achieved without ideal nutrition for specific microbe communities (Figure 5.6).

To elucidate the changes in the bacterial community that might have contributed to the difference in the distance run by the Groups 2 and 3 after one week of the treatment, we used Metagenomics Longitudinal Differential Abundance (MetaLonDA) to study the time series changes for these two groups. Among the genera that were significantly increased with transplantation for the HF/CH+ group after one week of treatment were *Akkermansia* and *Alistipes,* genera which are recognized to increase with physical exercise (37, 54). *Alistipes* genus contain species that are indole producers (239) and that are bile acid tolerant (61), hence play a major role in health and disease. MaAsLin (Multivariate Association with Linear Models) was used to identify the associations between the bacterial communities to the physiological parameters measured (distance, speed, and body mass). An OTU corresponding to Lachnospiraceae NK4A136 was found to be positively correlated with distance (Table 5.1). For body mass, an unclassified genera from *Clostridiales* was positively correlated and an unclassified genera from *Lachnospiraceae* was negatively correlated (Figure 5.1). There was no significant correlation found for speed.

5.4.6 Metabolites

The partial least sums discriminate analysis of the cecal metabolome of each group at baseline, after one week of treatment, and after two weeks of treatment indicated diet was the primary determinate of metabolite composition (Figure 5.13). However, while Group 2 (purple) and Group 3 (green) were simliar after one week of

treatment, both groups were had significantly different metabolomes compared to Group 1 (red). This difference inidcates differences in metabolite presense to factors other than nutrient intake alone. However, the addition of a fecal transplant in Group 2 did not improve the simliarity to Group 1 any more than Group 3.

5.4.7 Cecum Weight and Liver Histology

At dissection, wet cecal tissue mass was not different between Groups 1-3 and significantly lower in Group 4 (0.62 ± 0.14 vs. 0.23 ± 0.06 g; $p < 0.0001$). Livers of Group 4 were noticeably lighter in color compared to Groups 1-3 (Figure 5.14 A-D). Approximately 15.76% of the left liver lobe volume of Group 4 animals were fatty steatosis (Figure 5.14 I). Group 1 had no signs of steatosis, while Group 2 and 3 both averaged under 1% each (Figure 5.14 I).

5.5 Discussion

We have repeated the findings from our previous study (265) where a high fat diet with fructose drinking solution dramatically decreased wheel running. Our treatment results indicated physical activity levels can be recovered if mice are reverted to a CHOW diet. The addition of a fecal transplant to support the colonization of new microbes led to a quicker recovery in wheel running, but only if the diet change also occurred. We hypothesize the microbes from mice on a CHOW diet gavaged into the guts of animals on the HFHS did not have the proper nutrient supply to graft effectively. Findings from Sheperd *et al.* support this claim (231). Even if the microbes of highly active mice were present in the gut of Group 4 animals, the gut metabolome underwent

minimal changes suggesting metabolites that may influence physical activity level were not created and are dependent on dietary intake (Figure 5.13).

There were substantial calorie deficits in Groups 2 and 3 after switching from the HFHS diet to the CHOW diet (Figure 5.4). If the increase in wheel running was from purely from caloric restriction, we believe the increase would have occurred at the same time between groups. Pierce and Epling studied caloric restriction in rats which increased activity within three days of becoming diet restricted (75). However, the caloric restriction experienced by the mice in Groups 2 and 3 may have been unique from Pierce and Epling's rats as when Groups 2 and 3 switched to a CHOW diet, the mice ate the same total grams of food as they were eating with the HFHS. Since the CHOW is not as calorically dense, the caloric intake was much lower. Additionally, Groups 2 and 3 had nearly identical decreases in caloric intake and body fat, but the wheel running from Group 2 increased quicker than Group 3.

The fecal microbial analyses indicated diet was by far the most influential determinate of richness and abundance. Group 2 had similar levels of richness and diversity as Group 3, but Group 2 had increases in multiple genera that were not increased in Group 3 such as *Clostridialesvadin* BB60, *Lachnospiraceaege*, and *Alistipes*. These microbes have had correlations with increased activity in previous studies. In order to test if these microbes are responsible for increasing activity or just in response to higher activity, these microbes should be depleted via antibiotics in future studies.

5.5.1 Limitations

The oral gavages (fecal or vehicle) reduced wheel running for one to three days in all mice likely due to stress. Due to stress-induced reductions in wheel running, only a single fecal transplant per week could be performed to allow wheel running to peak before another transplant was given. A single fecal transplant per week may not have been enough exposure for Group 4 (HF/HF+) to significantly alter the number of microbes from Group 1 (CH/CH) to engraft. Still, one transplant a week is more microbial volume than a typical human expose themselves to when taking probiotics such as yogurts or pills. Thus, if one transplant is not adequate, it is unlikely humans will manage to have greater exposure.

The exact diet chosen were not controlled for vitamins, protein, or fiber. This was chosen to best mimic a worse than average US citizen's diet. In this model, we attempted to produce the largest effect on physical activity possible. In future studies specific nutrients should be tested to determine what component of the diet is the most impactful. Initial work on this question has been conducted by Rhodes (213) who has shown that the fructose component is a major reducer of physical activity.

The mice in Group 4 had substantial levels of steatosis. Even though Groups 2 and 3 spent most of their lifespans on the HFHS diet, the presence of liver steatoses were nearly undetectable indicating recovery in under 4 weeks. In studies of overfeeding via added sugars in drinking water, sucrose added in drinking water did not decrease wheel running (113) while fructose did significantly (211). Of the two sugars, only fructose is known to generate severe steatosis in mice. It is possible that the removal of the fructose

in our study in Groups 2 and 3, and subsequent removal of excess energy storage in the liver, could be a primary factor leading to changes in physical activity.

5.5.2 Conclusion

The effects of diet on host health is widespread as physical activity behavior can be altered as well as a recharacterization of the gut microbiome. At least for mice, repairing poor gut health can be accomplished with proper nutrition. The repairing period may be supported with the addition of "healthy" microbes to ensure proper colonization but is not as critical as diet maintenance. Individuals should be wary that diets low in fiber and high in fructose can deplete the gut of beneficial microbes. Fecal microbes that are depleted with a HFHS diet or fecal microbes abundant in a CHOW diet may be critically important in modulating diet's effect on physical activity levels.

6. CONCLUSIONS

The results of this dissertation add insight on the evolution and regulation of physical activity levels. As there is a highly heritable component of physical activity levels, finding the proper genetic model is critical. However, Section 2 indicated there are many SNPs associated with physical activity level with no similarities between studies. The few SNPs found within exons were predicted to be older than anatomically modern humans and the rise of agriculture, but younger than the transition of primates to upright posture and endurance running. While a specific selection pressure cannot be pinpointed causing a benefit of the relevant SNPs, it seems engaging in physical activity was critical during this time period. The difficulty of applying these genetic findings to testable hypothesis is that the specific SNPs associated with physical activity are not shared among any other animals and are even different between animals of the same species. Thus, there is clearly a highly redundant system involved to control such a complex phenotype.

Sections 3-5 highlight some of the interconnected mechanisms that influence physical activity levels. Using the female SENCAR mice in Section 3 was the first study to test the effects of year-long diets on two mice cohabitating at a time. While wheel running was designed as the independent variable in this study on tumorigenesis, the lifelong maintenance of physical activity levels with mild caloric restriction has added strength to one paradigm proposed in this dissertation: the need for food reinforces high levels of physical activity. However, overfeeding via a HFHS did not significantly reduce wheel running compared to the *ad* libitum chow-like diet. It is

possible the added carcinogens altered the response to the overfeeding. Caloric restriction produces a stronger phenotype compared to overfeeding, at least within the SENCAR strain compared to the profound drop in the C57BL/6J mouse model.

While diet itself has a profound effect on physical activity, Sections 4 and 5 indicate the physical activity response to diet can be modestly modified with a fecal transplant. However, it is clear there is no single microbe or metabolite responsible for the changes in physical activity alone. In this model, the microbiome acts seemingly acts as a host's second set of genes interacting with the environment (diet). This extension of gene-by-environment interactions should be considered in future studies and translating to humans.

In conclusion, diet plays a significant role in the regulation of physical activity levels in many species. From an evolutionary prospective, diet's influence is likely to be shared as a basic biological need but modulated by specific environments between species. The gut microbiome should be considered part of the environmental factors adding to variation between individual's response to diet.

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APPENDIX A. FIGURES

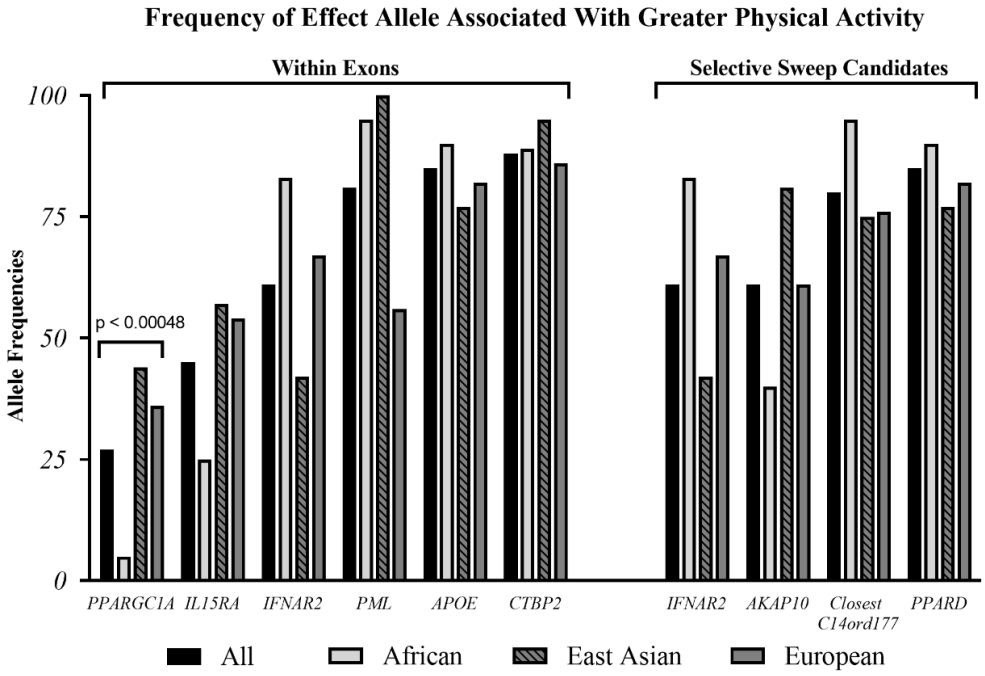

Figure 2.1: Effect Allele Frequencies of African, Eastern Asian, and European Populations. Chi-square statistics indicated a significant difference between population allele frequencies in *PPARGC1A.* All other SNPs located in exons or were strong candidates for selective sweeps had non-significant differences in population allele frequencies.

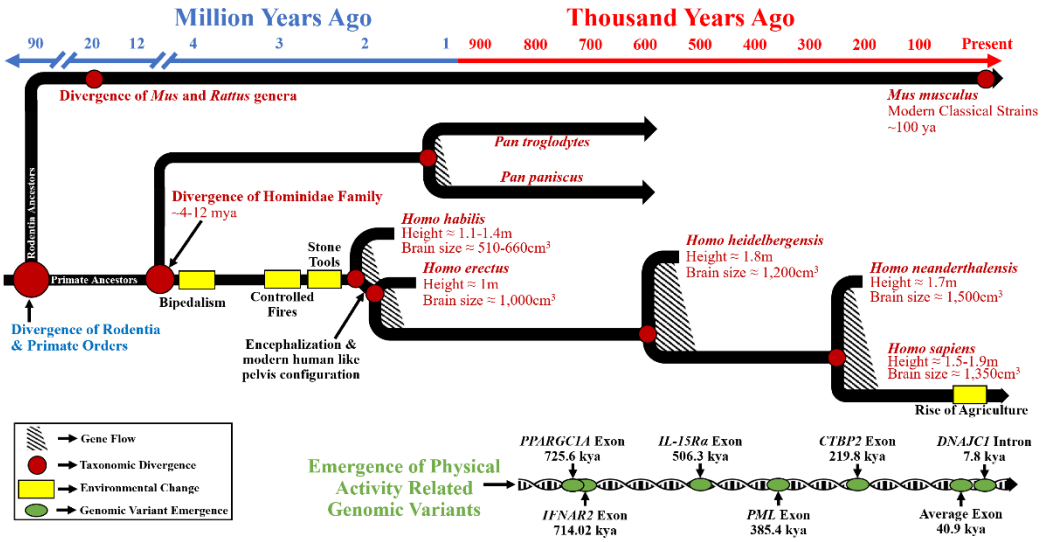

Figure 2.2: The Emergence of PA-Related SNPs and Potential Selection Pressures. PA-related SNPs in protein-coding genomic areas are as old or older than the hypothesized emergence of anatomically modern humans (~200-350 kya). The only intron (*DNAJC1*) was predicted to emerge around the time of the rise in agriculture.

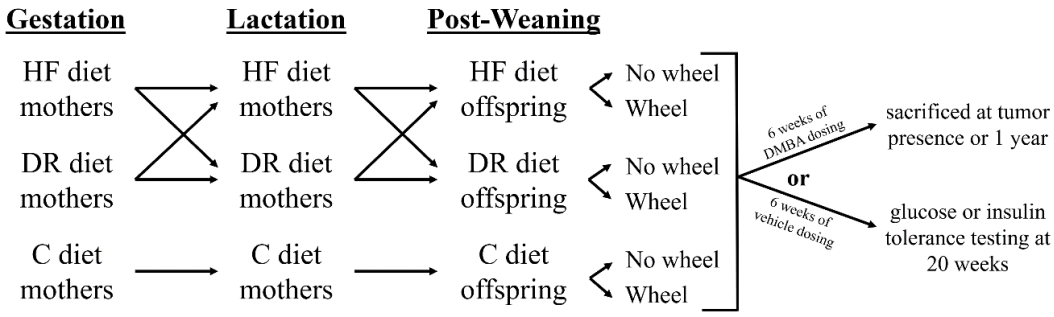

Figure 3.1: Development Scheme of Experimental Procedures. Mouse mothers were exposed to either a high fat/high sugar (HF) or restricted (DR) diet. Pups were then randomly culled with a mother on either a HF or DR diet. Post-weaning, at three weeks of age, pups were randomly co-caged, and assigned to a HF or DR diet. A control group was created using a chow-like diet (C) during gestation, lactation, and post-weaning. All pups were then randomly given two wheels or no wheels at four weeks of age. Starting at seven to nine weeks of age, pups were either given doses of DMBA or corn oil for 6 weeks. Animals given DMBA were either sacrificed at tumor presence or after 1 year. Animals given vehicle treatment had glucose and insulin tolerance testing at 20 weeks of age.

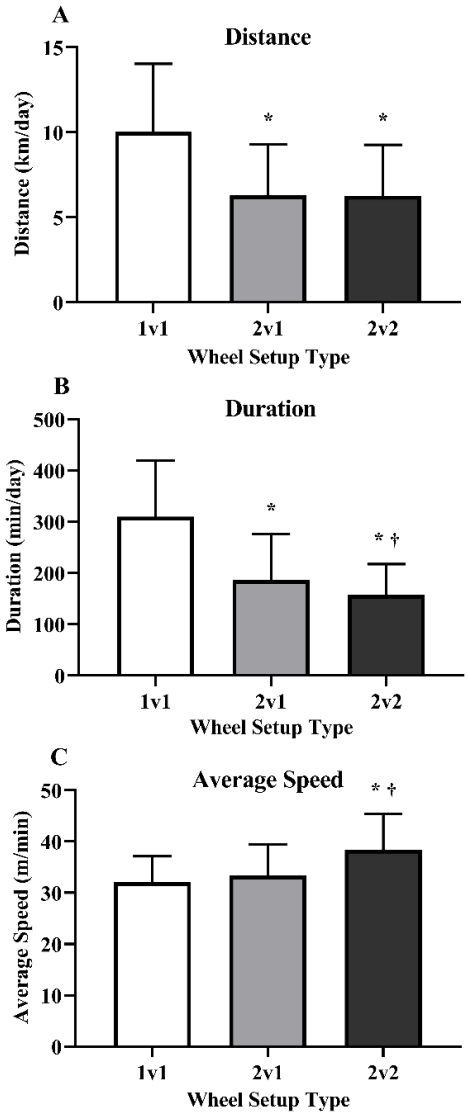

Figure 3.2: Daily Average Wheel Running (A) Distance, (B) Duration, and (C) Speed with Various Housing Densities and Wheel Setups. * $p < 0.05$ versus 1v1 setup. † $p < 0.05$ versus 2v1 setup.

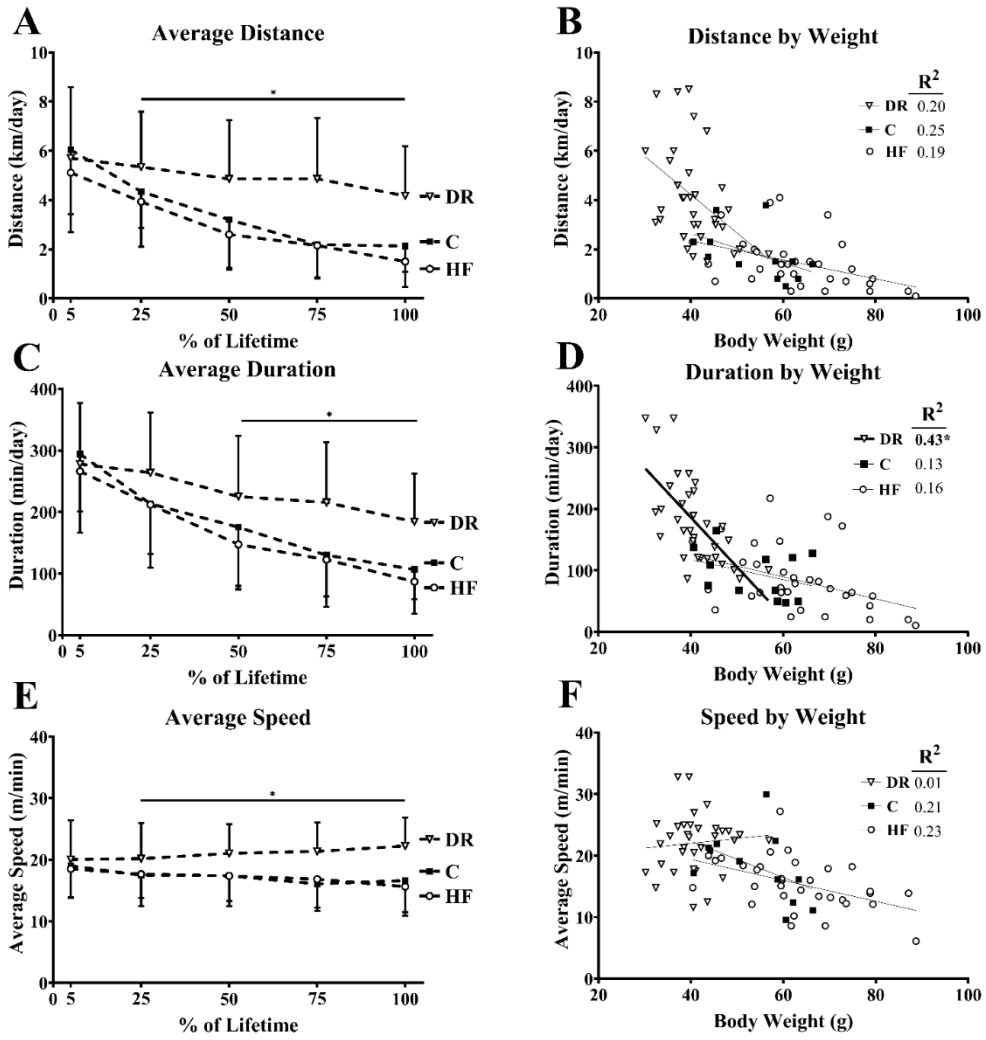

Figure 3.3: Wheel Running Across Lifespan and Body Weight. Post-weaning DR mice maintained higher distance (A), duration (C), and speed (E) throughout their lifespan compared to post-weaning C and HF mice. Points represent combined post-weaning diet groups ± SD. * $p < 0.01$ comparing post-weaning DR mice to post-weaning C and HF diet groups. Body weights of animals within post-weaning diet groups did not significantly correlate with distance (B), duration (D), or speed (F) except duration within the DR group. Points represent individual mice.

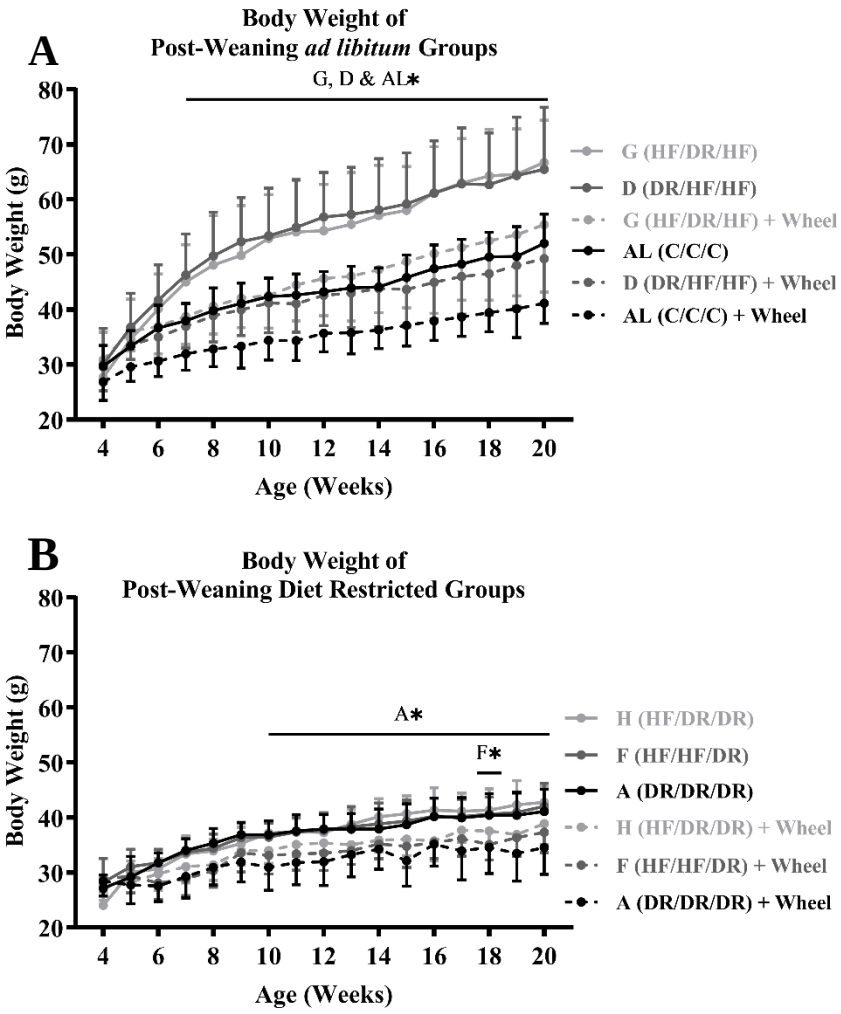

Figure 3.4: Body Weight of Mice Across 20 Weeks of (A) Post-Weaning *ad libitum* Diet Mice and (B) Post-Weaning Diet Restricted Mice. * $p < 0.05$ comparing listed diet group without wheel access to same listed diet group with wheel access. Points are average group values ± SD.

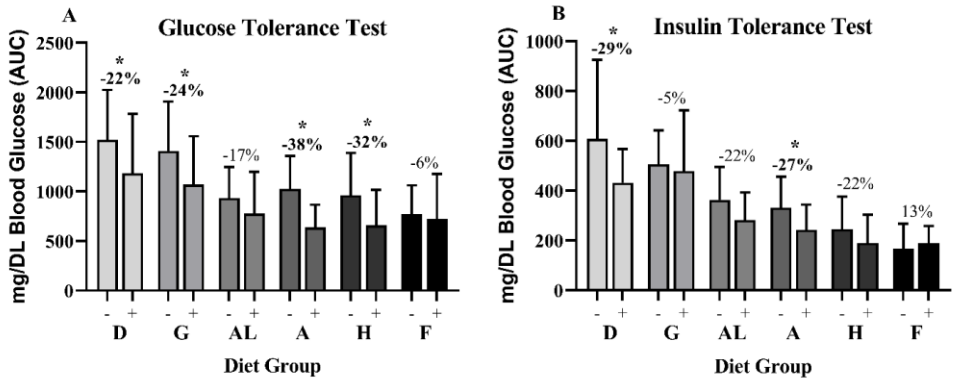

Figure 3.5: (A) Glucose Tolerance Test and (B) Insulin Tolerance Testing Without Wheel Access (-) and With Wheel Access (+). * $p < 0.05$ comparing groups with and without wheel access. Bars represent averages ± SD.

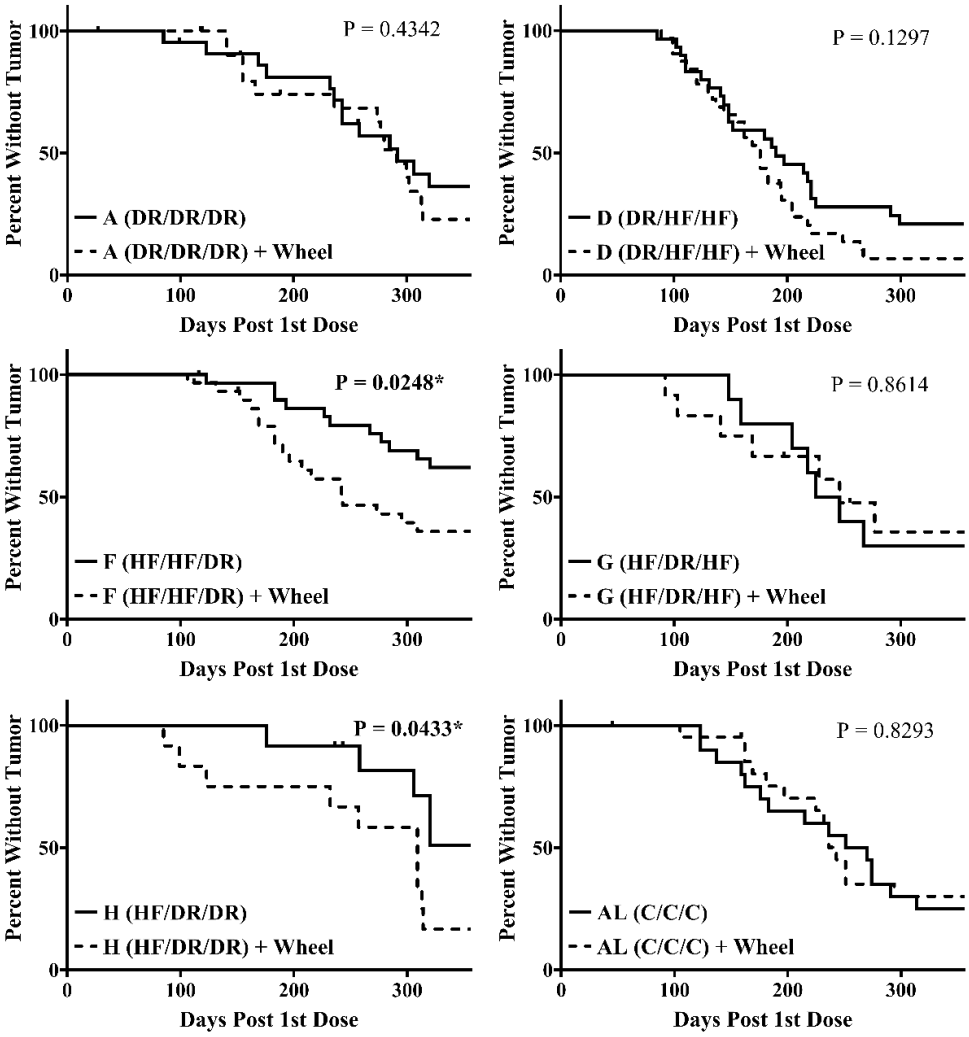

Figure 3.6: Tumor Latency of Diet Groups. Only diet groups F and H had significantly shortened latency with the addition of wheels.

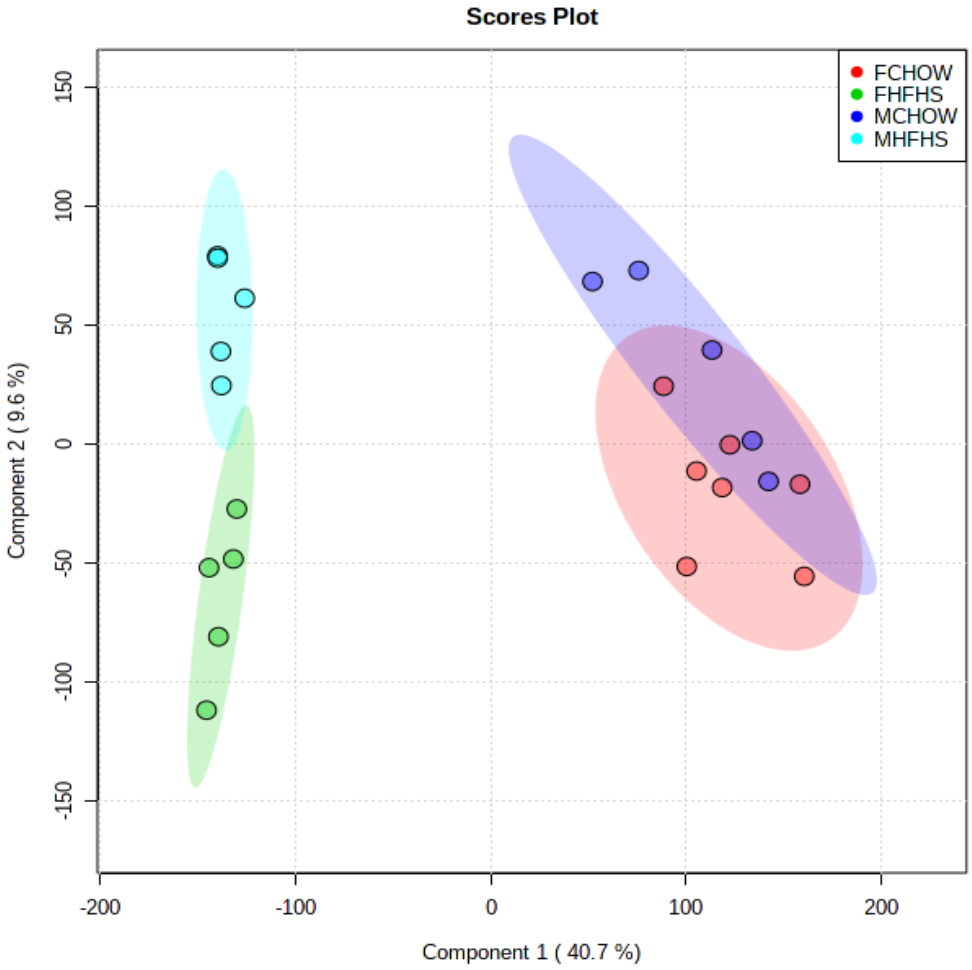

Figure 4.1: Partial Least Sums Discriminate Analysis of the Cecal Metabolome in Female and Male Mice on a CHOW or HFHS Diet. Cecal metabolome clusters were significantly different between mice on either diet. The metabolome of female and male mice on the HFHS diet also formed distinct clusters indicating a unique response to the diet dependent on sex.

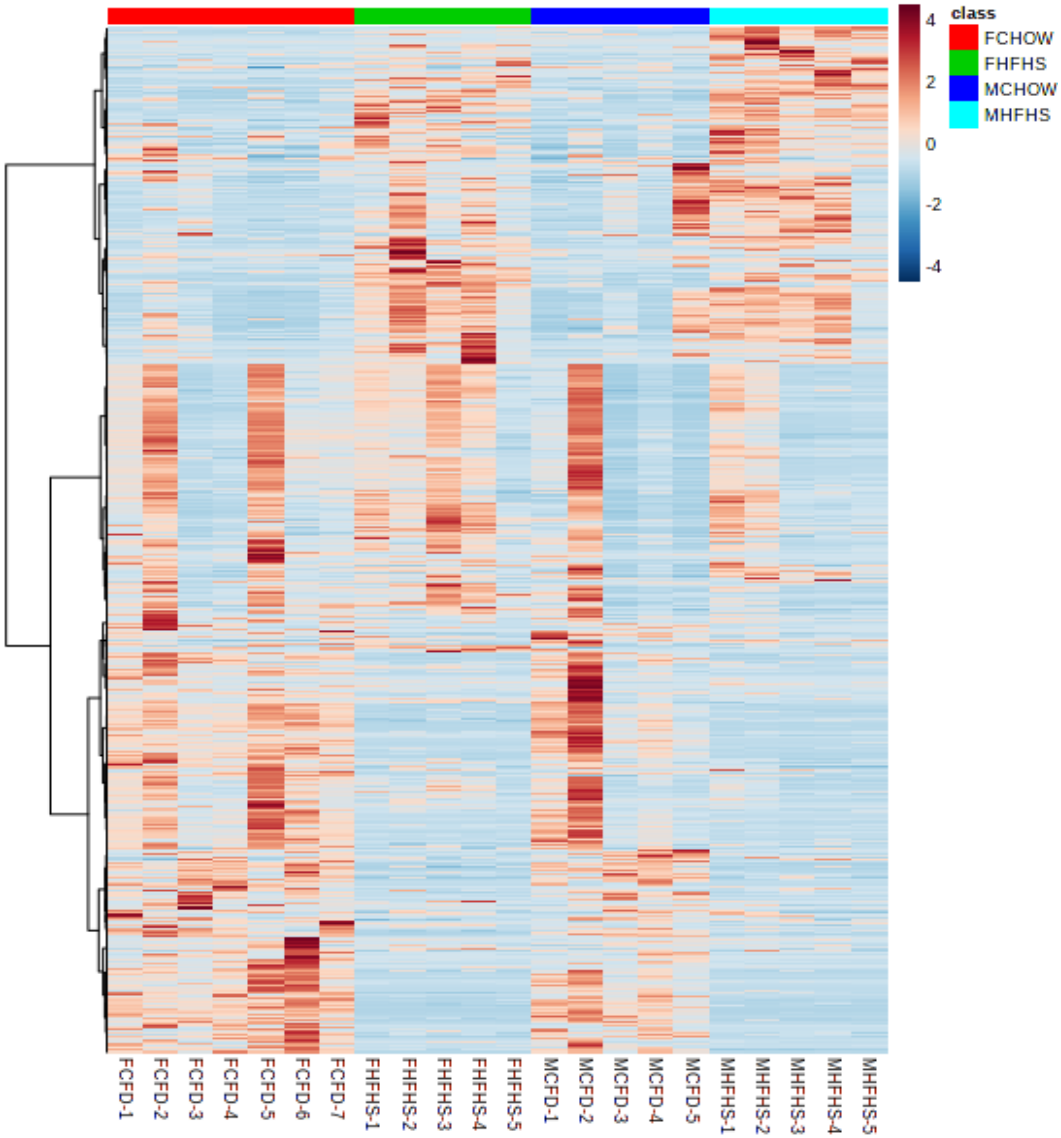

Figure 4.2: Heat Map of Top 1000 Differentially Expressed Features in Female and Male Mice on a CHOW or HFHS Diet. There were 10,961 features detected across all mice. Of these, 2837 were significantly different between female mice on either diet. 1669 were significantly different between male mice on either diet. 147 were significantly different between female and male mice on the HFHS diet. Columns represent specific mice from each group. Row are unique features detected via mass spectrometry. Darker red indicates relatively abundant features and dark blue indicates relatively depleted features.

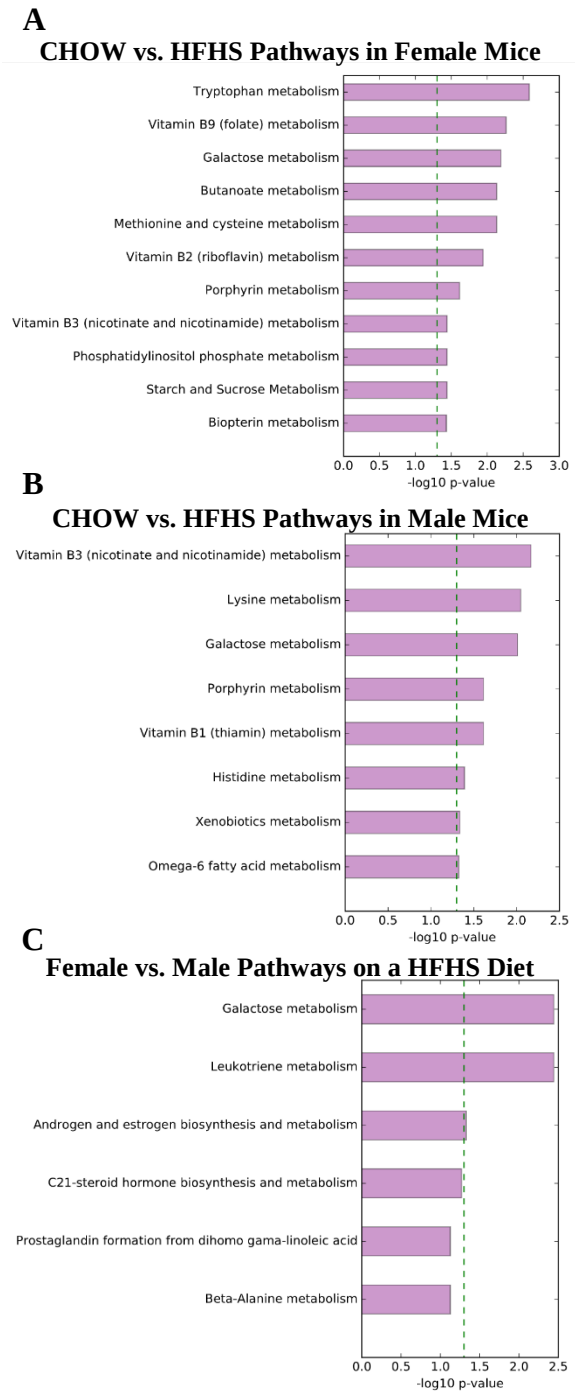

Figure 4.3: Metabolite Pathway Analyses. Bar graphs depict detected pathways that had altered metabolites at a significance level of 0.0025. A) Eleven detected pathways were altered between diets in female mice. B) Eight detected pathways were altered between diets in male mice. C) Three detected pathways were altered between sexes fed a HFHS diet.

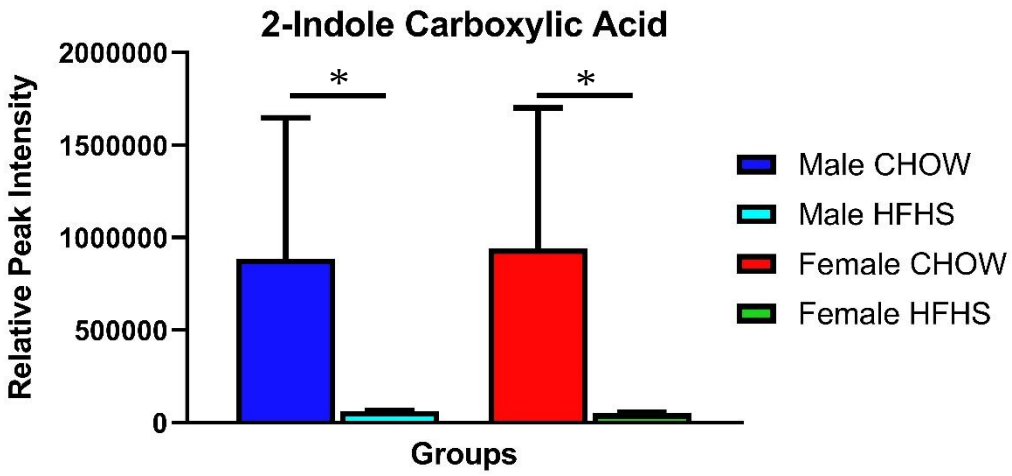

Figure 4.4: Indole Metabolite Expression. Bar graphs depict relative peak intensity of 2-Indole Carboxylic Acid from the cecum of each group.
* $p < 0.0001$.

Figure 5.1: Study Timeline. 40 male C57BL/6J mice were split evenly between groups. Green rectangles represent one week on the chow diet. Red rectangles represent one week on the high fat/high sugar diet. Treatment started at the beginning of week 14 and lasted four weeks. Fecal transplants from Group 1 were orally gavaged into Groups 2 and 4 one time at the beginning of each treatment week. Groups 1 and 3 received vehicle transplants at the same time. Microbes and metabolites were analyzed from pellets taken at the end of week 12 (baseline), week 14 (one week after treatment), and week 15 (two weeks after treatment).

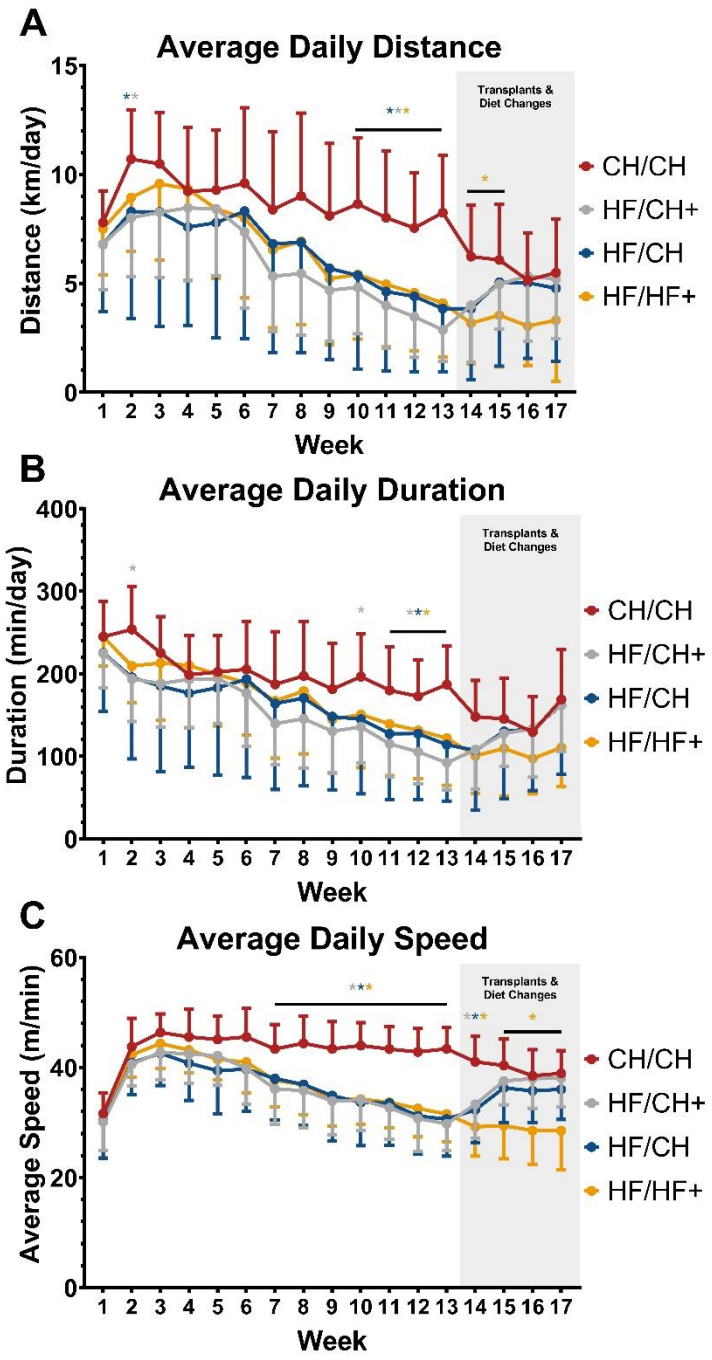

Figure 5.2: Absolute Wheel Running Indices. Values are average daily A) distance, B) duration, and C) speed across time ± SD. The grey panel indicates the inititation of treatment on the first day of week 14. Color of * represents that specific group compared to Group 1 (CH/CH) at $p < 0.05$.

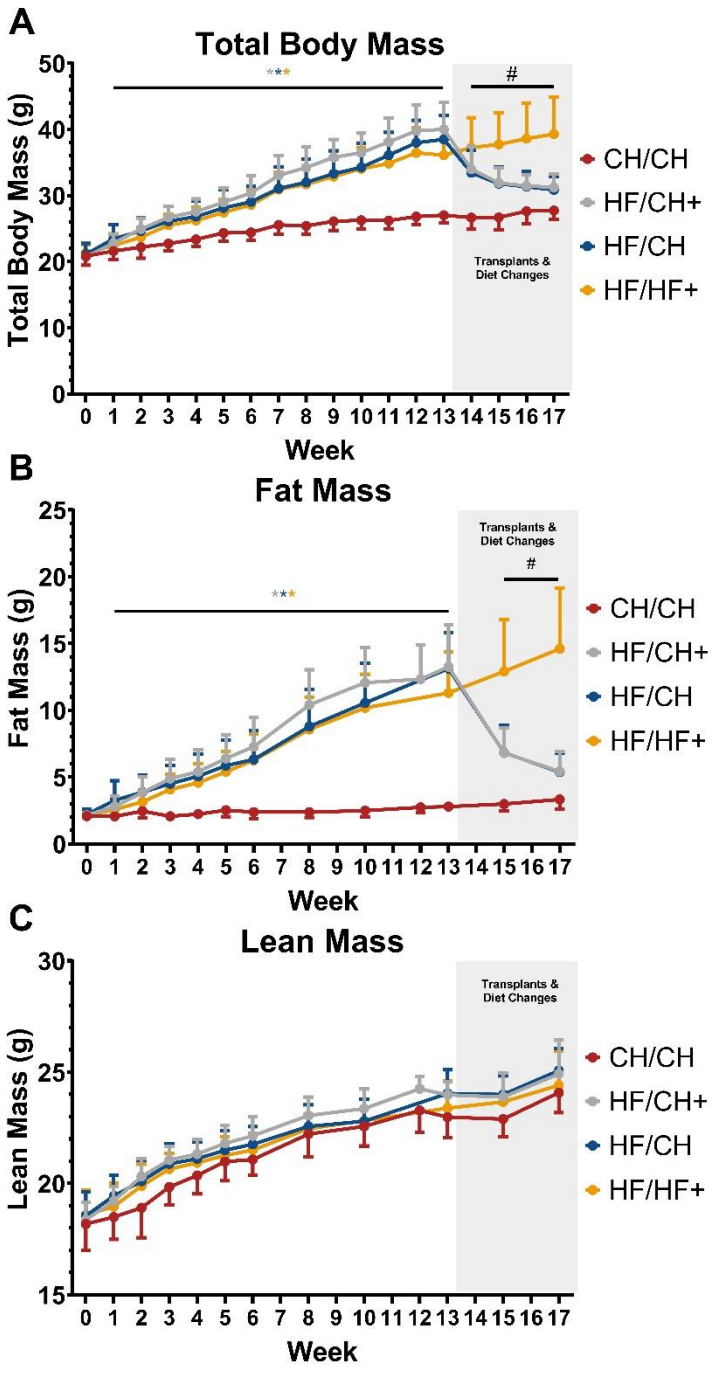

Figure 5.3: Body Composition. Values are average A) total body mass, B) fat mass, and C) lean mass as analyzed by an EchoMRI across time ± SD. The grey panel indicates the initiation of treatment on the first day of week 14. Color of * represents that specific group compared to Group 1 (CH/CH) at $p < 0.05$. # represents Group 2 and 3 are significantly different from Group 1 and Group 4.

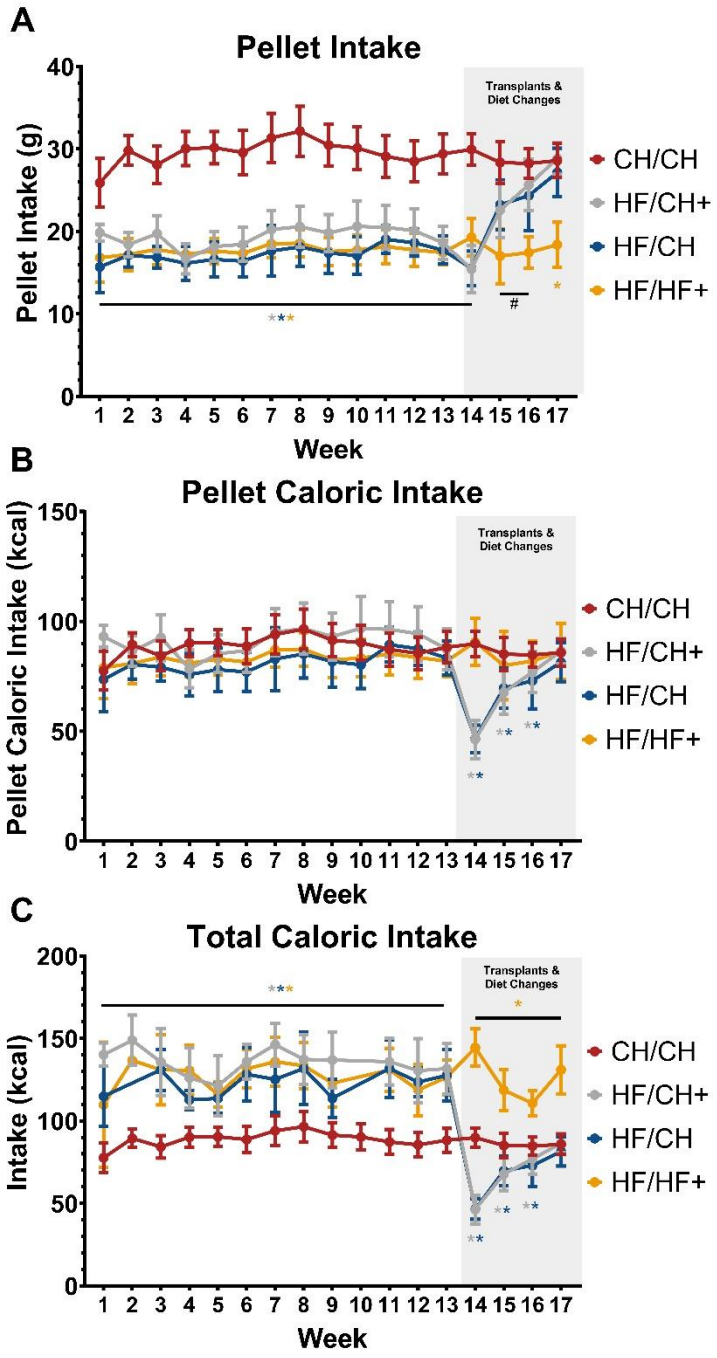

Figure 5.4: Diet Intake. Values are average weekly A) pellet intake, B) pellet caloric intake, and C) total caloric intake measured by scale once a week across time ± SD. The grey panel indicates the inititation of treatment on the first day of week 14. Color of * represents that specific group compared to Group 1 (CH/CH) at $p < 0.05$. # represents Group 2 and 3 are signifincanlty different from Group 1 and Group 4.

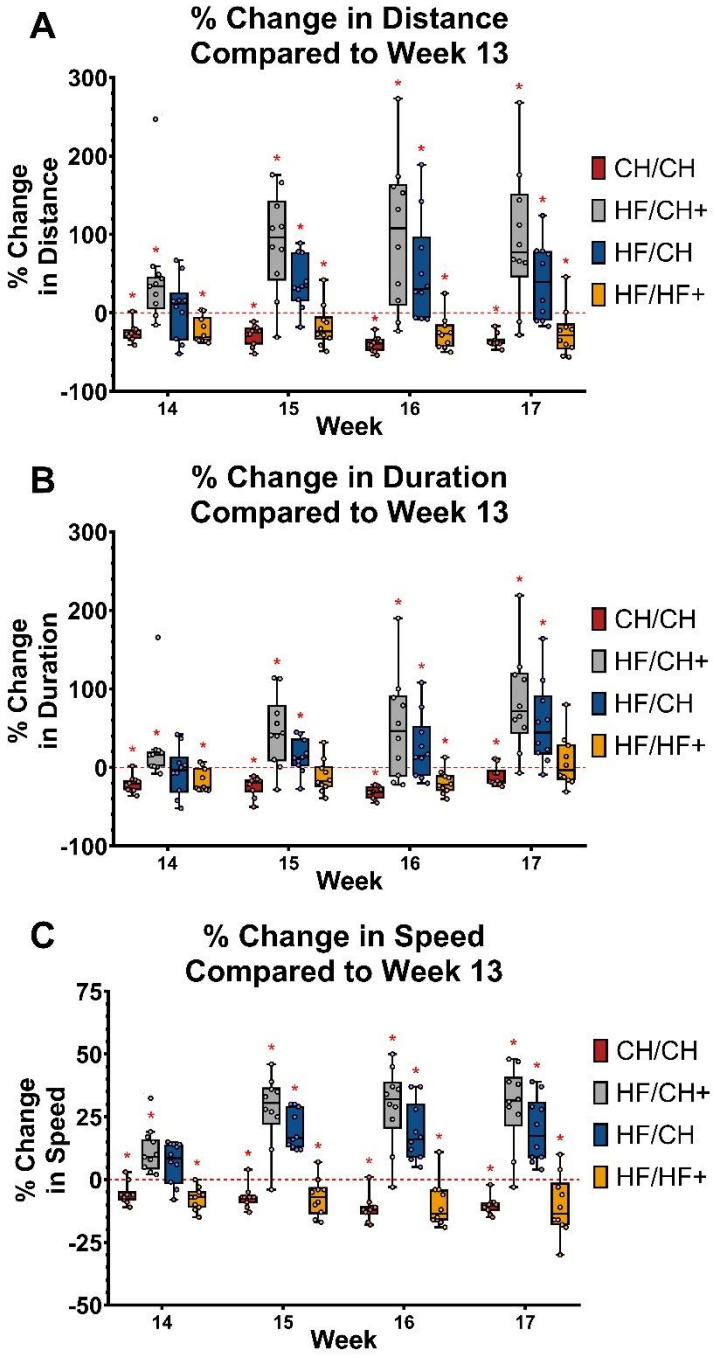

Figure 5.5: Relative Change in Wheel Running Indices Due to Treatment. Values are relative A) distance, B) duration, and C) speed generated by dividing individual mouse activity at week 14, 15, 16, or 17 by activity at week 13 and multiplied by 100. * represents that week as significantly higher than week 13 at $p < 0.05$.

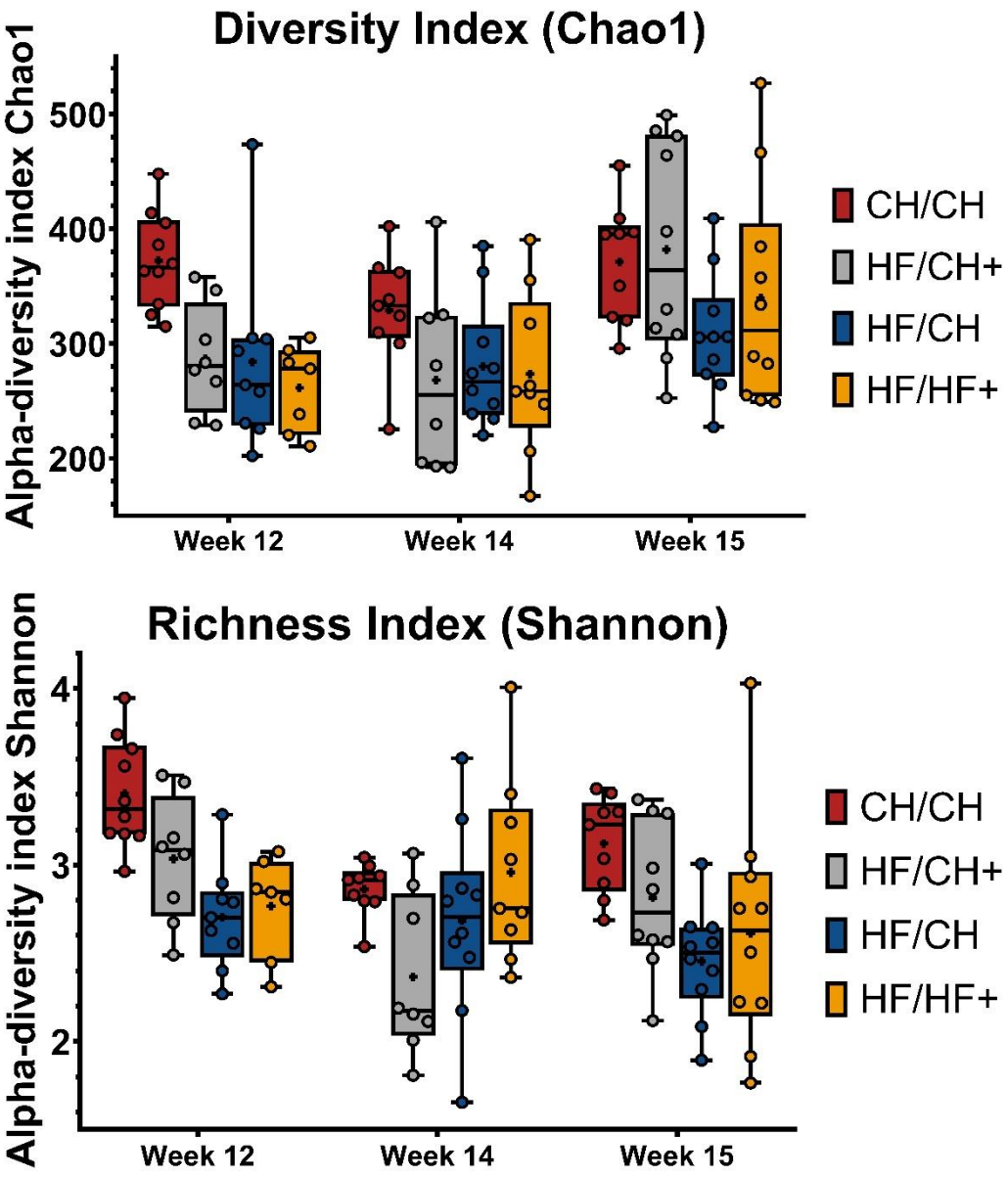

Figure 5.6: Diversity & Richness Index. Diversity and richness were significantly lower in groups fed a HFHS diet at baseline. Group 2 (HF/CH+) reached a similar level of diversity as Group 1 after two weeks of treatment. There was no consistent impact of richness in Groups 2-3 due to treatment.

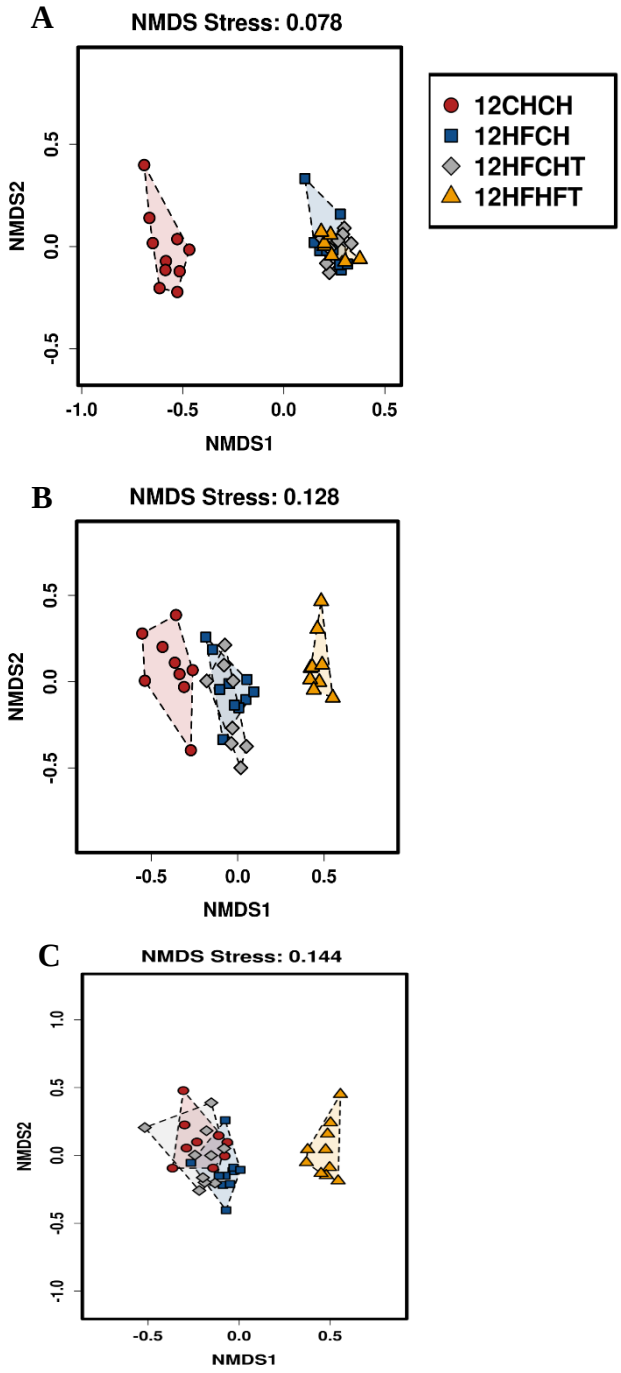

Figure 5.7: Non-Metric Multidimensional Scaling Analysis of Fecal Microboial Communities At Baseline, One Week of Treatment, and Two Weeks of Treatment. Groups 1-3 on a HFHS diet clustered seperetely from the Group 1 on a CHOW diet pre-treatement. After two weeks on treatment without or without a transplant, the clusters of Group 2 and 3 were simliar to Group 1 while Group 4 did not change.

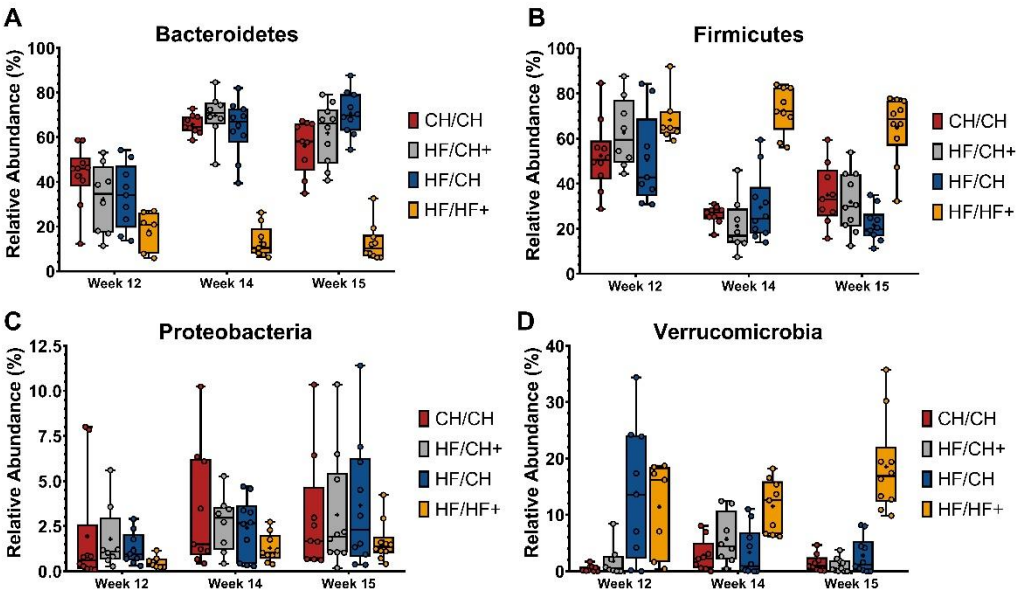

Figure 5.8: Relative Abundance of Phyla at Baseline, One Week of Treatment, and Two Weeks of Treatment. Boxplots represent individual mice with minimal to maximal relative abundance.

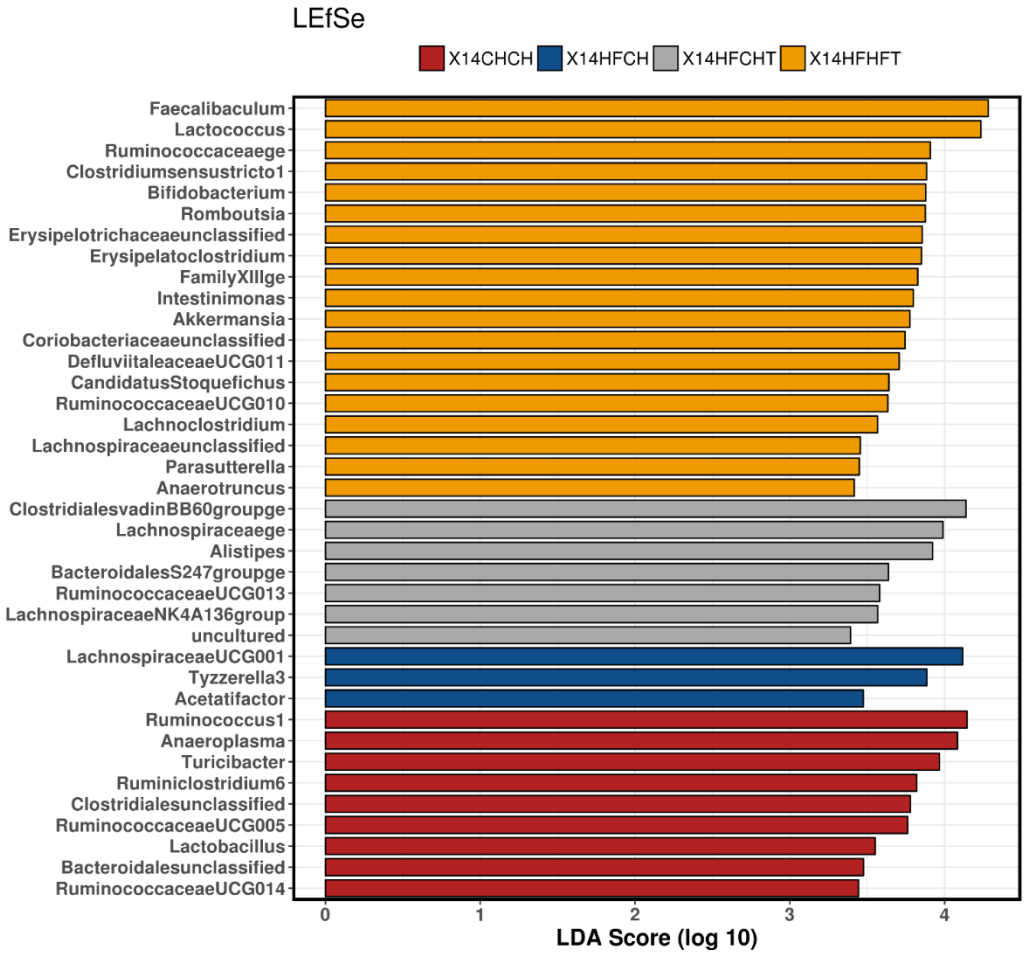

Figure 5.9: Differentially Abundant Fecal Microbes One Week After Treatment. Bars represent genera that were the most abundant in each group after one week of treatment (week 14).

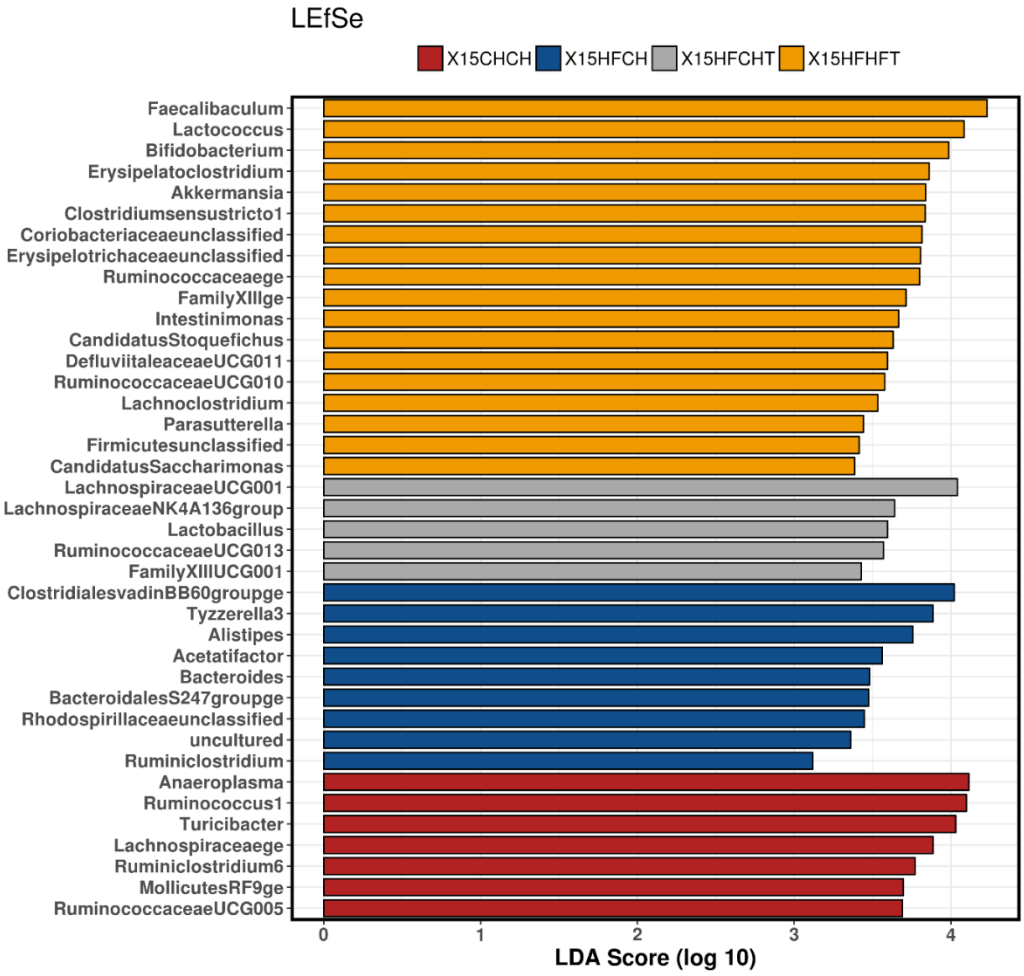

Figure 5.10: Differentially Abundant Fecal Microbes Two Weeks After Treatment. Bars represent genera that were the most abundant in each group after two weeks of treatment (week 14).

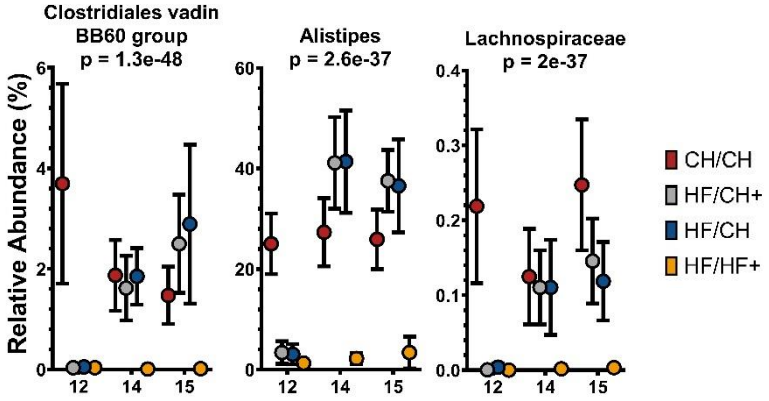

Figure 5.11: Predominate Genera with a CHOW Diet. Points represent group means with 95% confidence intervals.

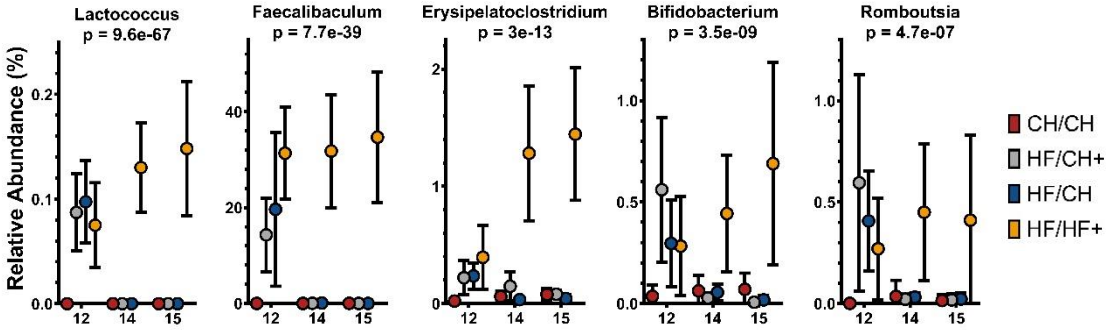

Figure 5.12: Predominate Genera with a HFHS Diet. Points represent group means with 95% confidence intervals.

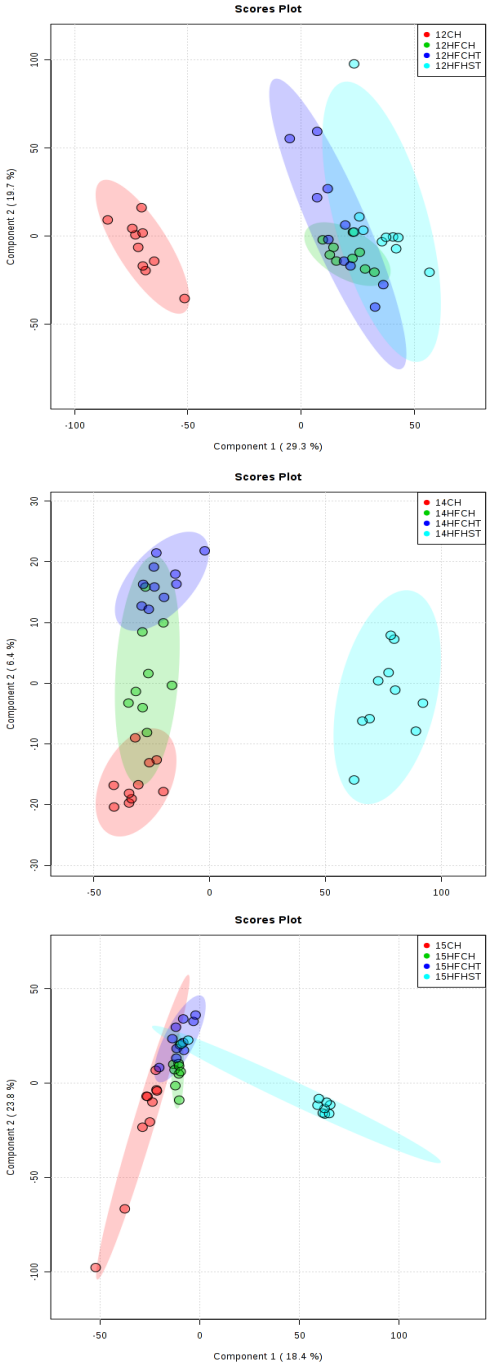

Figure 5.13: Partial Least Sums Discriminate Analysis of the Cecal Metabolome Between Groups at Baseline, One Week of Treatment, and Two Weeks of Treatment. Cecal metabolome clusters were significantly different primarily based on the current diet of the animal. However, while Group 2 (purple) and Group 3 (green) were simliar after one week of treatment, both groups were had significantly different metabolomes compared to Group 1 (red).

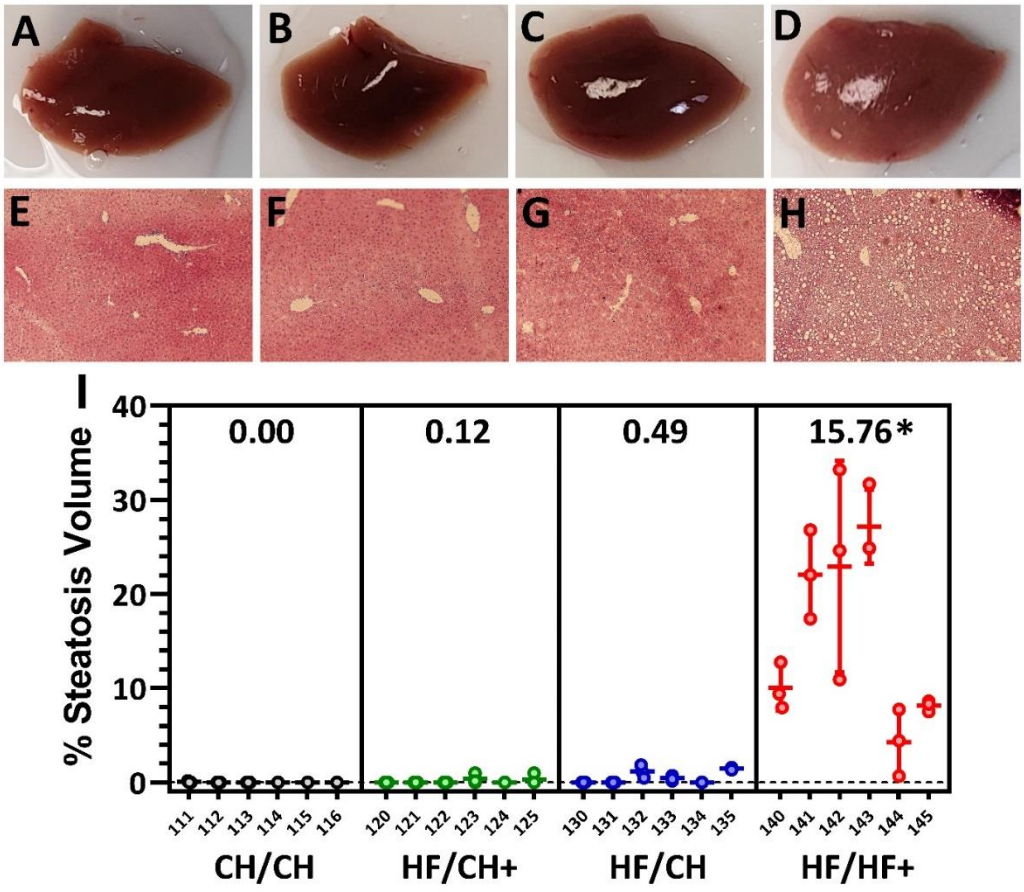

Figure 5.14: Liver Histology. (A-D) Photos taken at sacrifice. (E-H) Images closest to the group average percentage of steatosis. (I) Percentage of total liver area consisting of steatosis. * indicates significantly greater steatosis area compared to all other groups at $p < 0.05$

APPENDIX B. TABLES

Table 2.1: Human, Neanderthal, and chimpanzee alleles for physical activity phenotypes

rsID	Gene	Effect Allele[a]	Neanderthal[b]	Ancestral Allele[c]	Allele Frequencies[d]
rs16933006	Closest RPL7P3	A	AAADAA:0D2A	A>A/C	82/18
rs6025590	CTCFL	A	DAAAA_:0D1A	G>A/G	27/73
rs6454672	CNR1	T	AADAAA:1D0A	T>T/C	85/15
rs8066276	ACE	T	DAD_DA:0D1A	C>C/T	38/62
rs2267668	PPARD	A	DDDDAA:0D1A	A>A/G	85/15
rs1376935	CADM2	G	AAAAAD:0D2A	G>G/A	86/14
rs1638525	AKAP10	G	DADDAD:0D1A	G>C/G	61/39
rs35622985	MMS22L	G	AAAADA:0D1A	G>A/G	27/73
rs1959759	DCAF5	A	ADDDAA:0D2A	A>A/G	18/82
rs10851869	PML	T	D_A_AA:0D1A	C>T/C	57/43
rs2113077	Closest ISL1	A	DAADAD:0D2A	G>A/G	42/58
rs10145335	Closest C14ord177	G	DDDDAD:0D1A	A>G/A	80/20
rs113351744	Closest LINC01029	G	AADAAA:0D1A	G>G/A	98/2
rs12460611	Closest CCNE1	A	AA_DAA:0D3A	A>A/G	83/17
rs12438610	GABRA5	A	ADAAAA:0D1A	G>A/G/T	9/91/.002
rs12595253	GABRG3	A	A_DAAA:0D2A	G>A/G	13/87

[a] Allele associated with higher amount of physical activity

[b] First six characters represent the allele present in the following genomes: human reference, San, Yoruba, Han, Papuan, and French

[b] A - ancestral, D - derived, or _ if not known

[b] Following the colon are the amount of derived or ancestral alleles in Neanderthal genomes

Bold signifies a selective sweep in human lineage

[c] The first character represents the chimpanzee reference allele followed by human alleles

[d] UCSC allele frequencies of human genome found in previous column

SNPs with no effect allele given in primary study are not listed

Table 2.2: Estimated mutation age of PA-related SNPs

	African American	European American
All Exons SNPs[a]	47.6 ± 1.5	34.2 ± 0.9
PPARGC1A	785.2 ± 414.7	666.0 ± 402.3
IFNAR2	747.1 ± 411.8	681.0 ± 403.7
IL-15Ra	585.2 ± 391.8	427.4 ± 341.5
PML	221.7 ± 238.7	549.1 ± 385.5
CTBP2	210.5 ± 231	229.1 ± 263 .9
DNAJC1		7.8
Average Exon	**478.4 ± 327.5**	**542.1 ± 369.4**

Predictions are listed as predicted mutation age in thousands of years ago ± range

[a]All exon located SNPs predicted by Fu et al. (12)

Table 3.1: Diet composition

	Chow-Like Control		High Fat-High Sugar	
Ingredients	**Grams**	**% of calories**	**Grams**	**% of calories**
Casein	200	20%	200	20%
L-Cystine	3		3	
Cornstarch	575	57%	0	
Maltodextrin	125	12%	100	10%
Sucrose	0		245.6	24%
Cellulose	50		50	
Soybean oil	25	6%	25	6%
Lard	20	4%	177.5	39%
Minerals	45		45	
Vitamin mix	10		10	
Choline bitartrate	2		2	
	4,057 kcal/1,005 g		4,057 kcal/858.1 g	
	10% of kcal from fat		45% of kcal from fat	

Table 3.2: Diet group tumor incidence and latency

No Running Wheel							
Group	**n**	**Non-Tumor Death**	**Tumor**	**Tumor Incidence**	**Tumor Incidence *Post hoc***	**Median Latency**	**Median Latency *Post hoc***
D (DR/HF/HF)	30	1	23	79%	A	190	A
AL (C/C/C)	21	1	15	75%	A	261	AB
G (HF/DR/HF)	10	0	7	70%	AB	236	ABC
A (DR/DR/DR)	23	4	13	68%	AB	292	BCD
H (HF/DR/DR)	12	2	5	50%	AB	Undefined	CD
F (HF/HF/DR)	30	1	11	38%	B	Undefined	D

Running Wheel							
Group	**n**	**Non-Tumor Death**	**Tumor**	**Tumor Incidence**	**Tumor Incidence *Post hoc***	**Median Latency**	**Median Latency *Post hoc***
D (DR/HF/HF)	32	1	29	94%	A	176	A
H (HF/DR/DR)	13	0	11	***84%**	AB	***309**	B
A (DR/DR/DR)	23	5	14	78%	AB	291	B
AL (C/C/C)	22	1	14	67%	B	243	B
F (HF/HF/DR)	31	2	18	***62%**	B	***243**	B
G (HF/DR/HF)	13	1	7	58%	B	246	B

Diet groups are ordered by highest tumor incidence to lowest tumor incidence
* $p < 0.05$ versus diet group without access to running wheels
Tumor Incidence *Post hoc* testing was performed via multiple χ^2 statistics
Median Latency *Post hoc* testing was performed via multiple Kaplan–Meier statistics

TABLE 4.1: Mouse characteristics

	Females		**Males**	
Characteristic	**CHOW**	**HFHS**	**CHOW**	**HFHS**
N	7	5	5	5
Body Mass (g)	19.8± 0.9	23.0 ± 2.5*	24.5 ± 2.1	32.1 ± 4.1*
Fat Percentage (%)	12.6 ± 3.1	25.4 ± 9.3*	13.6 ± 4.6	27.4 ± 4.2*
Fat Mass (g)	2.5 ± 0.5	4.4 ± 1.8*	2.8 ± 21.0	8.9 ± 3.0*
Lean Mass (g)	16.3 ± 0.9	17.3 ± 0.9	20.4 ± 1.6	21.8 ± 1.0
Caloric Intake (kcal/day)	10.04 ± 0.73	12.51 ± 0.56*	10.51 ± 0.55	14.95 ± 0.94*

* $p < 0.05$ versus same sex on opposing diet

Table 5.1: Genera correlated with body mass or wheel running distance in Groups 2 and 3

Feature	Value	Coefficient	N	N.not.0	P.value	Q.value
Bacteria|Firmicutes|Clostridia|Clostridiales|Clostridiales_unclassified|Clostridialesunclassified.1	Body Mass	0.0076	109	51	0.0003935	0.0222
Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|uncultured.99	Body Mass	-0.0016	109	2	0.0008789	0.0403
Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|Lachnospiraceaeunclassified.503	Body Mass	-0.0013	109	3	0.001053	0.0458
Bacteria|Firmicutes|Clostridia|Clostridiales|Lachnospiraceae|LachnospiraceaeNK4A136group.36	Distance	0.0032	109	8	2.83E-05	0.0027