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Impact of Positive and Negative Health Behaviors on Female Mice and/or their Offspring

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IMPACT OF POSITIVE AND NEGATIVE HEALTH BEHAVIORS ON FEMALE MICE AND/OR THEIR OFFSPRING

DISSERTATION

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Kristen Mitchell Platt

Lexington, Kentucky

Co-Directors: Dr. Kevin Pearson, Associate Professor, Pharmacology and Nutritional Sciences
and Dr. Howard Glauert, Professor, Pharmacology and Nutritional Sciences

Lexington, Kentucky

2014

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ABSTRACT OF DISSERTATION

IMPACT OF POSITIVE AND NEGATIVE HEALTH BEHAVIORS ON FEMALE MICE AND/OR THEIR OFFSPRING

Obesity is an ever-growing concern in the developed world that carries with it a plethora of health issues. For example, obesity increases an individual’s risk for Type 2 Diabetes and cardiovascular disease. Pregnancy is a vital time for a woman to maintain optimal health, both for her own benefit as well as that of her offspring, and yet almost half of women in the United States who are of age to bear children are overweight or obese. In mice, we found that offspring born to dams fed a high fat diet did not have impaired glucose tolerance, contrary to our hypothesis. In addition, we challenged the offspring with a high fat diet, and found no difference in glucose tolerance as a result of maternal diet. Exercise is at the opposite end of the wellness spectrum – individuals who exercise experience many health benefits. Even overweight or obese individuals who exercise without losing weight have improved insulin sensitivity, for example. Studies have previously used voluntary running and found that offspring born to exercised dams have improved glucose tolerance. With the goal of controlling variable running times and distances, we developed a novel model of controlled exercise and have shown that it is a safe intervention that warrants further study. In addition, many individuals choose to take dietary supplements for various reasons. Branched chain amino acids (BCAAs) are a common dietary supplement that have been shown to increase lean mass, and may be implicated in glucose metabolism. We supplemented female mice with BCAAs for 16 weeks and found that exercise plus BCAAs improved body composition compared to sedentary control-diet fed animals, when exercise alone did not. In summary, we herein explore a number of health behaviors in female mice, both negative treatments such as consumption of a high fat diet and positive interventions such as exercise and BCAA supplementation, and the impact that they may have on the female animal and/or her offspring.

KEYWORDS: obesity, exercise, branched chain amino acids, pregnancy, high fat diet
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CHAPTER 1 INTRODUCTION

1.1 Obesity

1.1.1 The Obesity Epidemic

In the past half century, obesity has become an enormous health crisis. The standard definition of obesity is having a body mass index (BMI), as calculated by kilograms of weight divided by height in meters squared, of $\geq 30.0$ (1998). In 1997, obesity was officially recognized as an epidemic by the World Health Organization (2000). Obesity is extraordinarily prevalent in the United States, and healthcare costs associated with the condition have been projected to easily surpass $200$ billion annually (Cawley and Meyerhoefer 2012). Recent reports from the Centers for Disease Control and Prevention indicate that 34.9% of all American adults are obese (Ogden, Carroll et al. 2013). Alarmingly, we know from this same report that obesity is present in a remarkable 44.4% and 56.6% of Hispanic and non-Hispanic black women, respectively. However, of the 78.6 million obese adults, 50.2 million are non-Hispanic whites. Perhaps most striking is that this report does not include overweight individuals, with body mass indices in the range from 25.0 to 29.9. Even though BMI is an imperfect measure of body composition (Romero-Corral, Somers et al. 2008), these data give us important insight into the state of excessive adiposity of the population.

Obesity increases an individual’s risk for multiple health complications. For instance, it is well established that increased weight is paralleled by an increased risk for hypertension and cardiovascular incidents (Wilson, D'Agostino et al. 2002, Yusuf,
Hawken et al. 2005, Roger, Go et al. 2011). Arthritic individuals suffer more when they are overweight (Schoffman, Wilcox et al. 2013). Obese individuals are more likely to develop Type II Diabetes Mellitus (T2DM) (Kahn, Hull et al. 2006, Sanada, Yokokawa et al. 2012). Obese individuals also suffer a serum profile indicative of systemic inflammation, with high levels of inflammatory cytokines (Fain 2006). It is not a new or surprising finding that individuals with a high BMI are more likely to die than normal-weight individuals (Calle, Thun et al. 1999).

Impairment in glucose homeostasis, often due to decreased insulin sensitivity, is a key metabolic perturbation associated with obesity. The primary means by which blood glucose levels are lowered is through skeletal muscle uptake of the glucose from circulation (Figure 1.1). To accomplish this postprandial uptake of glucose, insulin is released from the pancreas after food is consumed and blood glucose levels begin to rise (Prentki, Matschinsky et al. 2013). Insulin then binds to its receptor on the skeletal muscle cell, resulting in autophosphorylation of the intracellular domain of the insulin receptor. Insulin receptor substrate 1 (IRS1) is phosphorylated and interacts with phosphatidylinositol 3 kinase (PI3K). PI3K is critical in activating Akt (Protein Kinase B) by phosphorylation. The culmination of the signaling cascade is recruitment of vesicles containing glucose transporter type 4 (GLUT4), to the cellular membrane (Beale 2013) by way of Tre-2, Bub-2, Cdc-16 Family Member Type 4 (TBC1D4), also known as Akt Substrate, molecular weight 160 (AS160). Recruitment of these vesicles, thus the presence of more glucose transporters on the cell surface, increases uptake of glucose into
the cells. The final result is lowered blood glucose. There are, however, other means, such as exercise, by which glucose uptake into cells can be promoted (see section 1.2.1).

Adipose tissue has only relatively recently become recognized as a metabolically active organ. Adipose is responsible for releasing various compounds, collectively referred to as adipokines, that act in a number of ways throughout the entire body (Trayhurn, Bing et al. 2006). For instance, leptin is a well-known adipokine that received much attention around the time of its discovery (Zhang, Proenca et al. 1994). Leptin is released proportionately to the amount of adipose a person has, and acts in the hypothalamus to signal satiation (Jequier 2002). However, resistance to leptin is a known issue – if the receptor is no longer sensitive to satiety signals, then the person is apt to consume more food, thus promoting obesity.

Other factors associated with insulin resistance are released from the inflamed adipose of an obese individual as well, e.g. retinol binding protein 4 (RPB4) and tumor necrosis factor α (TNFα) (Figure 1.1). RPB4 is increased in obesity and decreases insulin signaling by way of inhibiting PI3K and/or IRS1 (Yang, Graham et al. 2005, Christou, Tselepis et al. 2012). TNFα, similarly, is released in greater quantity from the adipose of an obese individual. This molecule works to impair glucose tolerance by means of inhibiting activation of the insulin receptor (Hotamisligil, Murray et al. 1994).

Another key adipokine is adiponectin (Gil-Campos, Canete et al. 2004). Adiponectin improves insulin sensitivity in the skeletal muscle by means of adenosine monophosphate kinase (AMPK) (Figure 1.1) (Yamauchi, Kamon et al. 2002). AMPK
increases recruitment of intracellular vesicles containing GLUT4 to the cell surface, thereby increasing cellular glucose uptake. Serum adiponectin levels are decreased in obese individuals (Tschritter, Fritsche et al. 2003). This decrease is concurrent with impairments in glucose tolerance and increases in inflammation. Adiponectin also plays a role in oxidation of fatty acids, and increased free fatty acids are associated with impaired glucose tolerance as well.

What has been appreciated for a longer time than the adipokine action on insulin resistance is that increases in free fatty acids, commonly found in obese individuals, act through various mechanisms to impair glucose homeostasis (Capurso and Capurso 2012). For instance, elevated serum free fatty acids and increased inflammation decrease insulin sensitivity through factors such as c-Jun n-Terminal Kinase (JNK) (Figure 1.1). JNK increases inhibitory phosphorylation of IRS1 (Hirosumi, Tuncman et al. 2002).

While there has been much discussion surrounding a ‘metabolically healthy’ obese phenotype, the general consensus is that it is still preferable to maintain a healthy body weight (Sims 2001, Bluher 2010, Kramer, Zinman et al. 2013). All considered, obesity is a disease that brings both debilitating health consequences to the individual in addition to imposing a heavy financial burden on the health care system.

1.1.2 Obesity during Pregnancy

Obesity in women is linked to infertility issues. An obese woman is more likely to have irregular cycles or polycystic ovary syndrome, increasing difficulty of conception (Norman, Noakes et al. 2004, Ogbuji 2010). One of the first suggestions a healthcare
provider will tell an obese woman struggling to conceive is to lose weight (Clark, Thornley et al. 1998). It has been shown that even a small weight loss improves chance of conception (Clark, Ledger et al. 1995).

Obesity and excessive gestational weight gain in a woman of childbearing age increases her risk for having a complicated pregnancy. Offspring born having been exposed to the milieu of maternal obesity also have increased risk for health issues, both perinatal and later-life complications (to be discussed in detail in section 1.4). In 1986, recommended weight gain was 22-26 pounds during pregnancy, and at this time there was concern about women not gaining enough weight during gestation (Taffel and Keppel 1986). This number does not account for the woman’s weight before pregnancy, nor is it corrected for her height. In the last three decades, this concern has shifted and more focus is now placed on women who begin pregnancy as overweight or obese, and appropriate gestational weight gain. Current recommendations are based on pre-pregnancy BMI, and range from as little as 11 pounds for obese women, to as much as 35 pounds for normal weight women (2009). There is emerging evidence that even current standards are too much to gain for obese women (Kominiarek, Seligman et al. 2013). These current recommendations, however, have the advantage of taking pre-pregnancy weight and height into account by means of BMI categorization.

Obesity and excessive weight gain during pregnancy increases women’s risk for enduring various complications. For example, obese women are at increased chance for experiencing preeclampsia and gestational diabetes (Sebire, Jolly et al. 2001, Bodnar,
Ness et al. 2005, Gaillard, Steegers et al. 2011). Labor is more likely to be complicated for obese women, including higher incidence of induction of labor, increased risk for hemorrhage, and more frequent use of caesarean section (Sebire, Jolly et al. 2001, Lynch, Sexton et al. 2008, Fyfe, Thompson et al. 2012, Graham, Brunner Huber et al. 2014). In addition to beginning pregnancy overweight or obese, excessive weight gain during pregnancy also carries many of the same complications (Chandrasekaran, Levine et al. 2014). One study found that almost half of women gained more than the recommended amount of weight during gestation (Raymond, Foureur et al. 2014). A large study found that gaining weight in excess of the recommendation based on pre-pregnancy BMI increased risk for many of the above-mentioned complications (Haugen, Brantsaeter et al. 2014). There is little doubt that being overweight or obese before conception and/or gaining excessive weight during pregnancy can be extremely detrimental to a pregnant woman. There should be more exploration of ways to minimize the complications that arise from obesity and excessive gestational weight gain before and during pregnancy, such as exercise.

1.2 Exercise

1.2.1 Exercise – Prevalence and Practicality

Exercise is an extremely efficacious means for improving health. A clear advantage of exercise is that it is completely accessible to any individual at no cost – for example, walking/running or body-weight resistance exercises require no monetary investment. A large survey in the United States showed that 55.7% of individuals
completed some type of exercise regularly (three or more times weekly) (Schaffer, Gordon et al. 2003). Individuals who are lean and also subjected to an exercise intervention exhibit improved insulin sensitivity (Reyna, Tantiwong et al. 2013). In addition, exercise may be a useful means by which to prevent weight gain over time: one study observed that individuals who increased their physical activity levels gained less weight over time than those who did not (Mozaffarian, Hao et al. 2011). As being sedentary is highly associated with increased risk for obesity, there is little doubt that exercise has strong potential to counter this.

Currently, the American College of Sports Medicine, as well as the American Heart Association, recommend 150 minutes of moderate exercise per week – for example, half an hour, five days per week (Haskell, Lee et al. 2007, Garber, Blissmer et al. 2011). Unfortunately, the number of individuals achieving this goal is far less than desirable (Centers for Disease and Prevention 2013). Exercise and physical activity are terms that are broadly defined. Individuals may have difficulty deciphering “moderate exercise,” and it certainly varies based on the fitness of the person.

Exercise acts on the skeletal muscle by means of adenosine monophosphate activated protein kinase (AMPK) to stimulate GLUT4 transport to the membrane – and thus glucose uptake from the bloodstream - independent of insulin (O’Neill 2013). The means by which this occurs is not well understood, but in general, muscle contraction increases adenosine monophosphate (AMP) levels, which activates AMPK, which then acts through AS160 to recruit GLUT4 vesicles (Richter and Hargreaves 2013).
Regardless, exercise acutely increases glucose uptake as well as insulin sensitivity. In fact, even one bout of exercise improves glucose tolerance in normal individuals (Knudsen, Karstoft et al. 2014). Conversely, data suggest that the insulin sensitizing effects of exercise last only around two to three days, so a structured regimen is ideal (Etgen, Brozinick et al. 1993, Mikus, Oberlin et al. 2012).

Not only is exercise useful for improving body composition and various metabolic measures, it is also becoming more apparent that it has many other benefits as well. For example, exercise is well shown to improve depressed individuals’ mood state, and the American Psychiatric Association now includes it in their recommendations for treatment approaches (Dunn, Trivedi et al. 2005, Alan J. Gelenberg, Marlene P. Freeman et al. 2010, Hoffman, Babyak et al. 2011). One study suggests that simply increasing steps taken per day may have potential to improve fibromyalgia outcomes (Kaleth, Slaven et al. 2014). There are other potential benefits of exercise as well, including decreased risk of some cancers and increased intellectual capacity (Friedenreich and Orenstein 2002, Hogan, Mata et al. 2013, Chang, Chu et al. 2014). There is clearly a wide array of advantages offered by exercise.

Conversely, individuals who are accustomed to exercising and subsequently cease physical activity suffer deleterious consequences. For example, men who were recreationally active suffered diminished vascular function upon cessation of their exercise routine (Boyle, Credeur et al. 2013). Similarly, individuals who did not increase their exercise regimen over time were not protected from longitudinal weight gain
(Mozaffarian, Hao et al. 2011). This suggests that exercise should be implemented as a life-long tool to improve health and fitness.

Finally, any exercise is better than none. Reports indicate that even small bouts of physical activity improve cardiovascular risk, for example (Sattelmair, Pertman et al. 2011). Simply minimizing sedentary time can have a dramatic impact on metabolic health.

1.2.2 Exercise in Obese Individuals

As exercise is extremely advantageous to health in the general sense, it is potentially even more beneficial for an obese individual. It has been observed that as the number of hours an individual watches television increases, so does that person’s risk for obesity (Cleland, Schmidt et al. 2008). It would be easy to then surmise that the opposite is also true – as has been shown – that there is an inverse relationship between the amount of leisure time physical activity a person undertakes and body weight (Ladabaum, Mannalithara et al. 2014). Even simple actions that increase activity levels are beneficial. For example, individuals who walk their dog are significantly less likely to be obese than both non-walking dog owners and people without dogs (Coleman, Rosenberg et al. 2008). Clearly even simple lifestyle attributes can help to determine health.

Even without weight loss, exercise has been shown to improve insulin sensitivity and blood pressure in individuals exhibiting excessive adiposity (Carroll and Kyser 2002, Duncan, Perri et al. 2003). People who simply accumulate 10,000 steps per day,
measured by pedometer, are less fat, less hypertensive and have improved glucose tolerance (Iwane, Arita et al. 2000, Tudor-Locke, Ainsworth et al. 2001, Swartz, Strath et al. 2003). Even a single bout of exercise acutely improves insulin sensitivity in obese individuals (Newsom, Everett et al. 2013). It would be ideal and potentially very efficacious if more physicians would ‘prescribe’ exercise as an intervention for pre-diabetic and pre-hypertensive individuals in an effort to stop them from developing the full-blown condition (Bushman 2014). By that same token, exercise could be prescribed as a treatment for the diseases, if people would adhere to the recommendation.

Even though physical activity has advantages without decreasing body weight, exercise is also an efficacious means by which to increase weight loss (Donnelly, Honas et al. 2013). In the simplest terms, weight gain occurs when calories consumed exceed calories spent. Exercise, of course, results in an increase in calories burned. This can help to swing the calorie balance back to a healthy level if the individual maintains an exercise regimen. It has been shown that individuals who are trying to lose weight have more success if they exercise in addition to dieting than people relying on diet alone (Foster-Schubert, Alfano et al. 2012). Muscle tissue plays an important role in whole body homeostasis and should not be disregarded as unimportant (Wolfe 2006). Small increases in muscle mass can accumulate to make a notable difference in health.

1.2.3 Supplementation

Diet and exercise may not be the only mechanisms by which health and metabolism may be improved. Dietary supplements – products for oral consumption
designed to add some nutritional component to a person’s diet – have become extremely popular in the United States. In the early 1990’s, there were around 4000 dietary supplements on the market – and in 2008 there were over 75,000 (Marcus, Williams et al. 2006). As of 2011, dietary supplements comprised a $30 billion industry (Bailey, Gahche et al. 2013). This is clearly a gargantuan enterprise that people are willing to participate in if they perceive some advantage of doing so.

In general, there are two classes of dietary supplements – vitamin/mineral supplements, and non-vitamin/non-mineral supplements. Non-vitamin, non-mineral supplements are a broad class of compounds. They may include herbs and botanicals, protein purifications, antioxidants, and other plant products (Radimer, Subar et al. 2000). There are many reasons that people choose to take non-vitamin, non-mineral supplements. Some reasons include: as an energy booster, e.g. maca; as a memory aid, e.g. ginkgo biloba; or in an attempt to treat a specific condition such as cancer, e.g. garlic or green tea (Neuhouser 2003). Whatever the rationale, and regardless of the scientific support (or lack thereof) for a given product, supplement use is widespread in the United States.

Many individuals who exercise may also choose to consume dietary supplements. For example, 68.6% of persons who reported exercising at least once per week also report that they consumed some non-vitamin, non-mineral supplement (Schaffer, Gordon et al. 2003). Another report found that 56.3% of individuals claiming high amounts of exercise consumed some supplement regularly (Bailey, Gahche et al. 2013). Interestingly, the
same report found that the highest prevalence of consumption (52.8%) based on BMI was among the adults in the healthy range. A common reason for supplementing during an exercise regimen is to increase building of lean mass (El Khoury and Antoine-Jonville 2012). Protein supplements are frequently used for this purpose.

The standard recommended consumption value for a non-pregnant woman is 0.8 grams of protein per every kilogram of body weight, or about 52 grams for a 65 kilogram individual (Trumbo, Schlicker et al. 2002). Contrast this value with the recommendation for endurance athletes, at 1.2-1.4 grams per kilogram of body weight daily, and strength athletes at up to 1.7 grams per kilogram (American Dietetic, Dietitians of et al. 2009). The current recommendation is that pregnant women consume 25 additional grams of protein daily compared to the pre-pregnancy recommendation (Trumbo, Schlicker et al. 2002). This means that a pregnant woman of 65 kilograms would ideally consume ~75 grams of protein a day. One can easily imagine that it may be difficult to obtain the high consumption value required during pregnancy, particularly if a woman is concerned about keeping her weight gain, and therefore food intake, to a healthy level. A protein supplement could help bridge this gap.

Unfortunately, research regarding protein supplementation during pregnancy suffered a major setback in the early 1980’s (Rush, Stein et al. 1980). Researchers were supplementing protein in the diets of pregnant women who were under- or malnourished. Negative outcomes were seen in infant survival with high protein supplementation and therefore the research was terminated. This has stunted the body of literature regarding
protein supplementation during pregnancy, though in recent years the field has begun to recover, with the realization that protein supplementation decreases risk for intrauterine growth restriction (Imdad and Bhutta 2011). It seems in the right circumstances, an appropriate protein supplement during pregnancy can be advantageous to the woman and fetus.

Protein is composed of building blocks called amino acids. The branched-chain amino acids (BCAAs), named thus because of their branching structures, are leucine, valine, and isoleucine. The BCAAs have been shown to promote protein synthesis and fat loss (Mourier, Bigard et al. 1997, Blomstrand, Eliasson et al. 2006). It has been suggested that the BCAAs may be key to the improvements in weight management purported by high protein diets (She, Van Horn et al. 2007, Qin, Xun et al. 2011). Similarly, athletes who consumed a BCAA supplement lost more fat in the abdominal region than their respective control group (Mourier, Bigard et al. 1997). BCAAs also seem to play a role in insulin sensitivity, but the mechanisms are not entirely clear (Lu, Xie et al. 2013).

BCAAs are implicated in satiety, as well. For instance, leucine is known to act in the hypothalamus by means of mammalian target of rapamycin, or mTOR (Morrison, Reed et al. 2012). mTOR has been referred to as a cellular sensor, receiving the status of energy and nutrient levels and triggering an appropriate response, i.e. signaling satiety when sufficient nutrients are present (Howell and Manning 2011). Rats, for instance, that receive an intracranial dose of leucine eat less food and weigh less than controls (Cota, Proulx et al. 2006). mTOR levels are reportedly increased by leucine and is also the
means by which BCAAs act to promote protein synthesis in skeletal muscle (Layman and Walker 2006, Rennie, Bohe et al. 2006). For instance, once mTOR is activated, mTOR activates effector molecules such as serine-6 kinase 1 (S6K) and eukaryotic initiation factor 4G (eIF4G) (Wang and Proud 2006). These translation factors increase protein synthesis. In addition, leucine purportedly promotes “protein sparing” (Layman and Walker 2006). Protein sparing is the concept that muscle breakdown is minimized after exercise; the protein is spared. Instead of the protein, other sources are used as fuel, e.g. fatty acids or glucose, preserving muscle mass.

BCAAs may have potential as a supplement to encourage growth of lean mass, maintenance of body weight and perhaps even glucose homeostasis. For instance, leucine acts as an insulin secretagogue in the β-cell (Figure 1.1) (Yang, Chi et al. 2010). Once in the β-cell, leucine is deaminated to ketoisocaprate, which increases activation of the ATP-sensitive potassium (K_{ATP}) channel. Once depolarization of the cell occurs, the influx of calcium into the β-cell results in release of the granules containing insulin, as it usually would (Henquin, Gembal et al. 1994).

1.3 Exercise during Pregnancy

1.3.1 Exercise in Pregnant Women

The female body undergoes dramatic physiological changes during pregnancy, reviewed by Moya et al (Moya, Phillips et al. 2014). Blood volume increases by up to 50% and cardiac output increases accordingly. There is up to a 400 mL increase in red blood cell volume. Kidneys increase in mass, peristalsis slows, and bone remodeling
increases. Respiration also increases early in pregnancy. These changes can certainly have an impact on the mental and physical state of any woman.

While pregnancy has historically been seen as a time for a woman to rest and relax, modern science suggests that this may not be the best approach for either the woman or her developing offspring. Exercise can prevent excessive weight gain and increase the chances for a woman to stay within the weight gain guidelines during her pregnancy (Lamina and Agbanusi 2013, Harris, Liu et al. 2014). Current standards of care recommend 30 minutes of moderate exercise most days of the week for pregnant women, unless contraindicated (Practice 2002). Typically, women should avoid more risky forms of exercise, like contact sports or scuba diving.

James Clapp is widely known for his work involving exercise during pregnancy. Some of his benchmark work has shown that women who continue a high level of exercise during their pregnancy have easier pregnancies and deliveries and lower incidence of caesarean section (Clapp 1990, Clapp and Little 1995). Work by others has further substantiated these findings, even in women who do not exercise at such intense levels as those in Clapp’s studies. For example, even modest exercise such as stretching or walking during pregnancy decreases incidence of preeclampsia (Yeo 2009). Also, initiating an exercise routine during gestation can improve oral glucose tolerance (Oken, Ning et al. 2006, Barakat, Cordero et al. 2011, Barakat, Cordero et al. 2012). Exercise can prevent excessive weight gain in pregnant women with normal pre-pregnancy BMI (Ruchat, Davenport et al. 2012). In addition, exercise during pregnancy appears to
prevent and improve gestational depression outcomes (Daley, Foster et al. 2014). Finally, exercise during pregnancy may simply help the pregnant woman feel better, have more energy and experience less discomfort (Montoya Arizabaleta, Orozco Buitrago et al. 2010, Robledo-Colonia, Sandoval-Restrepo et al. 2012).

One point of interest, however, is that women who exercise intensely during the first half of their pregnancy and then cease to exercise may increase placental and fetal growth (Clapp, Kim et al. 2002). The belief is that the exercise early on promotes placental growth and efficiency, but once the exercise ends, the baby receives nutrients in excess. This increase in placental efficiency may be useful for women at risk for intrauterine growth restriction. However, in a large woman, this could increase risk for macrosomia. Macrosomia, or an exceedingly large neonate, is highly correlated to later-life incidence of obesity, T2DM, and heart disease. This will be further explored in section 1.4.

1.3.2 Exercise in Obese Pregnant Women

There is less basic scientific research regarding exercise in the obese pregnant woman, even though around half of women of childbearing age are obese or overweight. One can imagine the difficulty of instigating an exercise regimen in a normal pregnancy of a woman with healthy weight, and thus the exceedingly difficult task of initiating that same regimen in an ‘at risk’ pregnancy. However, exercise may help to minimize excessive weight gain in obese pregnant women, which is important for the health of the woman and her developing child (Mottola, Giroux et al. 2010). There is also evidence
that even mild exercise during the obese woman’s gestation may decrease pregnancy-associated discomfort (Mottola 2013). In addition, physical activity may improve cardiovascular parameters during pregnancy (Stutzman, Brown et al. 2010).

As the research regarding exercise in obese women during pregnancy is relatively limited at current, animals are being used to explore these effects. For instance, obese pregnant mice/rats that exercise have improved lipid profiles and increased conception success (Vega, Reyes-Castro et al. 2013). However, even the animal data regarding exercise, obesity and pregnancy is minimal. This field will continue to grow in the coming years, and more insights will be available and substantiated by primary research data.

Typical recommendations for exercise in the normal-weight pregnant woman also apply to the obese pregnant woman. That is to say, at least 30 minutes of activity, most days of the week, unless contraindicated. Recent work has suggested that even previously sedentary women can initiate an exercise regimen during pregnancy as long as they begin at a low intensity and subsequently work their way up in intensity and duration (Mottola 2009).

1.3.3 Obesity, Exercise, and Pregnancy in the Mouse

Since it is often difficult to study lifestyle interventions during pregnancy in humans, it is important that we use rodent models to explore the health complications and ramifications during this critical developmental window. Although relatively few pregnant women adhere to recommendations for physical activity during pregnancy
(Evenson, Savitz et al. 2004), perhaps with more empirical evidence of the benefit, and more concrete standards of practice, they may be swayed.

Obesity is frequently induced in mouse models in order to study the condition. Several means are used to induce obesity. For one, genetic manipulation is common (i.e. disruption of the genes encoding either leptin or its receptor, resulting in hyperphagia and thus excessive weight gain) (Ingalls, Dickie et al. 1950, Friedman and Halaas 1998). There are, alternatively, chemical means by which to induce obesity in mice. One example of chemical induction is by injection of monosodium glutamate (MSG) (Andreazzi, Scomparin et al. 2009, Miranda, Branco et al. 2013). Another frequently used option is to feed the animals a high fat diet (Samuelsson, Matthews et al. 2008, Elahi, Cagampang et al. 2009, Ornellas, Mello et al. 2013). Herein we will utilize high fat diet as the means to induce obesity, as we posit that it is a more physiologically relevant model for our research. It is also simple and quick to use high fat diet feeding to cause obesity in the mouse.

Obesity in the mouse carries with it many of the same consequences as obesity in humans or other mammals. Alongside of rapid weight gain, mice fed a high fat diet also become intolerant to glucose as well as insulin resistant in only a few weeks. They also have a dramatic increase in fat mass when measured by magnetic resonance imaging (MRI), which is not surprising.

Exercise is also a common intervention in the mouse. Voluntary running is a frequently employed method (Brown, Naples et al. 2012, Carter, Lewis et al. 2012,
Martin, Dantzer et al. 2014). In voluntary running models, an exercise wheel is placed into the home cage of the animal and the mouse may run as much or as little as it desires, whenever it chooses. Often, the running wheel rotations are recorded electronically for analysis. While it has been previously proposed that wheel running may be a form of obsessive compulsive type disorder in captive rodents, more recent work has shown that even animals in the wild will choose to run on exercise wheels when they are provided (Altemus, Glowa et al. 1993, Meijer and Robbers 2014). Even as such, it is impossible to control the amount (duration or distance, or both) that the animals are running.

Alternatively, the animal may be removed from its cage and subjected to forced running, such as on a treadmill. The treadmill usually has a negative stimulus at its rear aspect, such as an electric shock grid, to encourage the animal to exercise. Arguably, this negative stimulus would be stressful for the mouse, and often it is undesirable to stress the pregnant females unduly. A third running model using a rotating wheel, called controlled exercise in the current work, is less widely employed at large (Khazaei, Moien-Afshari et al. 2008, Shearer, Ross et al. 2011, Vadhavkar, Golbidi et al. 2011). This model involves removing the mouse from her home cage and placing her into a wheel that rests upon a motorized platform that rotates at a set speed. The models of exercise utilized herein include both a controlled model, such that the animal is removed from her cage and placed into a rotating wheel that lacks any form of negative motivation, as well as voluntary home-cage running.
Other forms of exercise do exist for the mouse. For instance, swimming is a fairly common choice (Almeida, Lima et al. 2011, Horckmans, Leon-Gomez et al. 2012, Liu, Wang et al. 2014). One drawback of swimming, however, is that the animal is, of course, wet after the exercise bout. This may leave her with a chill, and she will be forced to groom herself abundantly after every swim session. It is even used as a model of stress (Ghasem, Majid et al. 2013). As we did not want to stress the breeding females, we decided to avoid swimming as a model in the current work.

Finally, mice are well reputed to be good breeders. The average gestation for a mouse is 21 days, though in our hands litters consistently arrive beginning on day 19. ICR (CD1) mice average 11 pups per litter and tend to be good dams (i.e. do not frequently reject pups). Having a large number of pups allows for culling the litter to a consistent number; 8 is a common choice. This is in an attempt to standardize nutrient delivery to all pups: as a female mouse has 10 nipples, there should be no contest for nursing and no pup should be denied nourishment.

1.4 Developmental Programming

1.4.1 The Barker Hypothesis

In the late 1980’s, David Barker was studying the incidence of cardiovascular disease in Great Britain. He observed that there was much higher incidence of heart disease in the relatively impoverished areas of the country. As he began to delve further into this finding, it became apparent that the real correlation was between the individual’s birth weight and their increased risk for disease. Now it is widely accepted that
individuals with low birth weight are at an increased risk for developing coronary disease later in life (Barker 1990, Barker, Gluckman et al. 1993). This has been labeled the Barker Hypothesis. With this work, Barker is frequently given credit for laying the foundation upon which would be built the field of developmental programming.

Developmental programming is the concept that exposure to a given maternal milieu in utero predisposes the offspring to certain health outcomes later in life (Hales and Barker 1992). If the fetus is exposed to a healthy, stable milieu, then they have a greater chance of being healthy as a mature adult. Conversely, if the fetus is exposed to malnourishment or excessive nourishment in utero, then they have an increased risk for suffering disease later in life (Barker, Gluckman et al. 1993, Roseboom, de Rooij et al. 2006). This is especially true when the postnatal environment into which the fetus is born does not match the milieu it was exposed to prenatally.

The concept that the in utero environment plays an important role in offspring health was rather well accepted before it had a name. For example, it has been known for quite some time that consumption of alcohol and drugs during pregnancy is extremely detrimental for the offspring long-term (Jones, Smith et al. 1973, Smeriglio and Wilcox 1999, Bailey and Sokol 2008). Clinicians have long known that women should eat a healthful diet and gain an appropriate amount of weight during their pregnancy for the sake of the developing fetus (Suitor 1994, Rasmussen and Yaktine 2009). Historical studies and cohort analyses have lent insight into many facets of this maternal impact on offspring health.
1.4.2 The Dutch Famine and other Studies

During World War II, the Netherlands suddenly cut ties with Germany in the winter of 1944. Germany, in retaliation, set a blockade on their enemy. This incited a sudden, severe food shortage in the Netherlands, which forced them to ration every individual’s calorie intake to severely low levels, well below 1000 calories daily (Schulz 2010). As it turned out, they kept exceptional records of every person’s calorie allowance – including that of pregnant women. This offers an excellent opportunity to study calorie restriction during pregnancy, even during specific windows of development as the famine only lasted around 3 months.

Retrospective studies have followed the offspring of those women exposed to famine during pregnancy for over half a century (Roseboom, de Rooij et al. 2006). Exposure to such deprivation in utero correlates with increased risk of T2DM, cardiovascular disease, and certain types of cancer in middle age. Interestingly, these studies have also lent insight into the different impact of famine exposure during the different trimesters of pregnancy (Roseboom, de Rooij et al. 2006). Exposure to famine very early in pregnancy, for example, increased risk of impaired glucose tolerance and stress response, as well as increased risk for heart disease and breast cancer later in life. In contrast, exposure to famine very late in gestation resulted only in increased risk for glucose intolerance half a century after birth. This lends support to the idea that there are key developmental windows during which the fetus will be impacted more or less by a given stressor.
In addition to the study of famine during pregnancy, more recent work has begun to investigate other conditions during pregnancy. For instance, the Pima Indian population is often studied for the fact that they have an extremely high incidence of T2DM (Franks, Looker et al. 2006). This offers a prime opportunity to study diabetes during pregnancy, and offspring consequences thereof. These studies have shown that exposure to a diabetic maternal milieu increases offspring risk for obesity and diabetes (Gautier, Wilson et al. 2001, Franks, Looker et al. 2006, Clausen, Mathiesen et al. 2008). It has even been observed that when a woman has gestational diabetes, the offspring is at an increased risk for cardiovascular disease (West, Crume et al. 2011, Marco, McCloskey et al. 2012).

1.4.3 **Maternal Obesity and Offspring Health**

We have previously discussed maternal obesity and the impact on pregnant women, labor, and delivery. In addition, lack of proper nourishment during pregnancy is known to predispose offspring to disease. In recent decades, however, more women of childbearing age are obese than ever before. Therefore, the number of neonates having been exposed to the milieu of obesity *in utero* is growing.

Maternal obesity predisposes offspring to a number of health concerns throughout their life (Tenenbaum-Gavish and Hod 2013). Obesity during pregnancy is positively correlated with infant macrosomia (Ehrenberg, Mercer et al. 2004). Some sources define macrosomia as birth weight of greater than 4.0 kg, while others raise it to 4.5 kg (Oral, Cagdas et al. 2001, Ehrenberg, Mercer et al. 2004). Regardless, macrosomia carries with it a number of concerns. For instance, macrosomic babies are more likely to suffer trauma
and/or need intervention during delivery (Oral, Cagdas et al. 2001, Weissmann-Brenner, Simchen et al. 2012). Their Apgar score, a measure of the health of a newborn using appearance, pulse, grimace, activity, and respiration, is likely to be lower as well (Charles R. B. Beckmann 2013). Infants born to obese women are also insulin resistant (Catalano, Presley et al. 2009). Extreme cases may even suffer hypoglycemia (Linder, Lahat et al. 2014).

During childhood and adolescence, offspring born to an obese mother are at risk as well. They are more likely to be obese, for instance (Whitaker, Wright et al. 1997). Childhood obesity has increased dramatically in the last 30 years and is a prevalent health issue in the United States (Ogden, Carroll et al. 2010, Ogden, Carroll et al. 2014). It has been observed that obesity specifically in the first trimester increases risk for obesity in the child (Whitaker 2004). Recent studies have shown that offspring born to obese women are also more likely to suffer attentive disorders, such as attention deficit hyperactivity disorder (Rodriguez 2010). Children born to obese women also have up to a 30% increased chance of experiencing asthma (Forno, Young et al. 2014). Clearly, there are several ways that maternal obesity may impact the offspring.

Adults who were born macrosomic are also predisposed to several health issues. For example, they are more likely to be obese (Oken and Gillman 2003). It is therefore easy to imagine the cycle of obesity – an obese woman bears a child who is more likely to be obese as an adult, who then becomes an obese pregnant woman, and so on. We know maternal obesity increases risk for gestational diabetes, and offspring born to diabetic mothers have an increased risk for developing T2DM (Dabelea 2007). Finally,
young adult offspring of women who were obese before pregnancy are more likely to have elevated blood pressure (Laor, Stevenson et al. 1997).

Rodent models are frequently used to explore the consequences of maternal obesity on the offspring. As previously mentioned, a high fat diet is often fed to the animals to induce an obese phenotype. Through these experiments, others have shown that offspring born to high fat-fed dams are at increased risk for impaired glucose tolerance and insulin resistance (Gniuli, Calcagno et al. 2008, Nivoit, Morens et al. 2009, Rother, Kuschewski et al. 2012). Maternal high fat diet also promotes an increase in formation of mammary tumors in rat offspring and leads to an increased risk of fatty liver disease in mouse offspring (Hilakivi-Clarke, Clarke et al. 1997, Gregorio, Souza-Mello et al. 2010). Similarly, consumption of a high fat diet during murine gestation results in altered bone structure and body composition in offspring (Lanham, Roberts et al. 2010, Krasnow, Nguyen et al. 2011).

1.4.4 Maternal Exercise and Offspring Health

As exercise during pregnancy had rather the opposite effect on the woman compared to obesity, the same holds true for the offspring. Maternal exercise appears to be a boon for the offspring’s long term health. While this is a fledgling area of research, there is evidence to suggest that exercise during gestation improves several outcomes in offspring.

Neonates born to women with higher levels of physical activity typically have an easier delivery (less need for vaginal operative assistance) and are able to depart the
hospital sooner (Morgan, Rahman et al. 2014). Clapp has also followed offspring born to exercised women until they are five years of age (Clapp 1996). They have higher scores on cognitive tests and remain leaner than their counterparts born to sedentary women. This is exciting evidence that demonstrates the potential for offspring advantages of maternal exercise in young children.

Currently, the field has minimal data regarding long-term human offspring outcomes of maternal exercise to offer. For such information, we must turn to rodent and other animal models. Maternal exercise improves aged offspring glucose tolerance in both mice and rats (Carter, Lewis et al. 2012, Carter, Qi et al. 2013). Swine have been used to demonstrate improved offspring vascular function as a result of maternal exercise (Bahls, Sheldon et al. 2014). Maternal exercise also improves offspring metabolic parameters rodent models and may protect offspring from the consequences of maternal high fat feeding (Laker, Lillard et al. 2014, Stanford, Lee et al. 2014). It has even been shown that maternal exercise improves outcomes in offspring of a transgenic mouse line that develops an Alzheimer’s-like condition (Herring, Donath et al. 2012). These animal findings suggest that the advantages of maternal exercise for aged human offspring may have potential to be dramatic, and further research is warranted.

In conclusion, the long-term human offspring effects of maternal exercise are currently understudied. Mice, rats, and other mammals demonstrate the apparent benefit of maternal exercise on offspring health long term. These benefits include improved
metabolic parameters, improving cognitive scores, and evidence of decreasing the incidence of neurologic disease.

1.5 Scope of Dissertation

1.5.1 Goals of Dissertation

Pregnancy is a vital developmental window, not only for the growing fetus, but also for pregnant women. Health behaviors during pregnancy may impact both the child for the duration of his or her life and that of the mother for the remainder of hers. For reasons discussed, animals are a useful model to explore the impact of health behaviors both short- and long-term. In light of this background information, this dissertation has three primary goals, outlined in Figure 1.2:

Aim 1. To elucidate the maternal and offspring effects of consumption of several different high fat diets by the pregnant ICR (CD1) mouse. Maternal obesity was achieved along with impaired glucose tolerance. Although the offspring did not have health consequences because of the maternal diet, several insights can be gleaned from the data. This chapter will include a discussion of the reasons that the offspring did not have impaired glucose tolerance, even when challenged by a high fat diet themselves.

Aim 2. To develop a novel model of exercise in the pregnant ICR mouse. This section will explore the safety of the model as a pregnancy intervention. In addition, offspring were observed for one year for differences in glucose tolerance. Subsequent studies within this section will utilize an increased speed for the maternal intervention, as
well as employing an additional group to elucidate the effect of the sedentary wheel exposure, if any.

Aim 3. To explore the combined effect of BCAA supplementation and exercise on body composition in female C57BL/6 mice with and without high fat diet. Exercised animals consuming the control diet did not have an increased lean to fat mass ratio compared to sedentary control diet animals, but exercised animals plus BCAA supplement did. This supplement regimen could be explored in the future as a pregnancy intervention.

1.5.2 Impact

It warrants little discussion that obesity is a prevalent and costly health concern in modern society. Within this dissertation we will explore one mouse model of high fat diet-induced obesity and the impact on the offspring long-term. In addition, exercise during pregnancy is a fledgling area of study. Therefore, we present a novel model of exercise during pregnancy for mice that is safe for both the dam and her offspring, and offers some advantages compared to other exercise models. We demonstrate the application of a BCAA supplement in female mice that improves body composition in exercising animals, both with and without the concurrent consumption of a high fat diet. This is important, as the dietary supplement industry in the United States is experiencing explosive growth, and women need large amounts of protein during pregnancy.
Figure 1.1 Diagram of insulin signaling pathway and associated factors.

Standard insulin signaling pathway is indicated by blue arrows. The negative impact of obesity on the pathway is indicated by red arrows. Factors that increase insulin release and/or sensitivity are indicated by green arrows. Insulin is released from the pancreatic β-cell postprandially (Prentki, Matschinsky et al. 2013). Insulin binds its receptor on the surface of the skeletal muscle cell. The intracellular domain is phosphorylated, which activates insulin receptor substrate 1 (IRS1). IRS1 activates phosphatidyl inositol 3 kinase (PI3K), which then activates Akt. Akt acts through Akt substrate, molecular weight 160 (AS160) to recruit vesicles containing glucose transporter type 4 (GLUT4) to the cell membrane, resulting in increased glucose uptake from the bloodstream. In lean individuals, adiponectin promotes insulin action by means of adenosine monophosphate kinase (AMPK) (Gil-Campos, Canete et al. 2004). Obesity impairs insulin signaling through several mechanisms, including increases in tumor necrosis factor α (TNFα), c-Jun n-terminal kinase (JNK), retinol binding protein 4 (RPB4), and decreases in adiponectin (Hotamisligil and Spiegelman 1994, Christou, Tselepis et al. 2012). Exercise promotes GLUT4 vesicle recruitment by increasing the adenosine monophosphate (AMP) present in the cell, activating AMPK (Richter and Hargreaves 2013). The branched-chain amino acid leucine acts as an insulin secretagogue in the β-cell (Yang, Chi et al. 2010).
This project focuses on female mice. The three legs of the project involve exploration of different high fat diets and the effect on offspring long term, the use of a novel model of exercise in the mouse, and consumption of a BCAA supplement on the body composition of the female mouse. The overarching goal of this work is to study the consequences of maternal actions during pregnancy, both on the pregnant female and on her offspring, and ultimately improve the health of future generations. Blue color indicates Aim 1 scope, hashed lines include Aim 2, and red color indicates Aim 3. Darker shading for “Diet” in the center includes both Aim 1 and 3.
CHAPTER 2 MATERNAL HIGH FAT DIET

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2.1 Introduction

Obesity is rampant in the United States with potential for annual economic ramifications that could surpass $209.7 billion (Cawley and Meyerhoefer 2012, Ogden, Carroll et al. 2012). No intervention has been identified that can effectively decrease obesity rates in a large proportion of the population, and an overweight/obesity prevalence approaching 90% has been predicted should trends continue (Wang, Beydoun et al. 2008). Among other complications, obesity increases the risk for developing type 2 diabetes (Kissebah and Peiris 1989, Balkau, Deanfield et al. 2007). Since up to half of women of childbearing age in the United States are overweight or obese (Vahratian 2009), countless future generations could suffer deleterious consequences because of in utero exposure to maternal obesity and diabetes.

Developmental programming, recognized for over 20 years, is the concept that the uterine environment to which a developing fetus is exposed can impact its response to stimuli throughout the duration of its postnatal life (Hales and Barker 1992, Barker, Gluckman et al. 1993). Retrospective studies have indicated that babies exposed to under-nutrition during gestation have increased incidence of diabetes, cardiovascular disease,
and certain cancers later in life (Roseboom, de Rooij et al. 2006). Conversely, in utero exposure to excess nutrients leaves offspring more susceptible to obesity, diabetes, and other complications (Whitaker, Wright et al. 1997, Tenenbaum-Gavish and Hod 2013). One can easily visualize the cycle of obesity that can be created.

Animal studies, particularly in rodents, are widely used to explore the offspring consequences of maternal high fat diet consumption, a common model for over-nutrition. Studies report a plethora of detrimental findings in mouse offspring as a consequence of maternal high fat feeding, including increased risk of fatty liver disease, bone malformation, glucose intolerance, and insulin resistance (Gniuli, Calcagno et al. 2008, Gregorio, Souza-Mello et al. 2010, Lanham, Roberts et al. 2010, Rother, Kuschewski et al. 2012). Maternal obesity and high fat diet consumption and their influence on offspring obesity outcomes are less clear. Some studies suggest an increase in rat offspring obesity (Buckley, Keseru et al. 2005, Bayol, Farrington et al. 2007, White, Purpera et al. 2009), while another indicates no effect (Khan, Dekou et al. 2004). Experiments in mice, in general, suggest an increase in offspring weight due to maternal high fat diet intake and/or obesity (Samuelsson, Matthews et al. 2008, Elahi, Cagampang et al. 2009, Ornellas, Mello et al. 2013). Several studies indicate that impaired glucose tolerance can be induced in offspring as a consequence of maternal high fat diet feeding in both rats (Taylor, McConnell et al. 2005, Nivoit, Morens et al. 2009, Ainge, Thompson et al. 2011) and mice (Samuelsson, Matthews et al. 2008, Masuyama and Hiramatsu 2012).
Many studies compare non-purified, cereal-based diet (commonly referred to as laboratory chow) fed control animals to purified high fat diet-fed animals (Srinivasan, Katewa et al. 2006, Dunn and Bale 2009, Hartil, Vuguin et al. 2009, Howie, Sloboda et al. 2009, Liang, Oest et al. 2009, Chechi, Herzberg et al. 2010, Giraudo, Della-Fera et al. 2010, Gout, Sarafian et al. 2010, Sun, Purcell et al. 2012). Cereal-based diets are those that are primarily sourced from natural ingredients such as grains and meals (1977, Reeves 1997, Heindel and vom Saal 2008). Cereal-based diets often have variable composition, therefore introducing nutrients and phytochemicals not represented in a purified high fat diet (Jensen and Ritskes-Hoitinga 2007). There is even notable variability between different batches of the same formula cereal-based diet (Mead 2006). Conversely, many purified diets, including the ones used in the current study, substitute sucrose in place of fat when formulating the control diet. High sucrose diets fed perinatally in mice may introduce offspring complications of their own (Samuelsson, Matthews et al. 2013). Therefore, a perfect diet may be difficult to find, particularly when interested in commercially available products.

For this study, we set out to find a readily available commercial high fat diet that could be fed to female ICR mice before and during pregnancy and nursing that would impair glucose tolerance and/or alter body composition in offspring when compared to animals fed a purified control diet with the same fat source as the high fat diet. We chose two lard-based high fat diets (45 and 60% by calories) and two butter-based high fat diets (32 and 60% by calories) from Research Diets, Inc. (New Brunswick, NJ). Control animals received either the lard- or butter-based diet containing 10-11% fat calories. We
hypothesized that male and female offspring born to high fat diet-fed dams would have impaired glucose tolerance and show increased body weight and obesity during adulthood. Male and female offspring from all dietary groups were followed for at least one year with minimal effects on glucose tolerance, body weight, and body composition.

2.2 Methods

2.2.1 General

The current study was carried out according to a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. Fourteen week old female ICR mice (n = 120) were purchased from the vendor (Taconic Farms, Germantown, NY) after they had delivered one litter at their facility. Animals were acclimated to controlled temperature (21-24°C), humidity, and light/dark cycle (14h/10h) for one week after arrival to the University of Kentucky and maintained under these conditions for the duration of the study. All cages contained standard chipped wood substrate (Sani-Chip, Harlan-Teklad, Madison, WI), a cotton Nestlet™ for bedding (Ancare, Bellmore, NY), and a plastic mouse igloo (Bio-Serv, Frenchtown, NJ). After acclimatization, the females were housed four per cage and separated into six groups approximately balanced by body weight (n = 20 per group). Animals received one of six commercially available purified diets (Research Diets, Inc., New Brunswick, NJ). The three lard based diets were D12450B (10% fat calories, 3.9 kcal/g), D12451 (45% fat calories, 4.7 kcal/g), and D12492 (60% fat calories, 5.2 kcal/g). The three butter based diets were D12489B (11% fat calories, 3.9 kcal/g), D12266B (32% fat calories, 4.4
kcal/g), and D02101801 (60% fat calories, 5.3 kcal/g). See Table 2.1 and Table 2.2 for detailed dietary information. There are some key differences in the diets. For instance, while the lard diet used consistent soybean oil contents for all three diets, the butter diets used widely variable amounts of corn oil instead. They also contained two different mineral mixes. Dam body weight and food consumption were recorded weekly for the duration of the study. Food consumption data was collected per cage and averaged based on the number of mice in that cage to obtain a daily intake value.

During the fourth week of diet treatment (days 24-25 of diet), animals were subjected to glucose tolerance testing and body composition analysis (EchoMRI™, Echo Systems, Inc., Houston, TX). After the fourth week of diet treatment, animals were bred to ICR males. For breeding purposes, two females were introduced into a cage containing one male to cohabitate continuously for six days and nights before removal of the male (thus the male also consumed the respective diets of the female mice during mating). One week after removal of the male, females were split into individual cages and remained single housed for the duration of pregnancy and suckling. Offspring were counted (Table 2.3) and culled to 8 pups per litter on postnatal day 2. Alternatively on day 2, pups in litters of less than 8 were cross-fostered after marking for identification in order to balance litters. Offspring weights were recorded on postnatal days 7, 14, and 21. All dams were switched to the control diet within the fat source group of their current diet (60% lard were switched to 10% lard; 32% butter were switched to 11% butter, etc.) at postnatal day 14. This was in an attempt to minimize the opportunity for the offspring to consume the high fat diet themselves. Offspring were implanted with microchip
transponders (BioMedic Data Systems, Inc., Seaford, DE) for identification between days 17-19 and weaned onto the control diet consumed by their dam on postnatal day 21. Offspring body weight was recorded biweekly for more than one year. Body weight data represents one randomly chosen offspring per sex per treatment group.

A separate cohort of male and female offspring born to lard fed dams was placed on the 60% lard diet when they were 4 months of age. They were fed this diet ad libitum until they reached the age of 10 months. At this time, they were subjected to glucose tolerance testing and body composition analysis.

2.2.2 Glucose Tolerance Testing

During the fourth week of diet treatment, female mice were fasted for three hours and blood glucose was recorded via tail nick using a standard glucose testing meter (Bayer Breeze2 Bayer HealthCare, LLC, Tarrytown, New York). Animals received a bolus of 2 g dextrose (Bimeda®, Oakbrook Terrace, IL) per kg body weight via oral gavage. Blood glucose readings were repeated from the same tail nick 15, 30, 60, and 120 minutes after glucose was administered. At 6 weeks and 3 months of age, offspring from all groups were fasted for three hours and subjected to the same glucose tolerance testing protocol, except dextrose was delivered by intraperitoneal injection. At 8, 10, and 12 months of age, offspring were subjected to glucose tolerance testing with dextrose administered via oral gavage, but otherwise following the same procedure as described. At 15 months of age, offspring were fasted overnight (18 hours) and underwent the intraperitoneal glucose tolerance testing procedure but were injected with 2.67 g dextrose
per kg body weight. Varied fasting times, doses, and alternate routes of delivery were used in order to elucidate any potential differences in glucose tolerance. Offspring glucose tolerance represents no more than one pup per litter per sex at each time point, though not necessarily all the same animals at every time point.

2.2.3 Body Composition Analysis

Dams were subjected to EchoMRI during the fourth week of dietary treatment. Briefly, animals were gently restrained in a plastic tube designed specifically for the EchoMRI. The tube was inserted into the EchoMRI and the animal was analyzed for fat and lean mass as well as free and total water content. A portion of the animal’s mass is unaccounted for (hair, bone, and connective tissue). The analysis for one mouse is complete in approximately two minutes. Offspring body composition was analyzed at 7 weeks, 3, 10, and 12 months of age following the same procedure. Offspring body composition represents no more than one pup per litter per sex at each time point, though not necessarily all the same animals at every time point.

2.2.4 Statistics

Data analysis was completed using version 9.3 of SAS (SAS Institute, Cary, NC) and version 11.0 of SigmaPlot (SigmaPlot Software, San Jose, CA). Fisher’s exact test was employed to analyze the number of litters born and weaned out of those bred (Table 1). The number of pups per litter was analyzed using one-way analysis of variance followed by Bonferroni-adjusted post-hoc test where applicable (Table 2.3). Dam lean to fat mass ratio and 15 month offspring area under the curve (AUC) was analyzed via one-
way analysis of variance with Bonferroni-adjusted post-hoc testing (Figure 2.1 E,F, and Figure 2.6). For outcomes assessed repeatedly over an experimental time-course (Figure 1.2 C,D) or age (Figures 2.1 A,B, 2.2, 2.3, and 2.7), linear mixed models were fit (via PROC MIXED in SAS) to relate outcome trajectories to the different doses of lard and butter in the diets, with random effects for individual mice (or litters for pup weights) to account for possible correlations among repeated assessments on the same mice (or litters). Bonferroni-adjusted post-hoc tests sought to identify pairwise differences between doses with respect to the change in the trajectory from baseline (Figure 2.1 A,B and Figure 2.3) or the AUC of the trajectory (Figures 2.1 C,D, 2.2, and 2.7). The glucose tolerance tests for offspring yielded doubly repeated measures data: the tests were conducted at multiple ages, and at each age there were multiple data points acquired over the two-hour time course. The data points at each age were summarized by an AUC score (via the "Area Below Curves" function in SigmaPlot), and then the AUC scores at various ages were treated as outcomes in linear mixed modeling. Bonferroni-adjusted post-hoc tests then sought to identify pairwise differences between doses with respect to the area under the curve of the trajectory (in effect, an AUC of an AUC; Figure 2.4). Statistical significance was defined by a p-value less than 0.05.

2.3 Results

2.3.1 Dams

There was a significant dose effect for change in dam body weight over time as analyzed by linear mixed model ANOVA for the lard and butter based high fat diet
groups (p < 0.001 and p = 0.016 for lard and butter, respectively). Dam body weight increased significantly as a consequence of consumption of either of the 60% fat diets compared to their respective 10-11% control diet (p < 0.001 for lard; p < 0.001 for butter by post hoc) (Figure 2.1 A,B). Sixty percent lard-fed animals were also heavier than 45% lard (p = 0.002 by post hoc), though neither the 45% lard nor 32% butter were heavier than their respective controls. Body weight differences were not as a consequence of differential calorie consumption as all animals consumed similar numbers of calories on average compared to their respective control diet group when analyzed by linear mixed model ANOVA for dose effect change over time (p = 0.551 for lard; p = 0.879 for butter) (Figure 2.2). Neither resting energy expenditure nor activity levels were measured, but changes in these characteristics could have led to increased body weight in the 60% fat fed female mice.

During the fourth week on the diets, glucose tolerance and body composition were measured in the female mice. The glucose tolerance test data were analyzed by linear mixed model ANOVA, and there were trends for dietary dose effects toward increased blood glucose in lard high fat diet fed females both at the initial time point and over time, although neither reached significance (p = 0.060 for fasting; p = 0.083 for change over time) (Figure 2.1 C). There was a significant dietary dose effect for an increase in glucose over time in the butter based high fat diet fed female mice (p < 0.001 by linear mixed model ANOVA). Following post hoc analysis, it was determined that the 32% and 60% butter based diet fed animals had impaired glucose disposal compared to the 11% fed controls (p = 0.011 for 32%; p = 0.002 for 60%) (Figure 2.1 D). Lard
animals AUC was not significantly affected by the diet treatment, but butter animals consuming the 60% diet had increased AUC compared to 11% controls (p = 0.006). Both lard high fat diets decreased lean to fat mass ratio in an incremental fashion in the female mice prior to mating when compared to their respective control group (p = 0.029 for 45% and p < 0.001 for 60% compared to 10%; p = 0.001 for 45% compared to 60%) (Figure 2.1 E). The same incremental decrease was present in the butter diet fed animals (p = 0.002 for 32% and p < 0.001 for 60% compared to 11%; p = 0.004 for 32% compared to 60%) (Figure 2.1 F).

Data concerning litters born, number of pups born per litter, and litters weaned are presented in Table 2.3. There were no significant decreases in number of litters born or weaned out of those bred in the high fat lard or butter diets compared to their respective control diets, but the 10-11% fat diets did have the highest number of litters born and weaned. There was a significant dose effect for number of pups per litter for lard diet fed animals (p = 0.044), but the difference did not reach significance upon post hoc analysis (p = 0.053 for 60% lard compared to 10% lard). The number of pups born per litter in the butter cohort was not significantly affected by the fat percentage in the diet (p = 0.565). It is likely that a larger number of breeding females would have established negative litter outcomes for the high fat diet-fed dams compared to the controls.

2.3.2 Offspring

Pre-weaning pup weights per litter were significantly affected by dose of high fat diet over time for female and male offspring born to lard based diet fed dams (p = 0.008
and \( p < 0.001 \) for male and female lard, respectively, by linear mixed model ANOVA). Pup weight was significantly increased before weaning for pups born to 45% lard based diet fed dams compared to 10% controls (\( p = 0.007 \) for lard male pups and \( p < 0.001 \) for lard female pups), but those from 45% lard fed dams did not significantly differ from pups born to 60% lard fed dams (Figure 2.3 A,B). Male pups born to 60% lard fed dams did not weigh significantly more than male offspring born to 10% lard fed dams (Figure 2.3 A). There was a strong trend toward increased weight in the female offspring born to 60% lard fed dams compared to female offspring born to control diet lard fed dams (\( p = 0.050 \)) (Figure 2.3 B). Male and female offspring born to butter based diet-fed dams had no change in mean pup weight per litter before weaning compared to the 11% controls (\( p = 0.109 \) and 0.263 for butter based males and females, respectively) (Figure 2.3 C,D). Body weight over the first year is shown in Figure 2.4. No differences were observed in male or female lard offspring by linear mixed model ANOVA (\( p = 0.821 \) for males; \( p = 0.260 \) for females) (Figure 2.4 A,B). Similarly, male butter offspring weights were not significantly different (\( p = 0.182 \)) (Figure 2.4 C). Female butter offspring born to dams that consumed 32% fat diet had increased body weight as they aged compared to offspring from 11% or 60% dams (\( p < 0.001 \) for both comparisons) (Figure 2.4 D) even though the body weights for this group did not differ significantly pre-weaning. The cause of this increased body weight in the adult female offspring is unknown but was not due to increased food consumption as average daily food intake was not significantly different in those females born to the 32% fat fed compared to control dams (Figure 2.5).
It could have resulted from a decrease in metabolic rate and/or activity in the offspring born to the 32% fat fed dams, but these parameters were not measured.

AUC was calculated for each of the glucose tolerance tests up to one year of age, and the overall differences were assessed. There were no significant differences in AUC for lard males ($p = 0.138$), lard females ($p = 0.876$), butter males ($p = 1.00$), or butter females ($p = 0.159$) compared to the respective control diet groups (Figure 2.6). In order to determine whether a short fasting time could have masked any differences, a glucose tolerance test was completed with a higher dose of glucose after an overnight fast in the offspring when they were 15 months of age (Figure 2.7). Area under the curve was not significantly different in any sex or dietary group at this advanced age as determined by one-way ANOVA. Similarly, lean to fat mass ratio was analyzed across all time points, and no significant effects were observed for lard males ($p = 0.904$), lard females ($p = 0.457$), butter males ($p = 0.249$), or butter females ($p = 0.833$) (Figure 2.8). Of note, the offspring glucose tolerance tests and body composition data represent no more than one pup per litter per sex at each time point, although not all of the same animals were included at each time point in Figures 2.6 and 2.8. Thus, these data do not represent trajectories of progressions for individual animals but rather estimates of mean-level trajectories of progression within populations of animals.

2.3.3 Offspring high fat challenge

Since the offspring fed control diet did not have differences in glucose tolerance or body composition as a consequence of maternal high fat diet, we attempted to stress
the offspring with high fat diet in an attempt to determine differences in glucose metabolism or adiposity. The cohort of offspring that was challenged with a high fat diet beginning at 4 months of age gained weight as a consequence of the diet (the graphs includes the line for the offspring of 10% lard fed animals that remained on the 10% lard diet for comparison). However, they did not gain different amounts of weight ($p = 0.585$ for males, $p = 0.326$ for females) as a consequence of maternal diet (Figure 2.9). Once they were 10 months old, they were subjected to glucose tolerance testing and EchoMRI. The high fat diet consumption did not indicate a difference in glucose tolerance ($p = 0.383$ for males, $p = 0.472$ for females) as a consequence of maternal high fat diet (Figure 2.10). Similarly, there was no significant difference in lean to fat mass ratio as a consequence of maternal diet in the male offspring challenged with high fat diet (Figure 2.11A). Interestingly, there was a significant decrease in lean to fat mass ratio as a consequence of maternal 45% lard diet in the female offspring challenged with this very high fat diet (Figure 2.11B).

### 2.4 Discussion

The current study found minimal effects in offspring body weight, glucose handling, and body composition as a consequence of maternal consumption of a variety of commercially available high fat diets. Although some differences in lard pup weight before weaning were apparent, these differences disappeared after weaning. After weaning, the female offspring born to 32% butter dams were the only animals to have a significantly increased weight, but this increased weight did not correspond to any significant change in glucose tolerance or body composition. We do not have an
explanation for the increased weight in this group since we did not monitor energy expenditure or activity levels. While the lack of detrimental effects was surprising, we propose a number of explanations. We will discuss several of these possibilities, including: the use of purified diet for the control groups, the use of the ICR stock of mice, the use of primiparous females, and the removal of the high fat diet at postnatal day 14.

Irrespective of rodent species or outcome of interest, it is common practice to use cereal-based diets as the control diet for high fat maternal feeding studies (Srinivasan, Katewa et al. 2006, Dunn and Bale 2009, Hartil, Vuguin et al. 2009, Howie, Sloboda et al. 2009, Liang, Oest et al. 2009, Chechi, Herzberg et al. 2010, Giraudo, Della-Fera et al. 2010, Gout, Sarafian et al. 2010, Sun, Purcell et al. 2012). Cereal-based diets have some characteristics that researchers should consider when using them. For example, they contain phytoestrogens from the plant material used in their production. Phytoestrogens have been shown to impact many body systems, including cardiovascular, reproductive, skeletal, and immune systems (Burton and Wells 2002, Douglas, Armitage et al. 2006, Masilamani, Wei et al. 2012, Chiang and Pan 2013). They have been studied in the context of various disease states, including obesity (Orgaard and Jensen 2008). The consequence of maternal/perinatal isoflavone exposure has been an area of active study (Hilakivi-Clarke, Cho et al. 1998, Klein, Wisniewski et al. 2002, Jefferson, Padilla-Banks et al. 2005, Souzeau, Belanger et al. 2005). Articles have been published warning of the potential impact that phytoestrogens may have on animal health in research studies (Jensen and Ritskes-Hoitinga 2007). While diet manufacturers minimize the phytoestrogen content of their cereal-based diets when possible, the only way to entirely
eliminate them is to use a purified diet. Future studies should directly compare cereal-based diet fed animals to fat matched purified diet fed animals to explore the impact, if any, on glucose tolerance and body composition. It is entirely possible that many of the notable offspring effects in the literature are due to cereal-based versus purified diet comparisons rather than dietary fat or lean versus obese dam comparisons.

In the current study, we chose to use purified, commercially available control diets (10% lard diet, 11% butter diet) as the controls for the high fat diets. One notable shortcoming of the control diet used in the current study is that the fat difference was made up by adjusting the sucrose concentration (among other adjustments) in the diet. High sucrose diets have been shown to cause an increase in pup weight in pre-weaning rats and mice (Sedova, Seda et al. 2007, Samuelsson, Matthews et al. 2013), as well as impairments in female mouse offspring glucose tolerance (Samuelsson, Matthews et al. 2013). The lard based 10% diet contains around 35% sucrose by calories, while the 45% and 60% fat diets contain around 17% and 7% sucrose, respectively. The 11% and 32% butter based diets both have approximately 25% sucrose, where the 60% butter diet offers only 9% sucrose. It is interesting that the two groups with comparable sucrose content (the 11% and 32% butter animals) are the groups that exhibited significant differences in female offspring body weight. Regardless, the variation of sucrose in the diets used in the current study is notable and could have masked any offspring differences. The manufacturer currently offers formulations for the 10% fat diet (product numbers D12450H and D12450J) that are sucrose matched to the 45% and 60% lard diets, and they also offer a control diet that eliminates sucrose and features corn starch instead
Future studies should use sucrose-matched diets or custom designed, content-matched diets instead of commercially available ones.

The ICR (CD1) mouse is a large, outbred stock of albino animals. We have previously described some of the breeding differences between ICR and C57BL/6 mice (Platt, Charnigo et al. 2013). The ICR animals are better breeders that deliver large litters (~11 pups per litter) and provide excellent maternal care. These are among the reasons that we use this strain frequently, and the large litter size allows for culling litters to control for postnatal food availability. However, some differences have been documented between the ICR stock compared to other strains regarding glucose tolerance, lung function, and drug response (Weizman, Paz et al. 1999, Schlenker, Shi et al. 2006, Shimizu, Sakazaki et al. 2012). Future studies should consider the impact that mouse strain/stock may have on various outcomes of interest.

The current study used ICR females that had previously delivered one litter at the vendor prior to delivery to the University of Kentucky. This choice was made because we would anticipate that experienced breeding females would viably reproduce. Further, one would expect that dams delivering their second litter would provide superior care and nutrition to their pups (Miller, Harrison et al. 2007). However, studies have shown deleterious effects of multiple pregnancies, both on the dam and on the offspring born to that dam (Rebholz, Jones et al. 2012). It is therefore possible that the controls and high fat diet-fed female mice were already heavier than virgin females would have been, thus limiting detectable effects in the offspring. Future studies should be aware of the parity of
the animals in use. While this is commonplace in developmental programming studies, other fields do not always account for the number of times a mouse has birthed litters.

It is known that maternal high fat diets can have differential effects on offspring depending on the timing of exposure (Sun, Purcell et al. 2012). Many high fat diet and developmental programming studies leave the dams on the experimental diet throughout nursing until the pups are weaned (Samuelsson, Matthews et al. 2008, Elahi, Cagampang et al. 2009, Masuyama and Hiramatsu 2012, Ornellas, Mello et al. 2013). In our study, we chose to remove the high fat diet from the dams when the pups reached 14 days of age. Our intent was to minimize the offspring opportunity to consume the high fat diet directly, as mouse pups begin nibbling solid foods before weaning. Future studies should investigate outcomes based on the time at which high fat diet feeding to the dams is terminated during the early postnatal period.

In summary, the lack of effects that we have observed in the offspring in the current study is important. While butter high fat diet-fed dams had impaired glucose tolerance, and all high fat diet-fed dams exhibited decreased lean to fat mass ratio when compared to their respective control group, the male and female offspring in the current study had no significant impairments in long-term glucose tolerance and no significant differences in body composition. Challenging the lard offspring with a long term high fat feeding did not elucidate a difference in glucose tolerance regardless of the fact that their dams consumed high fat diet. This is interesting because other studies have reported an increase in impaired glucose tolerance in offspring as a consequence of maternal high fat
diet (Tamashiro, Terrillion et al. 2009). While we did not see differences in lean to fat mass ratio in male offspring challenged with high fat diet, the females whose dams consumed 45% fat lard diet did exhibit a decrease in lean to fat mass ratio when challenged with a high fat diet. This suggests that the maternal 45% high fat lard diet did increase the propensity for the female offspring to develop obesity. It would be potentially useful to feed a cohort of the butter offspring a high fat diet in an attempt to decipher any potential differences in glucose tolerance or body composition in that group, particularly since the 32% butter female offspring were already exhibiting increased weight as a consequence of maternal diet.

In conclusion, future studies should take into account the variables discussed herein that may have contributed to the results of the current study. Additional considerations, including the age of the breeders and the duration of dietary treatment before mating could also play a role in the lack of offspring effects. Female mice in our study were fed the high fat diets for a relatively short period prior to mating (4 weeks) and then throughout pregnancy and the majority of nursing. A longer pre-feeding prior to mating could possibly contribute to detrimental effects in the offspring that we did not observe as part of our study. In addition, the same maternal study design could be used with more sensitive measures of glucose handling in the offspring (such as hyperinsulinemic euglycemic clamp). This study highlights important questions that should be posed during the design of future developmental programming studies in mice and other models, and researchers should be cognizant of these potential issues as we move forward.
Figure 2.1 Maternal Characteristics

Body weight (A,B) for dams that weaned litters is shown. Weight was increased in both 60% lard and 60% butter high fat diet-fed animals compared to their respective control group (n = 11 – 17 for lard, n = 10 – 16 for butter). Glucose tolerance was not significantly impaired in lard animals (C) the week before mating, but glucose disposal was impaired in both 32% and 60% butter animals (D) (n = 16 per group). Lean to fat mass ratio was decreased in an incremental fashion before mating for both 45% and 60% lard (E) and 32% and 60% butter females (F) (n = 20 per group). Groups not sharing a common letter (‘a’, ‘b’, or ‘c’) in the legend of the graph or on the graph itself are significantly different. Error bars indicate S.E.M.
Figure 2.2 Maternal Food Consumption

Animals were fed a known amount of food and food remaining was weighed, subtracted from the starting value, and divided by the number of days to obtain a daily food intake value. This number, in grams, was multiplied by the calories per gram of food to obtain a daily calorie intake. Neither lard (A) nor butter (B) animals consumed different amounts of food in calories. Error bars indicate S.E.M., n = 20 per group.
Figure 2.3 Offspring Weight before Weaning

Pups were weighed 7, 14, and 21 days after birth, and the average pup weight by sex was calculated for each litter. Weights were increased for male and female offspring born to 45% lard fed dams before weaning compared to controls (A,B). Weight was not significantly changed for male or female butter offspring before weaning (C,D). n = 10-17 lard litters per group and n = 10-16 butter litters per group. Groups not sharing a common letter (‘a’ or ‘b’) in the legend of the graph are significantly different. Error bars indicate S.E.M.
Male and female offspring were weighed biweekly and results from the first year are shown. There was no significant effect on lard offspring weight as a consequence of maternal diet. Male butter offspring weight was not significantly affected \((C)\), but maternal 32% butter diet increased female offspring weight \((D)\). \(n = 9-15\) for male lard \((A)\), 11-17 for female lard \((B)\), 10-17 for male butter \((C)\), and 9-14 for female butter \((D)\). Groups not sharing a common letter (‘a’ or ‘b’) in the legend of the graph are significantly different. Error bars indicate S.E.M.
Figure 2.5 Female butter offspring food intake

Offspring food consumption was monitored throughout the course of the study. Although there was a difference in body weight, there was not a significant difference in food intake between the three female butter offspring groups. Error bars indicate S.E.M. n = 2-6 cages of animals per group.
Glucose tolerance in the mice was measured at multiple time points and is summarized in the graphs by showing AUC at each age. Values to the left of the vertical line (6 weeks, 3 months) were obtained after an intraperitoneal glucose tolerance test, while those on the right of the line (8, 10, and 12 months) were obtained from an oral glucose tolerance test. Neither maternal lard (A,B) nor butter (C,D) high fat diet significantly altered offspring glucose tolerance AUC over time. n = 6-10 per sex per group at each time point. The AUC at each age was calculated using all of the data points acquired at that age over the two-hour time course of the glucose tolerance test administered at that age. Error bars indicate S.E.M.
Offspring were subjected to an intraperitoneal glucose tolerance test at 15 months of age. Animals received a dose of dextrose at 2.67 g/kg body weight after an 18 h overnight fast. AUC was calculated to summarize the two-hour time-course for the blood glucose trajectory. n = 5-10 per sex per group. Neither lard (A,B), nor butter (C,D) AUC was significantly altered by maternal high fat diet. Error bars indicate S.E.M.
Figure 2.8 Offspring lean to fat mass ratio

EchoMRI body composition analysis was completed at 7 weeks, 3, 10, and 12 months of age. There was no significant impact on lard (A,B) or butter (C,D) offspring lean to fat mass ratio for the duration of the study. n = 7-17 per sex per group at each time point. Error bars indicate S.E.M.
Figure 2.9 Offspring body weight when challenged with a 60% lard diet

Offspring born to lard fed dams were maintained on 10% lard diet until four months of age. They were then switched to the 60% lard high fat diet until they reached 10 months of age. Body weight is presented here for males (A) and females (B). Note that the legend indicates maternal diet, and all of these animals were consuming 60% lard diet. The dotted line, labeled “offspring consuming 10% lard,” indicates the body weight of the offspring born to 10% dams that remained on the 10% lard diet. This was included for purposes of visual comparison. Error bars indicate S.E.M. n = 8-13 per group for males, 11-16 per group for females.
Offspring were fasted for 3 hours prior to an oral glucose challenge after being fed high fat diet for 6 months. There was no difference in glucose tolerance area under the curve as a consequence of maternal diet in males (A) or females (B) at 10 months of age. Bars labeled “10% lard on 10% diet”, indicated by bars hashed with diagonal lines, are the offspring born to 10% lard fed dams that remained on the 10% lard diet for the duration of the study, shown for comparison purposes. Error bars indicate S.E.M. n = 6 - 10 per group.
Figure 2.11 Lard offspring lean to fat mass ratio when challenged with high fat diet.

Offspring that had been challenged with high fat diet feeding were subjected to EchoMRI for body composition analysis. There was no difference in lean to fat mass ratio in male offspring at 10 months of age (A). Female offspring whose dams consumed 45% lard diet had significantly decreased lean to fat mass ratio when challenged with high fat diet compared to those offspring born to dams consuming 10% lard diet (B). Bars labeled “10% lard on 10% diet,” indicated by diagonal lines, represent the lean to fat mass ratio of the offspring born to 10% lard fed dams that remained on the 10% lard diet for the duration of the study. This group is shown for visual comparison purposes only. Error bars indicate S.E.M. $n = 9-15$ per group.
Table 2.1 | Detailed dietary information for lard diets

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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Potassium Citrate (H₂O)</td>
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<td>17</td>
</tr>
<tr>
<td>Vitamin Mix (V10001)</td>
<td>10</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>FD&amp;C Yellow Dye #5</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td>FD&amp;C Blue Dye #1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Total | 1055 | 4057 | 858  | 4057 | 774  | 4057 |

* indicates ingredient in butter but not lard diets
10%, Research Diets D12450B
45%, Research Diets D12451
60%, Research Diets D12492
All values were obtained from Research Diets product data sheets and rounded to the nearest whole number where possible.
Table 2.2 | Detailed dietary information for butter diets

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<thead>
<tr>
<th>Ingredient</th>
<th>11%</th>
<th>32%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g%</td>
<td>kcal%</td>
<td>g%</td>
</tr>
<tr>
<td>Protein</td>
<td>16</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>71</td>
<td>73</td>
<td>57</td>
</tr>
<tr>
<td>Fat</td>
<td>5</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td><strong>kcal/g</strong></td>
<td></td>
<td>3.9</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>11%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>32%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>60%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ingredient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>kcal</strong></td>
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<td></td>
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</tr>
<tr>
<td><strong>g</strong></td>
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<td></td>
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</tr>
<tr>
<td><strong>kcal</strong></td>
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<tr>
<td>Casein (80 mesh)</td>
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<td>760</td>
<td>190</td>
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<tr>
<td>L-Cystine*</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
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<tr>
<td>Corn Starch</td>
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<tr>
<td>Maltodextrin 10</td>
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<tr>
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</tr>
<tr>
<td>Corn Oil</td>
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<td>354</td>
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</tr>
<tr>
<td>Lard*</td>
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<td>Vitamin Mix (V10001)</td>
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<td>44</td>
<td>11</td>
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<tr>
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<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>FD&amp;C Red Dye #40</td>
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<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>FD&amp;C Blue Dye #1</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td>1178</td>
<td>4596</td>
<td>1043</td>
</tr>
</tbody>
</table>

* indicates ingredient in lard but not butter diets

11%. Research Diets D12489B
32%. Research Diets D12266B
60%. Research Diets D02101801

All values were obtained from Research Diets product data sheets and rounded to the nearest whole number where possible.
Table 2.3 | Number of litters born, average pups per litter, and litters weaned out of those bred

<table>
<thead>
<tr>
<th>Group</th>
<th>Females Bred</th>
<th>Litters Born</th>
<th>Pups/Litter (mean ± SEM)*</th>
<th>Litters Weaned</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Lard</td>
<td>20</td>
<td>18</td>
<td>10.9 ± 0.6</td>
<td>17</td>
</tr>
<tr>
<td>45% Lard</td>
<td>20</td>
<td>15</td>
<td>10.6 ± 0.6</td>
<td>15</td>
</tr>
<tr>
<td>60% Lard</td>
<td>20</td>
<td>14</td>
<td>8.6 ± 0.9</td>
<td>11</td>
</tr>
<tr>
<td>11% Butter</td>
<td>20</td>
<td>17</td>
<td>10.1 ± 0.4</td>
<td>16</td>
</tr>
<tr>
<td>32% Butter</td>
<td>20</td>
<td>12</td>
<td>9.4 ± 0.6</td>
<td>10</td>
</tr>
<tr>
<td>60% Butter</td>
<td>20</td>
<td>12</td>
<td>9.8 ± 0.4</td>
<td>12</td>
</tr>
</tbody>
</table>

* One-way analysis of variance revealed a significant dose effect regarding pups per litter within the lard fed animals, but this difference was not significant upon post-hoc analysis.
3.1 Introduction

Pregnancy is a vitally important time, not only for pregnant women but also for the developing offspring. It is well accepted that women should make certain lifestyle changes in order to maximize the chances for a successful pregnancy and healthy baby. For example, alcohol consumption during pregnancy is well known to have negative consequences for the fetus (Bailey and Sokol 2008) and a large majority of women eliminate its use throughout gestation. Reports indicate that 51.5% of non-pregnant women consume alcohol compared with 7.6% of pregnant women in the United States (2012). Data also indicate that only 12% of pregnant women in the United States smoke tobacco, compared to 23-25% of non-pregnant women (Cnattingius 2004). Taken together, these observations may suggest that many women are willing to forego pleasurable or addictive behaviors in the hopes of having a successful and healthy pregnancy. There are also other, more mundane things that women in the general population should avoid during pregnancy, such as cat litter and cold deli meats (Kapperud, Jenum et al. 1996, Delgado 2008). It seems clear that women will often eliminate behaviors from their lifestyle in order to maximize their health during gestation.

Further, women will often add behaviors to their daily routine with the goal of improving their own health during pregnancy, as well as the health of their growing baby.
For example, women hoping to conceive are more likely to consume a folic acid supplement (Chuang, Hillemeier et al. 2011). In one study in the United Kingdom looking at smoking and alcohol cessation, caffeine limitation and fruit/vegetable consumption, 81% of pregnant women were willing to comply with health recommendations (Crozier, Robinson et al. 2009). These factors seem to suggest that women are willing to cease or adopt behaviors during pregnancy that will improve both her health and that of her unborn child. We therefore posit that women may be willing to initiate an exercise routine during gestation if there are clear benefits to doing so.

Exercise has been reported to improve mood, body composition, and glucose tolerance as well as decrease cancer incidence (Friedenreich and Orenstein 2002, Annesi 2005, Weiss and Holloszy 2007). Exercise during pregnancy has been shown to offer an array of positive outcomes for the pregnant woman, including decreased maternal weight gain and decreased body fat in the second half of gestation (Clapp and Little 1995). Exercise during pregnancy also improved oral glucose tolerance and reduced gestational diabetes risk (Oken, Ning et al. 2006, Barakat, Cordero et al. 2011). Maternal exercise is becoming a highly studied area, and recently the focus has turned to potential beneficial effects on offspring outcomes. For example, maternal exercise resulted in lighter, leaner human offspring, (Clapp and Capeless 1990, Hopkins, Baldi et al. 2010) and maternal exercise enhanced oral and cognitive skills in 5 year old offspring (Clapp 1996). There are clearly great potential benefits of exercise during pregnancy for both the pregnant woman and her baby. At current, this area is still one of growing interest, and data
regarding the long-term human outcomes as a consequence of maternal exercise are not available.

Rodents, however, are a model through which the long-term offspring benefits of exercise during pregnancy can be explored. Using mouse and rat models it has been shown that voluntary exercise during pregnancy and nursing improved glucose tolerance and insulin sensitivity in adult offspring (Carter, Lewis et al. 2012, Carter, Qi et al. 2013). Others have shown that voluntary running during mouse pregnancy increased offspring neural development (Bick-Sander, Steiner et al. 2006). Additionally, voluntary exercise during mouse pregnancy protects transgenic offspring from an Alzheimer’s type pathology (Herring, Donath et al. 2012). Clearly, exercise during pregnancy has exciting potential offspring benefits and this is an area of active research.

It is important to be aware, however, that voluntary exercise as a model has some limitations, such as long and variable running distances. For example, one mouse may run 14 kilometers a day, while another may average only 8 kilometers daily. Some mice run fast, others slow. Therefore, it would be ideal to develop a novel model of maternal exercise to control for the limitations of voluntary running that still imparts maternal and offspring benefits. Herein, a controlled exercise model in which the mouse is removed from her home cage and allowed to exercise for a known speed and duration is utilized. Admittedly, alternative models could have been used, such as swimming or treadmill running. However, swimming models leave the animal wet afterward, and then she would be expending energy to warm, dry, and groom herself, in addition to being, arguably,
stressed. Treadmill running models often rely on an electric shock to keep the animal running, which could in theory impart excess stress on the pregnant female. The controlled exercise model does not rely on a negative stimulus to keep the animal moving.

The aim of study 1 in this chapter was to employ the paradigm of controlled exercise in order to assess the safety of the model during mouse pregnancy as an alternative to voluntary wheel running. We also explored the differences between the ICR and C57BL/6 strains as they related to pregnancy outcomes. In study 2, we examined the impact of the controlled exercise intervention on pregnancy glucose tolerance and body composition in the ICR mouse, as well as the effects of the exposure to the sedentary wheel compared to a group that remained in their home cages. In study 3, we utilized the controlled exercise strategy for investigating offspring health implications of this exercise intervention.

3.2 Methods

3.2.1 Study 1: Controlled Exercise is a Safe Intervention in Pregnant Mice

3.2.1.1 General Husbandry

All animal experiments were carried out according to an approved Institutional Animal Care and Use Committee protocol at the University of Kentucky. The University of Kentucky Division of Laboratory Animal Resources is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Female ICR (CD1) and C57BL/6 mice were bred and produced one litter at
the vendor (Taconic Farms, Inc., Germantown, NY) prior to shipment to the University of Kentucky. Upon arrival, the 3-4 month old primiparous (having delivered pups one time) females were single housed in individually ventilated cages (ACE, Allentown, NJ) with Sani-Chip bedding (Harlan-Teklad) and maintained on a standard chow diet (Teklad Global 18% Protein Rodent Diet #2018) for the duration of the study. Plastic shelters (Mouse Igloos, Bio-Serv, Frenchtown, NJ) and nesting pads (Nestlets, Ancare, Bellmore, NY) were provided for environmental enrichment. All animals were maintained on a 14:10 hour light:dark cycle at temperatures between 21-24°C. Quarterly testing was completed on sentinel mice from related racks. Sentinels were found to be negative for mouse hepatitis virus, mouse parvoviruses, Sendai virus, Mycoplasma pulmonis, Theiler's murine encephalomyelitis virus, epizootic diarrhea of infant mice, pneumonia virus of mice, reovirus, lymphocytic choriomeningitis virus, Ectromelia, mouse adenovirus 1&2, polyomavirus, Encephalitozoon cuniculi, cilia-associated respiratory bacillus, Clostridium piliforme, mouse cytomegalovirus, fur mites and pinworms.

3.2.1.2 Study Design

After a one-week acclimatization period, females of each strain were assigned to control and exercise groups (ICR control, n = 20; ICR exercise, n = 20; C57BL/6 control, n = 20; C57BL/6 exercise, n = 19) so that the cohorts were approximately balanced by body weight. Once per day, five consecutive days per week, females were removed from their home cage and placed into a wheel positioned upon a motorized platform for 60 min (Mouse Forced Exercise/Walking Wheel System 80800A, Lafayette Instrument,
Lafayette, IN). Photographs are provided in Figure 3.1 A and B. The wheel bed of the control (sedentary) group was not activated and remained completely stationary. Exposure to the wheel beds occurred during the light cycle, between approximately 1 and 3 hours after lights-on. After a five day initial training period (3 m/min for 30 min and 3.5 m/min for 30 min on day one; 4 m/min for 30 min and 4.5 m/min for 30 min on day two; 5 m/min for 30 min and 5.5 m/min for 30 min on day three, 6.0 m/min for 60 min on days 4 and 5) the wheel bed of the exercise group was activated at a speed of 6 m/min for the duration of the study. Dams were not removed from their home cages on the day of or the day after parturition (delivery) in an attempt to maximize successful rearing. Dam body weight was recorded twice weekly (once after five days of wheel exposure and once after two days without wheel exposure). Animals were fed a known amount of food at the beginning of the study. Food remaining was recorded, discarded, and replaced with a fresh, known amount of food on a weekly basis. Weekly intake was divided by 7 to achieve a daily intake value. Daily food intake was divided by 2 while the male mouse was in the cage for mating. Exercise continued throughout pregnancy and nursing.

3.2.1.3 Breeding

After two weeks of controlled exercise, including the five day training period, females were introduced to males for breeding. One male was housed with one female for two weeks. At no point during the study did the males exercise. ICR mice that did not deliver litters within the first 4 days were removed from analyses in Figure 3.2 A and C due to their postponed body weight gain relative to the rest of the cohort. The delay was
most likely caused by a delay in mating and/or conception but we cannot state this with certainty because copulatory plugs were not monitored during the study. The day that pups were found was designated postnatal day 0. On postnatal day 2, litters were standardized to 8 and 6 pups for ICR and C57BL/6 mice, respectively. The C57BL/6 litters were culled to 6 because of the smaller litter size compared to the ICR mice. Pups were cross-fostered from other litters of the same group if they did not have at least 8 or 6 pups for ICR and C57BL/6 dams, respectively. Pups were weighed on postnatal day 7, 14, and 21. Food intake, body weight, and litter weight data were not included in the analyses for females that did not successfully wean their litters. One exercise group ICR litter was not weighed on postnatal day 21, but the data were included for days 7 and 14. In addition, one C57BL/6 dam refused to walk in the controlled exercise wheel after delivery so her body weight, food intake, and litter weight were not included in Figure 3.2 and Figure 3.4. She did participate prior to delivery so her litter data were included in Figure 3.3 B and D.

### 3.2.1.4 Statistics

Analyses were completed using version 9.3 of SAS, version 11.0 of SigmaPlot, and/or IBM SPSS 22, and figures were made using version 11.0 of SigmaPlot. Within strain strata (ICR or C57BL/6), control and exercise groups were compared on proportions of litters born and weaned using Fisher's exact test (Table 3.1), on repeated measures of body weight and food intake using linear mixed models with time and group as categorical predictors (Figure 3.2), on day of birth and pups per litter using a Mann-
Whitney rank sum test (Figure 3.3), and on repeated measures of pup weight using linear mixed models with time and group as categorical predictors (Figure 3.4). Also, strains were compared on proportions of litters born and weaned using Fisher's exact test in study 1 (Table 3.1). Significant overall differences in linear mixed modeling were followed by Bonferroni-adjusted post-hoc tests to compare groups at specific time points, but no significant overall differences were observed. One-way ANOVA was used for non-repeated measures comparisons of 3 groups. A p value of < 0.05 was considered significant.

3.2.2 Study 2: Impact of Controlled Exercise on Pregnant Female Mice

3.2.2.1 General Husbandry

For general animal husbandry, see section 3.2.1.1. Note that this study utilized only primiparous ICR animals and no C57BL/6 animals. Animals were fed LabDiet #5008 (LabDiet, St. Louis, MO).

3.2.2.2 Study Design

Three cohorts of female ICR animals were established (n = 20 per group) such that body weight was approximately balanced between groups (p = 0.996). Once per day, 7 days per week, females were removed from their home cage and placed into the controlled exercise wheel system (Figure 3.1). The wheel was activated on day 1 at a slow speed (4.5 m/min) for one hour. Each day the speed was incrementally increased (5.5, 7.5, 8, 9, and 9.5 m/min) such that maximum speed (10 m/min) was achieved on day 7. Every subsequent day, the wheel was activated at 10 m/min (referred to as ‘exercise’
A second group of females was removed from their home cage daily and placed into the wheel, but the platform was not activated (referred to as ‘sedentary wheel’ group). A third group was taken to the room where the exercise wheels were housed, but they were not removed from their cage (referred to as ‘sedentary home cage’ group). Animals were weighed daily except on the day of parturition, when they were not disturbed.

On day 23 of the study, all bedding was collected and frozen. This was a 72 hour collection. Later, the feces were removed from the bedding and underwent extraction for corticosterone analysis. Fecal pellets that had been separated from the bedding (72 hour collection) were subjected to a corticosterone extraction protocol. Feces were placed into a 15 mL conical tube with the cap loosely affixed so that they may equilibrate to ambient temperature and humidity for 3 days. 1 mL of 80% methanol was added per 50 mg of fecal material. The mixture was mechanically homogenized for 2 minutes per sample, then vortexed for 30 minutes before centrifugation (10 minutes, 12,000 x g, 22°C). Feces were stored at -80°C until they were shipped and analyzed for corticosterone via radioimmunoassay (MP Biomedical, Santa Ana, CA) by a core facility at the University of Alabama Birmingham after appropriate dilution (50 uL extract to 500 uL diluent).

On day 26 of the study (after one training week and three weeks at 10 m/min), female cages received a handful of bedding from male cages to stimulate the estrous cycle in the females. Females were visually evaluated on subsequent days and mated when proestrous was suspected between study day 28-30. For mating, one female was
placed into a cage housing one male and remained together for one night. The male never
exercised or underwent any wheel exposure at any point during the study.

3.2.2.3 Glucose Tolerance Testing

On gestation day 14, or the equivalent study day for non-pregnant females, animals were removed from their home cage and placed into a clean, dry cage with no food. After 3 hours, the tail was nicked with a sharp blade and fasting blood glucose was recorded as time zero using an Ascencia Breeze2 Glucometer (Bayer HealthCare, Tarrytown, NY). Subsequently, the animal was scruffed and the bolus of glucose (2 g/kg body weight) was delivered by oral gavage, a timer was activated, and blood glucose was recorded 15, 30, 60, and 120 minutes after delivery using blood from the same tail nick. The next animal was begun immediately after injection of the previous. This allows for testing up to ~20 animals per session. Following the testing, the animal was returned to its home cage.

3.2.2.4 EchoMRI

On gestation day 16, or equivalent study day, body composition was analyzed by use of EchoMRI™ (Echo Systems, Inc., Houston, TX). This system is designed to analyze fat mass, lean mass, and water content of a conscious animal in only ~2 minutes. Some parts of the animal are unaccounted for (bone, fur, nails). The animal is placed into a long cylindrical tube with a plunger that is gently compressed to restrain the animal. The tube is inserted into the machine which then provides a readout of the animal’s body composition. After the testing, the animal was immediately returned to its home cage.
3.2.2.5 Offspring

When pups were delivered, no action was taken on day 0. Dams were not exercised or removed from home cage on day of delivery, or on postnatal day 1. Exercised resumed on postnatal day 2 and continued until the end of the study. On postnatal day 2, pups were counted, weighed, and culled to 8 per litter. For litters of less than 8, pups were taken from other litters and fostered into the litter to bring it up to 8. The study was terminated on postnatal day 7.

3.2.2.6 Statistics

See section 3.2.1.4.

3.2.3 Study 3: Long Term Offspring Consequence of Maternal Controlled Exercise

3.2.3.1 General Husbandry

For general husbandry, see section 3.2.1.1. This study used LabDiet #5008 for the maternal diet and ICR females that had previously delivered one litter at the vendor facility.

3.2.3.2 Study Design

Female ICR animals were separated into two cohorts (n = 20 per group) such that body weight was approximately balanced between groups (p = 0.984). Females were removed from their home cage daily and placed into the controlled exercise wheel system (Figure 3.1). The wheel was activated on day 1 at a slow speed (4.5 m/min) and the speed
was incrementally increased each subsequent day (5.5, 7.5, 8, 9, and 9.5 m/min) such that the experimental speed of 10 m/min was achieved on day 7. Every following day, seven days per week, the wheel was activated at this experimental speed (referred to as ‘exercise’ group) with the exception of one day of rest after mating concluded. A second group of females was removed from their home cage on a daily basis and placed into the wheel, but the motor was not activated (referred to as ‘sedentary’ group). Before mating, females were subjected to glucose tolerance testing after a 3 hour fast period as well as EchoMRI for body composition analysis. Animals were weighed daily and mated after four weeks of intervention (one training week plus three weeks at 10 m/min) after exposure to male bedding to stimulate the estrous cycle. Males remained housed with females for 7 nights, and the subsequent day no exercise or wheel exposure occurred (no animals were removed from their cages or exposed to the wheel this day). On the day that females gave birth, the dams with pups were not removed from their cage or disturbed in any way and the wheels were only activated at 6 m/min for the 1 hour duration in order to minimize stress on the remaining pregnant females. Normal exercise protocol of 10 m/min resumed on postnatal day 2 and continued for the duration of the study.

3.2.3.3 Offspring

Pups were weighed and culled to 8 per litter on postnatal day 2. If litters were born with less than 8 pups, then pups were removed with litters of more than 8 (within the same treatment group) and fostered into the litter with fewer pups. Offspring were also weighed on postnatal day 7, 14, and 21, on which day they were weaned. Weaned
offspring were given *ad libitum* access to a low fat purified rodent diet (Research Diets D12450B, 10% fat calories, 3.9 kcal/g).

### 3.2.3.4 Glucose Tolerance Testing

Offspring were aged to 3 months and then subjected to glucose tolerance testing. Animals were fasted in a clean cage with no access to food. After 3 hours fasting, a sharp blade was used to nick the tail and blood glucose was recorded as time zero using an Ascencia Breeze2 Glucometer (Bayer HealthCare, Tarrytown, NY). Subsequently, the animal received the bolus of glucose (2 grams per kilogram body weight, oral dosing), a timer was activated, and blood glucose was recorded 15, 30, 60, and 120 minutes after glucose delivery. Glucose tolerance testing was repeated at 6, 8, and 18 months of age.

### 3.2.3.5 Statistics

See section 3.2.1.4.

### 3.3 Results

#### 3.3.1 *Study 1: Controlled Exercise is a Safe Intervention in Pregnant Mice*

Body weights of ICR and C57BL/6 females remained steady over the two weeks prior to mating (weeks 0-2 in the graphs) and were not significantly affected by the exercise regimen (Figure 3.2 A, B). There was no significant difference between body weights of sedentary and exercise ICR (p = 0.48) or sedentary or exercise C57BL/6 (f = 0.11) females. The body weights began to increase steadily around gestation day 10 (weeks 3.5-4 in Figure 3.2 A, B) and continued to rise as pregnancy progressed. Food
consumption followed a similar pattern, though the dams did not begin to eat substantially more until nursing (weeks 6-8, Figure 3.2 C, D). The exercise intervention did not significantly affect ICR (p = 0.93) or C57BL/6 (p = 0.31) food consumption. Figures 3.3 A and B show litter delivery day was not significantly delayed due to maternal exercise in either ICR (chi-square = 1.95 on 1 df, p = 0.16) or C57BL/6 mice (chi-square = 1.41 on 1 df, p = 0.23), respectively. While ICR dams had significantly larger litters than C57BL/6 dams (p < 0.001), panels 3.3 C and D show that exercise did not significantly alter litter size in either strain (chi-square = 0.12 on 1 df, p = 0.73 for ICR; chi-square = 0.14 on 1 df, p = 0.71 for C57BL/6).

Neither the number of delivered litters nor those that were successfully reared to weaning was significantly different between control and exercise groups in either strain of mice (Table 3.1). All 40 ICR mice (100%) delivered litters, and 90% of each group successfully reared litters to weaning (p = 1.0 for both comparisons). Out of 20 C57BL/6 control dams, 13 delivered (65%) and 11 successfully reared their pups (55% of total bred and 85% of litters born). In the C57BL/6 exercise group, 15 of 19 delivered (79%) and 14 raised pups to weaning (74% of total bred and 93% of litters born). For C57BL/6 dams, the exercise intervention did not cause any significant differences between number of litters born (p = 0.48), proportion of those weaned among females bred (p = 0.32), or those that delivered litters (p = 0.58). Interestingly, the ICR strain had a larger proportion of litters born (p < 0.001) and weaned (p = 0.0075) among females bred without regard to control or exercise designation when compared to C57BL/6 mice. However, the
proportion of litters weaned among those born was not significantly different when comparing the two strains of mice directly (p = 1.0).

Offspring body weight at postnatal days 7, 14, and 21 was recorded, and the average pup weight was calculated for each litter (Figure 3.4). Pup weights in both strains were not significantly affected by the exercise intervention at any of the time points (p = 0.26 for ICR; p = 0.65 for C57BL/6).

### 3.3.2 Study 2: Impact of Controlled Exercise on Pregnant Mice

Non-pregnant female mouse and dam body weight was decreased as a consequence of the exercise intervention (p = 0.002 for non-pregnant exercised animals, p = 0.021 for pregnant exercised animals) (Figure 3.5). Glucose tolerance testing completed at gestation day 14 (study day 44-46 for non-pregnant animals) is summarized as area under the curve (Figure 3.6). Exercised animals that did not conceive had improved glucose tolerance as indicated by AUC (p = 0.024) (Figure 3.6 A). Pregnant animals had no difference in glucose tolerance as a consequence of the controlled exercise intervention (p = 0.801) (Figure 3.6 B). Two days after the glucose tolerance testing, all animals were subjected to EchoMRI for body composition analysis (Figure 3.7). Exercise increased lean to fat mass ratio in the non-pregnant animals (p = 0.006). Interestingly, both exercise (p < 0.001) and sedentary wheel exposure (p = 0.029) increased lean to fat mass ratio in pregnant animals.

There was no significant difference in the number of pups born per litter (p = 0.421) (Table 3.2). There was, however, a significant decrease in weight of the two day
old pups as a consequence of maternal exercise (p < 0.001) (Table 3.2). There was no decrease in the number of litters that survived to seven days of age out of those born as a consequence of maternal exercise (8/9 sedentary home cage, 6/6 sedentary wheel, 8/8 exercise). Offspring body weight was decreased by maternal exercise at postnatal day 7 (p = 0.025 for exercise compared to sedentary home cage) (Table 3.2).

Fecal corticosterone was not significantly different between groups, suggesting that the wheel exposure (neither sedentary nor exercise) was not significantly stressful to the female mice (p = 0.765) (Figure 3.8).

3.3.3 Study 3: Long Term Offspring Consequences of Maternal Controlled Exercise

Dam body weight was decreased by the exercise intervention compared to sedentary wheel animals (p < 0.05 at timepoints indicated by *), though food consumption was unchanged between groups over the course of the study (p = 0.789 by repeated measures ANOVA) (Figure 3.9). Glucose tolerance before mating was not impacted by the exercise regimen (p = 0.155 by repeated measures ANOVA) (Figure 3.10 A). The EchoMRI, however, revealed that the exercise intervention decreased fat mass after three weeks of controlled exercise (p = 0.024) (Figure 3.10 B). Similarly, there was no difference in the number of pups born per litter (p = 0.217 by t-test), nor in the body weight of the pups at 7, 14, or 21 days of age (p = 0.734 by repeated measures ANOVA) (Figure 3.11).

As offspring aged, significantly improved glucose tolerance was observed in males at 3 months and at 6 months (Figure 3.12 A, B) by oral glucose tolerance testing as
a consequence of the maternal exercise intervention \((p < 0.05\) at 30 and 60 minutes). There was no significant difference, however, in female offspring glucose tolerance due to maternal exercise at these time points \((p > 0.05)\) (Figure 3.12 C, D). This significant improvement in glucose tolerance due to maternal exercise was not seen from 8 months of age and on in males, though a strong trend toward improved glucose tolerance was present due to maternal exercise and lasted until 18 months of age \((p = 0.08\) at 30 minutes) (Figure 3.13 A,B). Unfortunately, females still did not even show a trend toward improved glucose tolerance as a consequence of maternal controlled exercise at any of these later time points (Figure 3.13 C, D).

3.4 Discussion

The controlled exercise regimen was completed in both ICR and C57BL/6 mice prior to and during pregnancy and lactation (study 1). This study used a very modest walking pace, chosen because we did not want to stress the animals until we had established the safety of the intervention at very slow speeds. There were no obvious negative consequences for the dam or young litter due to the controlled exercise intervention. There were no significant differences in litter size or weight. The number of litters successfully weaned was not significantly impacted by controlled exercise. A further strength of this study was that the intervention was tested in both the ICR and C57BL/6 strains. We did not find any negative consequences due to the controlled exercise intervention in either strain of mouse.
However, the differences that we observed between the two strains of mice in study 1 were important but not surprising. The ICR animals were more likely to conceive and had consistently larger litters than the C57BL/6 animals. The ICR dams and pups were also larger, and dams consumed more food than the C57BL/6 mice. Interestingly, one report showed that ICR mice are more sensitive to chemically induced diabetes than C57BL/6J mice (Shimizu, Sakazaki et al. 2012). This could have potential implications for strain choice for future studies exploring offspring glucose tolerance and insulin sensitivity.

Exercise improves body composition and cognition while decreasing cancer risk (Friedenreich and Orenstein 2002, Weiss and Holloszy 2007, Winter, Breitenstein et al. 2007). In study 2, the controlled exercise intervention improved lean to fat mass ratio and area under the curve for glucose tolerance in non-pregnant females. Additionally, exercise during gestation offers a wide array of benefits during pregnancy. In one study, maternal exercise decreased the need for surgical intervention during labor and resulted in earlier delivery compared to sedentary controls (Clapp 1990). Also, implementing an exercise regimen improved oral glucose tolerance in pregnant women (Barakat, Cordero et al. 2011). Although we did not see improved glucose tolerance in the pregnant exercising females in study 2, the wheel exposure did improve lean to fat mass ratio in pregnant animals. Exercise appears to be a potentially positive intervention that can be used during healthy pregnancy.
The prospect of further exploring the benefits of exercise during pregnancy is exciting. For example, the antidepressant fluoxetine yielded negative consequences on mouse pregnancy (Bauer, Monk et al. 2010). Since exercise is shown to improve mood (Annesi 2005), it would be interesting to determine whether exercise during pregnancy may be a viable non-pharmacologic intervention for depression during pregnancy. In one instance, insulin treatment throughout mouse pregnancy resulted in decreased fetal mass (Andersen, Buschard et al. 1991). Perhaps exercise during pregnancy may be an alternative treatment to insulin for hyperglycemia during gestation, unless contraindicated or insufficient.

Maternal exercise has also been shown to impart offspring benefits in a number of species. In humans, babies born to exercised mothers have decreased fat mass (Clapp and Capeless 1990). In fact, they are still leaner at five years of age (Clapp 1996). If this trend persists as the offspring age, then exercise during pregnancy could hold exciting promise as a means to curtail the obesity epidemic. Additionally, voluntary exercise during gestation significantly improved glucose tolerance in aged mouse and rat offspring (Carter, Lewis et al. 2012, Carter, Qi et al. 2013). When tested in a transgenic mouse model, offspring born to exercising dams had decreased Alzheimer’s pathology (Herring, Donath et al. 2012). Thoracic aortas from female offspring born to pigs exposed to exercise during pregnancy displayed greater endothelium-dependent vasorelaxation response than those isolated from control offspring (S. C. Newcomer 2012). So in addition to the maternal benefits, exercise during pregnancy has the potential to provide a number of benefits to the offspring as well.
Voluntary exercise by including a running wheel in the home cages is one intervention that has been tested regarding offspring outcomes (Bick-Sander, Steiner et al. 2006, Carter, Lewis et al. 2012, Herring, Donath et al. 2012). However, there are some weaknesses in this model that should be acknowledged. Female mice run long and variable distances. In fact, voluntary exercise has been proposed to be a model for obsessive-compulsive type behavior in the rodent (Altemus, Glowa et al. 1993), though recent work has suggested that even animals in the wild will utilize a running wheel (Meijer and Robbers 2014). It has also been suggested that animals will postpone or eliminate crucial activities, such as eating and drinking, in favor of running (Sherwin 1998). While we have seen no indication of dam neglect in the ICR strain, it is still an arguable point in favor of exploring an alternative model.

It is in this light that study 3 was executed with the goal of determining whether or not the small amounts of maternal exercise at a relatively slow pace for a short duration on a daily basis used herein can improve glucose tolerance or body composition in offspring, either short or long term. We found significantly improved glucose tolerance in the young male offspring that disappeared with age. It is not unlikely that the improvement would have persisted with a larger number of subjects, as there were still notable trends toward improved glucose tolerance at the more advanced ages. The females never exhibited improved glucose tolerance. Sex differences in offspring outcomes due to various maternal interventions are a frequent phenomenon (Carter, Lewis et al. 2012, Rashid, Carter et al. 2013).
In addition to eliminating the issues associated with free choice running, the controlled exercise model also allows for a more precise experimental design. For example, the potential discrepancy in the time that the dam spends away from pup care (nursing) and grooming is eliminated because control dams are also removed from their home cage while the exercise group is subjected to the running paradigm. We also used the sedentary home cage group in study 2 to show that the wheel exposure itself does not impact pregnancy outcomes. Also, running time and distance are equivalent for all mice assigned to the exercise group. As the dams have only limited and controlled access to the wheel, they do not have the ever-present distraction that may arguably draw them away from nursing and licking their pups.

The speed at which the motor driving the wheels operates is completely programmable. Here, a mild pace (6-10 m/min) was utilized to mitigate any potential stress to the dam and her unborn offspring. In historical data from our facilities at the University of Kentucky, non-pregnant ICR females utilize voluntary running wheels at a speed of ~15 m/min while C57BL/6 females run an average of ~19 m/min (unpublished data). Clearly, the speed utilized for the current study was well below these voluntary speeds, as we did not want to negatively impact pregnancy success. Additionally, female mice will dramatically decrease their voluntary running as pregnancy progresses (Carter, Lewis et al. 2012). That being the case, the choice of a low speed (very low speed of 6 m/min for study 1 and more moderate 10 m/min for studies 2 and 3) was imperative for maintaining a constant pace for the duration of the study. One could argue that the slow running speed used in these experiments may not have elicited a training response.
Regardless, these findings are still relevant even if a training effect was absent. For example, studies suggest that climbing as few as 1-4 flights of stairs daily may reduce incidence of preeclampsia (Sorensen, Williams et al. 2003) while walking may prevent excessive weight gain during gestation (Stuebe, Oken et al. 2009). Using the slightly faster pace (10 m/min for one hour per day, 7 days a week – study 2 and 3) resulted in an increased lean to fat mass ratio in exercising ICR females compared to control mice, and indeed this was a safe pregnancy intervention as well (Figure 3.7, Figure 3.10). Future studies should consider decreasing the speed of the exercise as pregnancy progresses in order to more mimic the routine that the mice choose when allowed voluntary exercise.

Restraint stress during pregnancy in rats has been shown to result in decreased term weight (Lesage, Del-Favero et al. 2004). Disruption of the light cycle may also decrease pregnancy success in C57BL/6 mice (Summa, Vitaterna et al. 2012). In humans, stress has been implicated in shortened gestational length (Tegethoff, Greene et al. 2010). Though we did not directly measure gestation length, the day of delivery was not significantly changed by the exercise intervention in study 1. Therefore, we can speculate that the controlled exercise paradigm did not significantly stress the pregnant dams more than the disruption of the light cycle and placement in the running wheel bed that occurred within the control groups. Examination of maternal corticosterone levels from the feces suggested that the wheel exposure is not significantly stressful to the females (Figure 3.8). Subsequent studies should also include animals exercising during the dark cycle in order to reduce stress and mimic the animal’s behavior during free choice running interventions. Stress could certainly play a role in the variability in a number of
parameters, but an important finding is that the exercise paradigm in these studies did not appear to cause further stress (or perceptible negative outcomes).

Finally, the motorized wheel system used herein is not unknown in the rodent exercise field. It has been used in mice for studies involving learning, vasoconstriction and bladder function (Khazaei, Moien-Afshari et al. 2008, Khazaei, Moien-Afshari et al. 2009, Shearer, Ross et al. 2011, Vadhavkar, Golbidi et al. 2011). To our knowledge, however, no others have explored the Lafayette Walking Wheel System as a perinatal intervention in mice. We have provided valuable data regarding the use of this particular mouse exercise system before and during pregnancy and lactation in two common strains of mice.

In conclusion, we have illustrated a model of controlled exercise in the mouse that is safe for use during pregnancy. Additionally, we have provided side-by-side data for two strains of mice (study 1). The intervention improves glucose tolerance in non-pregnant animals (study 2). Exercise improves lean to fat mass ratio in non-pregnant females, while somewhat surprisingly, any wheel exposure improves body composition in pregnant animals. Excitingly, male offspring born to exercise dams have improved glucose tolerance at young ages, and trends toward improvements at advanced ages (study 3). Future studies using the controlled exercise model will continue to optimize the intervention in order to maximize offspring benefits and to narrow down therapeutic targets (duration, intensity) for exercise interventions during pregnancy.
Figure 3.1 Controlled exercise system, studies 1, 2, and 3.

A photograph of the controlled exercise wheel platform containing ICR mice is provided (A). Two platforms are present side by side so that 20 control animals and 20 exercise animals may be exposed to the wheel bed simultaneously (B).
Breeding studies were completed in both the ICR and C57BL/6 strains. Body weights were recorded twice weekly and food remaining was weighed weekly for the duration of the study. Horizontal lines associated with male symbols show when a male was present in the home cage for mating. There were no significant differences in ICR or C57BL/6 control or exercising dam body weights (A,B) or food consumption (C,D) at any point during the study. The data from the dams that had litters survive until weaning is included for all groups. Further, only the dams delivering within the first four days are included for the ICR strain. One C57BL/6 dam refused to consistently participate in the exercise intervention after parturition and was excluded. ICR control, n = 18; ICR exercise, n = 14. C57BL/6 control, n = 11; C57BL/6 exercise, n = 13. Error bars indicate standard error of the mean (SEM).
Figure 3.3 Day of birth and pups per litter, study 1.

Cages were monitored for delivery and the first day that any litter was found was designated as day 1. Litters born on subsequent days were designated day of birth 2, 3, et cetera. The exercise intervention did not cause a significant delay in ICR or C57BL/6 day of birth (A, B). There was no significant difference in the number of ICR or C57BL/6 pups born per litter (C, D). ICR control, n = 20; ICR exercise, n = 20. C57BL/6 control, n = 13; C57BL/6 exercise, n = 15. Horizontal lines indicate median. Median was chosen because of the improbability of having a fraction of a pup.
Figure 3.4 Offspring body weight was not significantly affected by maternal exercise in study 1.

Pup body weight was recorded 7, 14, and 21 days after birth. Plotted points are averages for the various litters. ICR control, n = 18; ICR exercise, n = 18 except for 21 days (n = 17). C57BL/6 control, n = 11; C57BL/6 exercise, n = 13. Horizontal bar indicates mean.
Figure 3.5 Body weight for female mice in study 2.

Body weight was recorded daily for the course of the study. Weight was significantly decreased by the controlled exercise intervention for both non-pregnant (A) and pregnant (B) females. n = 11-14 for non-pregnant animals, 6-9 per group for pregnant animals. Error bars indicate S.E.M.
Figure 3.6 Glucose tolerance in non-pregnant and pregnant females in study 2.

After a 3 hour fast, animals were subjected to an oral glucose tolerance test. Area under the curve is used to summarize the timecourse of the experiment. Exercised animals that did not conceive had significantly improved glucose tolerance as indicated by a lower area under the curve (A). Neither wheel exposure nor exercise intervention caused a significant difference in glucose tolerance in pregnant animals at gestation day 16 (B). Groups with the same letter are not significantly different from one another. Error bars indicate S.E.M. n = 10-13 per group for non-pregnant animals, n = 7-10 per group for pregnant animals.
Figure 3.7 Non-pregnant and pregnant animal body composition, study 2.

EchoMRI was used to analyze body composition in all animals. Lean to fat mass ratio is used to summarize the body composition data. The controlled exercise intervention significantly increased lean to fat mass ratio in non-pregnant females (A). Interestingly, while exercise did significantly increase lean to fat mass ratio in pregnant females, so did wheel exposure even when the platform was stationary (B). Error bars indicate S.E.M. n = 10-13 per group for non-pregnant animals, n = 7-10 per group for pregnant animals.
Figure 3.8 Fecal corticosterone was not significantly impacted by wheel exposure or exercise intervention in study 2.

Feces were collected from all cages and subjected to radioimmunoassay for corticosterone. There were no significant differences between groups for fecal corticosterone, suggesting that neither the sedentary wheel exposure nor the controlled exercise intervention was significantly stressful. n = 19-20 per group. Error bars indicate S.E.M.
Figure 3.9 Dam body weight and food intake for study 3.

Dams were weighed throughout the course of the study (A). Beginning in the second week of the intervention, female mouse body weight was significantly lowered by exercise. This decrease persisted until mid-gestation, and then reappeared after delivery. This effect was not as a consequence of decreased food consumption, as there was no significant difference in food intake (B). Error bars indicate S.E.M. n = 20 per group.
Figure 3.10 Female mouse glucose tolerance and fat mass before mating in study 3.

Mice were subjected to oral glucose tolerance testing during the third week of the controlled exercise intervention, which revealed no significant differences (A). Fat mass data collected the same week by EchoMRI, however, was significantly decreased by the exercise intervention (B). n = 10 per group for glucose tolerance test, n = 20 per group for EchoMRI. Error bars indicate S.E.M.
Figure 3.11 Number of pups per litter and offspring body weight before weaning in study 3.

Pups were counted after birth (A). There was no significant difference in the number of pups per litter as a consequence of the controlled exercise intervention. Offspring were weighed 7, 14, and 21 days after birth (B). There was no significant difference in the weight of pups before weaning. Error bars indicate S.E.M. n = 18 litters per group (A), n = 11 and n = 14 litters for body weights for exercise and sedentary, respectively.
Figure 3.12 Offspring glucose tolerance at 3 and 6 months in study 3.

Offspring were subjected to oral glucose tolerance testing at 3 and 6 months of age. Males had improved glucose tolerance at 3 (A) and 6 (B) months of age. Females did not have improved glucose tolerance at these time points (C,D). * indicates $p < 0.05$ at indicated time points. Error bars indicate S.E.M. $n = 14$ per group males and $n = 10$ per group females for 3 months, $n = 13-15$ per group for males and females for 6 months.
Figure 3.13 Glucose tolerance in aged offspring, study 3.

The improvement in male offspring glucose tolerance seen at younger ages disappeared at 8 months of age (A) but there was a strong trend toward improvement at advanced age (B). Females never exhibited even a trend toward improved glucose tolerance (C, D). Error bars indicate S.E.M. n = 8-15 per sex per group.
Table 3.1 | Number of litters born and weaned out of those bred, study 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice bred</th>
<th>Number of litters born</th>
<th>Number of litters weaned</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR control</td>
<td>20</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>ICR exercise</td>
<td>20</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>C57BL/6 control</td>
<td>20</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>C57BL/6 exercise</td>
<td>19</td>
<td>15</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Although 14 litters were weaned successfully, only 13 were included in Figures 2 and 4 because one dam refused to consistently participate in the exercise intervention after parturition.
Table 3.2 | Number of pups per litter and pup weight at 2 and 7 days of age, study 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Pups per litter</th>
<th>Weight (g) at 2 days</th>
<th>Weight (g) at 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary Home Cage</td>
<td>8.0 ± 1.2</td>
<td>2.2 ± 0.07</td>
<td>5.1 ± 0.19</td>
</tr>
<tr>
<td>Sedentary Wheel</td>
<td>9.9 ± 0.7</td>
<td>2.2 ± 0.08</td>
<td>5.4 ± 0.23</td>
</tr>
<tr>
<td>Exercise</td>
<td>8.0 ± 1.2</td>
<td>2.0 ± 0.08</td>
<td>4.3 ± 0.14</td>
</tr>
</tbody>
</table>
CHAPTER 4 BRANCHED CHAIN AMINO ACID SUPPLEMENTATION IN FEMALE MICE

4.1 Introduction

Obesity is a growing epidemic in the United States (Flegal, Carroll et al. 2012), and it is critical that effective solutions are identified that can attenuate the negative health outcomes associated with obesity and high fat diet (HFD) consumption. It is well accepted that exercise is one way to promote a healthy calorie balance and body weight (Schmitz, Jacobs et al. 2000). This is, however, only a small component of the benefit that exercise offers. Even in the absence of weight loss, exercise can lower blood pressure and improve insulin sensitivity (Carroll and Kyser 2002). The American Diabetes Association and the American College of Sports Medicine have released a report emphasizing the utility of exercise in both prevention and management of type 2 diabetes (Colberg, Albright et al. 2010). Endurance exercise can reduce several of the parameters defining the metabolic syndrome (Pattyn, Cornelissen et al. 2013). Additionally, an exercise intervention improves insulin resistance and non-alcoholic fatty liver disease parameters (Bhat, Baba et al. 2012). Exercise is therefore an extremely important modality for the improvement and maintenance of glycemic control.

Voluntary wheel running is a frequently used model for exercise in rodents. Male mice provided with a free-choice running wheel have improved glucose tolerance and body composition (Bradley, Jeon et al. 2008, Yan, DeMars et al. 2012). Physical activity can protect male mice from diet-induced obesity (Huang, Li et al. 2010, Yan, DeMars et
While the physical and metabolic effects of voluntary exercise have been studied extensively in male mice, evidence in female mice is lacking. In that light, the current study utilizes female mice and free choice wheel running.

Individuals who exercise routinely often consume dietary supplements. Physical activity was associated with an odds ratio of 2.45 for dietary supplementation in a young adult population (Gardiner, Kemper et al. 2007). The number of supplements available in the United States jumped from approximately 4,000 in 1994 to 75,000 in 2008 (Office 2009). A report in 2009 indicates that $14.8 billion was spent on non-vitamin, non-mineral supplements (Nahin, Barnes et al. 2009). As the use of supplements is becoming ever more widespread, further research is warranted regarding their safety and efficacy. Studies report rates of protein or amino acid supplementation among exercising individuals from 42% to 77% (Radimer, Subar et al. 2000, Morrison, Gizis et al. 2004). Branched-chain amino acids (BCAAs) are widely available for purchase and are a popular choice. Goston and Correia found that 6% of people exercising in gyms were taking BCAAs, a similar number to a report by de Silva et al. in professional athletes (de Silva, Samarasinghe et al. 2010, Goston and Correia 2010).

The BCAAs - leucine, valine, and isoleucine - are touted for preventing muscle breakdown (Howatson, Hoad et al. 2012). Indeed, BCAAs – leucine especially - have been shown to activate enzymes in the protein synthesis pathway, particularly mTOR (Blomstrand, Eliasson et al. 2006). mTOR is a nutrient sensor that promotes translation in skeletal muscle and satiety in the hypothalamus (Cota, Proulx et al. 2006, Layman and
Walker 2006). A decrease in food intake, which may be seen due to increased satiety, could impact body weight. Interfering with BCAA metabolism induces exercise failure (She, Zhou et al. 2010), suggesting that the BCAA’s are vital for sustained physical activity. Well-trained individuals subjected to calorie restriction and BCAA supplementation lose additional abdominal fat compared to those restricting calories alone (Mourier, Bigard et al. 1997). Perhaps this could be due, at least in part, to the protein sparing effects of leucine. BCAAs may play a role in the weight-management benefits of high protein diets (Qin, Xun et al. 2011). Additionally, male mice receiving BCAAs are protected from virally-initiated impaired glucose tolerance (Utsugi, Yoshida et al. 2000), which is not surprising as the BCAAs are implicated in glucose metabolism (Doi, Yamaoka et al. 2007). D’Antona et al reported that BCAA supplementation increases mean lifespan in male mice despite no significant changes in food intake or body weight (D’Antona, Ragni et al. 2010).

The goal of this study was to investigate the effects of voluntary exercise and BCAA supplementation in combination with HFD consumption in female C57BL/6 mice. Ten week old animals were subjected to a regimen of HFD feeding, exercise, and BCAA supplementation for 16 weeks. Drinking water was supplemented with BCAAs at a concentration of 2%, which is similar to other studies (Zhang, Guo et al. 2007, Guo, Yu et al. 2010, Arakawa, Masaki et al. 2011, Macotela, Emanuelli et al. 2011). We hypothesized that voluntary exercise and BCAA supplementation, both in combination and separately, would improve body composition and glucose tolerance in HFD fed mice. Instead, we found that voluntary exercise significantly protected against obesity and
glucose intolerance. Although BCAA supplementation did not significantly alter glucose tolerance it did significantly improve lean to fat mass ratio in combination with voluntary exercise.

4.2 Methods

4.2.1 Animals

This study was executed according to a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee. Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and arrived at 9 weeks of age. Mice were individually housed in standard shoebox cages with Sani-Chips (Teklad 7115 Coarse Sani-Chip Bedding, Harlan Laboratories, Inc., Indianapolis, IN) and provided with nesting pads (Nestlets, Ancare, Bellmore, NY). The animals were housed with a 14:10 h light:dark cycle and ambient temperatures between 20–23°C. After a one week acclimation period, animals were assigned to one of eight groups: 1) control diet (CD), sedentary (SED), water; 2) CD, SED, BCAA; 3) HFD, SED, water; 4) HFD, SED, BCAA; 5) CD, exercise (EX), water; 6) CD, EX, BCAA; 7) HFD, EX, water; 8) HFD, EX, BCAA. Groups were randomly assigned and adjusted so as to have approximately equal body weights between groups. Animals were maintained on their respective diet/water/exercise regimen for 16 weeks. Glucose tolerance testing and body composition analysis was completed during the 5th, 10th, and 15th week of treatment. Animals were euthanized via carbon dioxide exposure followed by cardiac puncture for
serum collection during week 16. Tissues were harvested and immediately placed into liquid nitrogen prior to storage at -80 °C.

### 4.2.2 Diet and Water Treatment

The mice were fed either a 10 kcal% fat CD (D12450B Research Diets, Inc., New Brunswick, NJ) or a 60 % HFD (D12492; Research Diets, Inc., New Brunswick, NJ). The CD was 3.85 kcal/g, while the HFD was 5.24 kcal/g. A pre-weighed amount of food was given to each animal in such a quantity that they could eat ad libitum. Food remaining was weighed, discarded, and replaced twice weekly. BCAA supplement (NutraBio BCAA 5000, Nutrabio, Inc., Middlesex, NJ) was added to the drinking water at a concentration of 20 g/L, yielding a 2% solution. The BCAA consisted of a 2:1:1 ratio of leucine:valine:isoleucine. A sample of the supplement was sent to a third party lab (ALS Global, Salt Lake City, UT) to confirm composition, which indicated an approximate 2:1:1 ratio as claimed by the product label. 1 mL of this solution accounts for 0.08 kcal. This calorie intake by water consumption was included in the calorie consumption data in Figure 4.2. Animals were given a known amount of control water or BCAA supplemented water. Water remaining was measured via graduated cylinder, discarded, and replaced with fresh solution twice weekly. The mice had 24 hour access to BCAA water except for brief periods of glucose tolerance testing, EchoMRI analysis, and a 3 hour fasting period prior to euthanasia when they were given water without BCAAs.
4.2.3 Exercise

Exercise animals had free voluntary access to running wheels mounted within the home cages (Phenome Technologies, Inc., Lincolnshire, IL) lined with wood chip bedding. Sedentary animals were housed in similar shoebox cages with identical wood chips but no running wheels. Animals with access to running wheels were not given nesting pads to prevent wheel dysfunction, though sedentary animals were given them. Wheel rotations were recorded mechanically and sent to computer via Actimetrics ClockLab software (Actimetrics, Wilmette, IL). All four exercise groups had 10 animals per group, but two computer files from the exercise HFD water group were corrupted and therefore n = 8 for this group. Running distance was averaged across each group for each day, and then the average of 115 days was taken for the final value (Figure 4.5). The mice had 24 hour access to running wheels except for brief periods of glucose tolerance testing, body composition analysis and a 3 hour fasting period prior to euthanasia.

4.2.4 Glucose Tolerance Testing.

During the 5th, 10th, and 15th weeks of treatment, all animals were fasted in a clean cage lacking running wheels and provided water only. After 3 hours (5 and 10 weeks) or 6 hours (15 weeks), the tail was nicked and fasting blood glucose was recorded (Bayer Breeze 2 glucose meter, Bayer HealthCare, LLC, Tarrytown, NY) prior to intraperitoneal injection of D-glucose (Sigma-Aldrich Co., LLC, St. Louis, MO) at a concentration of 2 g/kg body weight. Blood glucose reading was repeated 15, 30, 60, and 120 minutes post-injection. Animals were immediately returned to their home cage after the experiment concluded.
4.2.5 *Body Composition*

Two days after glucose tolerance testing, body composition analysis was completed using EchoMRI-100 (Echo Systems, Houston, TX). After running an appropriate control sample, conscious animals were placed inside of a specifically designed tube and then inserted into the EchoMRI and subject to analysis (~2 minute duration). Although many tissues contribute to the lean mass, there are also undetectable components such as bone, hair, and claws. Animals were returned to their home cages after completion of the measurement.

4.2.6 *Serum leptin*

Whole blood collected at euthanasia via cardiac puncture was allowed to clot and subsequently centrifuged at 10,000 rpm (4°C) for 10 min, then stored at -80°C until use. An assay kit was completed according to manufacturer’s directions for leptin quantitation (Leptin ELISA Kit, Genway Biotech, Inc., San Diego, CA).

4.2.7 *Statistics*

Linear mixed modeling was utilized in figure 1 and 2, with structure of a 3-way ANOVA plus random effects for correlations among repeated measures, assuming the mean within groups as a quadratic function of time. Figure 3 employed 3-way ANOVA with observations weighted by reciprocals of within-group variances. For running distance, longitudinal progression was applied with a 2-way ANOVA structure, assuming a quadratic function of time. Three-way ANOVA was used for serum creatinine. Subsequent Bonferroni post-hoc, when applicable, was utilized to obtain between-group differences. Groups not carrying the same letter are significantly different from one
another (a bar labeled only with \( a \) is significantly different from a bar labeled only with \( b \), though neither is significantly different from a bar labeled with both \( a \) and \( b \)). Significance was considered as \( p < 0.05 \).

4.3 Results

4.3.1 General

All 80 animals completed the 16 week intervention. Body weight was recorded weekly for the duration of the study (Figure 4.1 A). Exercise significantly attenuated body weight increases that were associated with HFD consumption, though not to the levels of the CD fed mice. BCAA supplementation had no significant impact on body weight. HFD fed mice ate significantly less food in grams (Figure 4.2 A) but took in significantly more calories compared to the CD fed animals (Figure 4.2 B). Exercised animals consumed more food in grams and calories than their respective sedentary CD fed mice (Figure 4.2 A,B). BCAA supplementation did not significantly impact food or calorie consumption. The BCAA supplementation did, however, significantly increase water consumption (Figure 4.2 C). All BCAA supplemented animals drank more than their respective control water group. Further, sedentary CD BCAA drank significantly more than any other group. Serum was analyzed for creatinine (Figure 4.2 D) and we found no evidence of kidney damage as a consequence of the BCAA supplement.

4.3.2 Glucose tolerance

Glucose tolerance following an IP glucose challenge was measured in fasting mice, and the time course (Figures 4.3 A) and area under the curves (AUC) (Figure 4.3}
B) are shown at the 5 and 15 week timepoint. Glucose disposal was significantly impaired by HFD feeding during the 5th (Figure 4.3 A,B) and 15th weeks of dietary treatment (Figure 4.3 C,D). The impairment escalated over time. Exercise significantly improved glucose disposal under both dietary conditions (p < 0.0001 for main effect of exercise at both 5 and 15 weeks). The glucose tolerance impairment induced by the HFD was attenuated such that they were not significantly different than CD fed sedentary mice. BCAA supplementation did not significantly affect glucose tolerance (p = 0.93, p = 0.70 for main effect of BCAA at 5 and 15 weeks, respectively).

4.3.3 Body composition

Body composition data were collected two days after glucose tolerance testing during week 5 and 15 (Figure 4.4). HFD significantly increased fat mass (g) in the sedentary condition at 15 weeks (Figure 4.4 B). Exercise significantly decreased fat mass in both the CD and HFD fed mice. HFD resulted in a small but significant increase in lean mass (g) when compared to their respective controls at 15 weeks (Figure 4.4 C). Interestingly, the exercised CD BCAA animals had lean mass equal to that of exercised HFD animals. When looked at in terms of lean to fat mass ratio (LFR) (Figure 4.4 E,F), the differences in body composition are more notable. HFD dramatically decreased LFR in the sedentary condition. Exercise in combination with HFD served to significantly increase LFR over sedentary HFD animals, and exercise HFD BCAA had LFR recovered to levels of sedentary CD animals. Similarly, exercise CD water did not increase LFR beyond sedentary CD animals, but exercise CD BCAA did induce a significant increase therein.
4.3.4 Leptin

Serum collected at euthanasia was analyzed for leptin levels (Figure 4.6 A). HFD caused a significant increase in serum leptin, which was completely rescued by voluntary exercise.

4.3.5 Running Distance

Running distance was continuously recorded for the duration of the study and then averaged (Figure 4.5). Both HFD and BCAA caused an increase in distance ran ($p < 0.0001$ for main effect of diet, $p = 0.0056$ for main effect of BCAA). There was no significant interaction between diet and BCAA supplementation ($p = 0.63$).

4.4 Discussion

Obesity is an epidemic that must continually be acknowledged and awarded significant consideration. The consequences of the HFD-induced obesity in the current study were dramatic, including extreme weight gain, excessive fat mass and severely impaired glucose tolerance in female C57BL/6 mice. Other consequences likely exist that are beyond the scope of this report. Exercise, however, had a profound impact on the HFD-fed mice. For instance, body weight in exercising mice was significantly less than that of sedentary HFD animals and glucose tolerance was completely rescued to the level of sedentary control diet animals. Excitingly, where exercise in combination with control diet and water alone did not significantly increase lean to fat mass ratio, exercise plus the control diet with BCAA supplement did significantly increase it. Similarly, exercise HFD animals had significantly lower lean to fat mass ratio compared to sedentary control diet
animals, but exercise HFD BCAA animals had lean to fat mass ratio equivalent to the control group (sedentary CD water).

As previously discussed, the benefits of voluntary wheel running on glucose tolerance and body composition have been explored in several studies using male mice (Bradley, Jeon et al. 2008, Yan, DeMars et al. 2012). Exercise training improved cardiovascular outcomes and glucose tolerance in male C57BL/6 mice fed a HFD (Hafstad, Lund et al. 2013). Voluntary wheel running in female A/J mice decreased fat mass and liver triglycerides (Takeshita, Horiuchi et al. 2012). Therefore, it is not surprising that we found improvements in several outcomes due to free choice exercise in the current study. The degree of the improvements, however, is notable. For one, the exercise mice fed control diet consumed significantly more calories than sedentary control diet animals (in fact, as many calories as the sedentary HFD animals) and yet weighed the same as the sedentary control diet animals. In addition, exercise improved glucose tolerance in HFD-fed animals to the level of sedentary control diet animals, which is a profound improvement, especially considering the difference in body weight.

Of the three BCAAs, leucine especially has been proposed to impart important effects (Layman and Walker 2006). Several studies similar to the current study have used leucine supplementation in combination with HFD and/or exercise in mice. At least two studies have used 1.5% leucine in the drinking water and the same HFD as the current study and observed improved glucose tolerance as a consequence of supplementation (Zhang, Guo et al. 2007, Macotela, Emanuelli et al. 2011). Guo and colleagues also
showed decreased hemoglobin A1C levels in male mice of two strains, NONcNZO10/LtJ and B6.Cg-A\textsuperscript{v}/J, as a consequence of 1.5% leucine supplementation in the drinking water for 8 months (Guo, Yu et al. 2010). Contrary to these findings, we found no significant impact on glucose tolerance using female mice supplemented with 2% BCAA (1% leucine) in the drinking water. Perhaps this could be due to differences in BCAA supplement and dose, or sex differences between the studies. While we did not monitor estrous cycle in these females, it has been shown that glucose tolerance is not impacted by stage of the cycle (Shi, Strader et al. 2007). Exploration of different doses of BCAAs or different BCAA products, or leucine alone, is warranted to further investigate the notable lack of effect. Importantly, the 16 week duration for the BCAA supplementation did not negatively impact glucose tolerance or weight gain.

Freudenberg and colleagues found that a 6% leucine supplement in the diet for 20 weeks decreased food intake and partially ameliorated HFD (20% w/w) induced weight gain in male C57BL/6 mice (Freudenberg, Petzke et al. 2012). Using the same HFD as the current study and a comparable 1.5% leucine supplement in the water for a 14 week duration, another study suggested this same protection from weight gain with leucine supplemented water in male C57BL/6 mice (Zhang, Guo et al. 2007). To the contrary, the current study resulted in no significant decrease in food intake or in body weight as a consequence of BCAA supplementation.

Other studies have suggested a similar lack of effect as that found in the current study. Nairizi and coworkers found no difference in body weight or food consumption as
a consequence of 150 mmol/L (1.97%) leucine supplement in the water for 14 weeks in male C57BL/6 mice, and again, the same HFD used in the current study (Nairizi, She et al. 2009). Similarly, male C57BL/6 mice consuming a diet supplemented with 4.5% leucine did not have different weight or glucose handling response (Noatsch, Petzke et al. 2011). A similar study also found no change in male mouse food intake or body weight with BCAA supplementation in the water at 1.5 mg/g body weight (D'Antona, Ragni et al. 2010). These findings are in agreement with our lack of significant difference in body weight and food consumption due to BCAA supplementation in female C57BL/6 mice.

Both Noatsch et al and Nairizi et al found no difference in body composition of male C57BL/6 mice as a consequence of increased leucine intake (Nairizi, She et al. 2009, Noatsch, Petzke et al. 2011). On the contrary, Zhang et al reported a decrease in fat mass gained by C57BL/6 males as a consequence of leucine supplementation (Zhang, Guo et al. 2007). While the exercise control diet water group did not have significantly increased lean to fat mass ratio over sedentary control diet water in female C57BL/6 mice in the current study, exercise control diet plus BCAAs did. Additionally, while exercise HFD water did not rescue lean to fat mass ratio back to sedentary control diet water levels, exercise HFD BCAA did return lean to fat mass ratio to equivalent of control animals. These two findings are particularly interesting in that they acknowledge that BCAAs do not significantly increase lean to fat mass ratio within the exercise strata, but that they may increase lean to fat mass ratio above sedentary levels where water and exercise alone failed to do so.
Studies similar to the current study report no change in water consumption using BCAA or leucine supplemented water (Zhang, Guo et al. 2007, Nairizi, She et al. 2009). Similarly, a study specifically looking at the impact of various amino acids on water intake in rats reported no change in water consumption when the animals were provided BCAAs (Anderson, Luo et al. 1994). Of interest, however, exercising rats given the choice between plain water versus a BCAA enriched solution prefer the latter (Smriga, Kameishi et al. 2006). The impact that the BCAA supplement had on water consumption in the current study is notable. As we found no significant increase in serum creatinine as a consequence of BCAA supplementation, we can interpret this to mean that the increase in water consumption is not likely to be a consequence of kidney damage. However, more detailed investigation of kidney histology and other urinary measures would be necessary to confirm this interpretation.

Leptin is an adipokine involved in satiety, or lack thereof, and subsequent obesity (Feng, Zheng et al. 2013). As leptin is secreted proportionately to the amount of adipose present, it is not surprising that the SED HFD animals have dramatically increased leptin levels, in both serum and adipose. It is impressive, however, to note the degree of rescue that EX causes in the HFD-fed animals. The serum levels are completely recovered, while weight was only partially returned to SED CD levels.

Finally, we did observe a significant increase in running distance as a consequence of HFD consumption, and a further significant increase when HFD was combined with the BCAA supplement. It has been shown that HFD can cause this
increase in running activity (Meek, Eisenmann et al. 2010). Also, BCAA supplement may
decrease the buildup of lactate in the muscle and decrease muscle fatigue, perhaps due to
an increase in lactate threshold because of enhanced BCAA oxidation (Koba, Hamada et
al. 2007, Matsumoto, Koba et al. 2009). Therefore, it is plausible that the mice in the
current study who received the BCAA supplement ran farther because they did not
fatigue quite as quickly. Future study is warranted to determine the validity of this
supposition.

In summary, exercise had a dramatic impact on the weight gain and glucose
tolerance of female C57BL/6 mice fed a HFD. BCAA supplementation only impacted
water intake, and, excitingly, improved body composition when combined with exercise
when exercise alone did not. A positive note is that we found no negative consequence of
the BCAA intake in the parameters explored in this study, but increased water intake in
the BCAA groups should be further investigated to ensure that this was not a result of
kidney damage. Future studies should include different doses of BCAAs, and either
BCAA supplemented food or a choice-based water experiment. It would also be
interesting to examine the impact that the BCAA supplement may be having on skeletal
muscle, if any. As suggested by the body composition data, BCAA supplementation may
provide the most benefit in combination with exercise. However, future studies should
also focus on alternative forms of exercise, such as resistance training, in combination
with BCAA supplementation. This study further supports the idea that exercise is
extremely efficacious for ameliorating the weight gain and glucose intolerance induced
by HFD, while BCAA supplementation had no impact on these parameters in female
C57BL/6 mice. Future studies should also explore the supplement as a perinatal intervention in mice to determine its safety, and whether it may offer advantages for glucose homeostasis or body composition during pregnancy. Similarly, offspring born to BCAA-fed dams should be examined for differences in body composition or glucose tolerance as compared to offspring born to dams not exposed to BCAA supplement.
Female mice were divided into groups ensuring no initial body weight difference (p = 1.00). Biweekly animal body weight is presented (A). As time progressed, HFD fed animals gained significantly more weight, independent of water treatment. Exercise attenuated this weight gain. Exercise had no effect on the body weight of animals fed the CD. Different letters indicate p <0.05 between groups (i.e. a is different from b, b is different from c, et cetera). Error bars indicate S.E.M. n = 10 for all groups.
Figure 4.2 Female C57BL/6 mouse food, water, and calorie intake, and serum creatinine.

Food remaining was weighed and replaced twice weekly and calculated as grams per day (A). Calorie consumption was calculated by multiplying grams of food consumed by calories per gram (B). Water remaining was measured by graduated cylinder and replaced twice weekly (C). Sedentary control animals consuming HFD decreased the quantity of food consumed, but ingested more calories. Exercise animals consuming the HFD similarly decreased their quantity of intake, but received the most calories of any group. The BCAA water groups consistently drank more than their respective water-consuming group, independent of diet or exercise treatment. This is not likely a consequence of kidney damage as there was no increase in serum creatinine due to BCAA supplement (D). Different letters indicate p <0.05 between groups (i.e. a is different from b, b is different from c, et cetera). Error bars indicate S.E.M. n = 10 for all groups.
Figure 4.3 Glucose tolerance test during the fifth and fifteenth weeks of treatment.

Animals were fasted for three (5 weeks) or six hours (fifteen weeks), blood glucose was recorded, and animals received an IP injection of 2 g/kg D-Glucose. Blood glucose measurement was repeated 15, 30, 60, and 120 minutes post-injection (A,C). HFD consumption resulted in impaired glucose tolerance, independent of water treatment. While it did not quite reach significance at 5 weeks (B), exercise significantly attenuated these changes to the level of controls at 15 weeks. Exercise, in combination with CD, improved glucose tolerance compared to controls. BCAA water supplementation had no impact on glucose tolerance, regardless of diet or exercise treatment. Area under the curve was significantly increased for sedentary HFD fed animals and rescued by exercise (D). Letters indicate p < 0.05 between groups. Error bars indicate S.E.M. n = 10 for all groups.
Figure 4.4 Body composition analysis during the 5th and 15th weeks of treatment.

Animals were subjected to EchoMRI for body composition analysis. HFD resulted in an increase in fat mass (grams) compared to control animals (A,B). Exercise attenuated some of this increase. HF feeding plus exercise resulted in an increase in lean mass compared to CD animals (C,D). HFD significantly reduced lean to fat mass ratio (E,F). Exercise plus CD did not increase LFR compared to SED CD animals at 15 weeks, but EX CD BCAA did. Similarly, BCAA supplementation resulted in recovery of LFR to SED CD Water levels, when EX HF alone did not. Different letters indicate $p < 0.05$ between groups. Error bars indicate S.E.M. n = 10 for all groups.
Record of distance ran was recorded mechanically and logged via computer. Daily group distance ran was averaged, and the average was subsequently taken across all days for the four groups. HFD fed animals ran farther than CD animals. BCAA supplementation resulted in an increased distance ran over the course of the 16 week study, independent of diet treatment. Letters indicate $p < 0.05$ between groups. Error bars indicate S.E.M. $n = 10$ for all groups except EX HFD water group $n = 8$. 

Figure 4.5 Running distance.
Figure 4.6 Serum leptin.

Leptin was analyzed by ELISA (A). High fat diet consumption significantly increased leptin, and exercise ameliorated this increase. Error bars indicate SEM. n = 6-8 per group for serum ELISA.
CHAPTER 5 DISCUSSION

5.1 Maternal High Fat Diet

Obesity and the consumption of a high fat, obesogenic diet are common issues in modern society. Offspring born to obese women are more likely to be macrosomic, suffer trauma during delivery, and grow to be obese children. As almost half of women of childbearing age are overweight or obese, this is an issue that deserves much attention and, ideally, intervention.

It is in that light that further studies are warranted into the long-term offspring consequences of maternal high fat diet. Within chapter 2, we set out to develop a model of maternal high fat feeding that induced impaired glucose tolerance, poor insulin sensitivity, and increased obesity in offspring. The intent was then to use that model to explore protective maternal interventions in the high fat-fed female mouse, such as exercise or dietary supplements. We hypothesized that maternal high fat diet would impair glucose tolerance in offspring and lower offspring lean to fat mass ratio.

The dams did gain excess weight as a consequence of the high fat diet, which was to be expected. It was, however, surprising when most of the offspring groups did not gain more weight than controls, nor did they suffer impaired glucose tolerance, even at advanced ages. Even when a subset of the offspring from the lard-fed groups was challenged with consumption of a high fat diet, they did not, for the most part, experience increased weight or impaired glucose tolerance as a consequence of maternal high fat
diet. Only the female offspring fed the intermediate fat diet exhibited decreased body weight.

There are several possible reasons that we did not discover the offspring outcomes that we had expected and that have been reported elsewhere in the literature. For one, we fed the control dams purified diets as opposed to chow diets, which is common in other studies. This could have important impact because chow diets are made from plant sourced materials that may contain phytonutrients that could, theoretically, impart effects on the control group that the high fat fed group would not receive. Second, the mouse strain that we used is a large, heavy mouse. While we have seen important improvements in offspring outcomes as a consequence of maternal exercise in this strain, this could purportedly be because the mouse is naturally experiencing a relatively poor metabolism, which exercise then improves. Third, smaller factors are likely at play, such as length of maternal high fat feeding before breeding, removal of the high fat diet at offspring day 14, and perhaps the use of second-time dams.

In summary, this study sheds light on the importance of careful study design, not only in the developmental programming field but in any field where animals are bred or fed purified diets. Ideally, content-matched diets would always be used, as opposed to a chow diet on one hand and a purified diet on the other. Future studies should revisit and refine the butter based 11% and 32% diets in particular.
5.2 Maternal Controlled Exercise

Exercise is an excellent, irreplaceable part of any healthful life. Exercise during pregnancy is extremely advantageous for the pregnant woman, with potential to decrease risk for preeclampsia and gestational diabetes and improve labor and delivery. Similarly, young offspring born to exercised women are leaner, even until five years of age, and score better on cognitive tests. Long term offspring consequences of maternal exercise, however, are yet unsubstantiated by human studies.

Previous studies have shown long term improvement in offspring glucose tolerance as a consequence of maternal exercise in rodents using voluntary exercise in the form of wheel running. The voluntary exercise model is, however, imperfect. For instance, mice run long and widely varied distances, and in exorbitant quantities. In that light, we demonstrated in chapter 3 a novel model of maternal exercise that is a safe intervention in mice. In addition, the exercise intervention improved glucose tolerance in non-pregnant animals and significantly improved lean to fat mass ratio in pregnant animals. Finally, young male offspring born to the exercised dams exhibit significantly improved glucose tolerance, though this effect faded with time.

This novel model of exercise in the pregnant mouse offers several advantages. For instance, not only do the mice all run the same distances, but they are also away from their cage for the same amount of time (sedentary control animals are removed from their cage and placed into the wheel system, but the wheels remain stationary). In addition, the amount of running that the mice undertake is very modest, which is arguably more
relevant to the human condition than the essentially constant exercise during the waking period that mice do when given voluntary running wheels. There are other advantages as well, including the fact that the breeding males have no access to the exercise wheels and there are no differences in environmental enrichment between control and exercise groups.

That we have shown decreased body weight and improved glucose tolerance as a consequence of the controlled exercise intervention is promising. It is exciting to see differences in the exercise group when compared with the control group, even with such a mild exercise intervention for such a short daily duration. We also used the third group that stayed in their home cage as the ultimate control group. This allowed us to show that the wheel exposure per se was not stressful to the mouse, as substantiated by the fact that there were no significant differences in pregnancy outcomes or fecal corticosterone levels.

Finally, young male offspring born to exercised dams showed significantly improved glucose tolerance, though this effect did not persist into old age. We propose that the aged offspring would have continued to have improved glucose tolerance if a larger number of subjects had been used, as the trends were still present. This is exciting to show that even modest amounts of maternal exercise can significantly improve offspring glucose tolerance. Further study is warranted to refine this model of maternal exercise.
For instance, when mice are given access to voluntary running wheels, they predominantly run in the dark, which is their waking phase. We completed the studies in chapter 3 in the light. Future studies should exercise the mice in the dark. Similarly, when given voluntary running wheels, mice run large amounts before pregnancy, but steadily decrease their distance ran as pregnancy progresses, and then maintain a very low daily distance throughout nursing. Future studies using the controlled exercise model should mimic this pattern of decreasing running as pregnancy progresses.

In summary, these studies demonstrate the safety of a novel model of exercise in the mouse during pregnancy. In addition, this exercise intervention improves glucose tolerance in non-pregnant animals and body composition in pregnant animals. Finally, young male offspring have improved glucose tolerance as a consequence of the maternal controlled exercise intervention and continue to have a trend toward improved glucose tolerance even at advanced ages.

5.3 Branched-Chain Amino Acids

The supplement industry in the United States is massive, and large portions of the population consume some type of dietary supplement regardless of whether or not there is any scientific evidence for their efficacy or even safety. Protein supplements are especially popular among exercising populations, and the BCAAs have been reported to play key roles in the proposed advantages offered by protein supplements. In chapter four, we hypothesized that a BCAA supplement would improve glucose tolerance and body composition in female mice. The intent of this study was to lay a foundation of
safety and efficacy of the supplement for future use as a pregnancy intervention to explore effects on offspring outcomes.

High fat feeding induced significant weight gain in the female mice, and exercise protected from some of this weight gain. Similarly, exercise significantly improved glucose tolerance in control diet-fed animals compared with sedentary control diet fed animals, and improved it in high fat fed animals compared with sedentary high fat fed animals. While we saw no impact on body weight or glucose tolerance as a consequence of the BCAA supplement, it is important to note that we saw no negative consequences either. Excitingly, the BCAA supplement significantly increased lean to fat mass ratio in exercise control diet-fed animals, where exercise alone did not. In addition, exercise plus high fat diet did not rescue lean to fat mass ratio to sedentary control diet-fed animals, but exercise high fat diet plus BCAAs did.

This is an important finding to suggest that BCAAs may indeed be efficacious at increasing training effect when combined with exercise, at least in female mice. Studies are lacking in female mice regarding BCAA supplements, and we have provided one such study to add to the field. It would be interesting if future studies further explored the dose of BCAAs, the route of delivery (water versus food delivery versus gavage dosing), and the intensity of exercise combined with BCAAs that illicit the most robust effect on body composition. Future studies should supplement a BCAA product during pregnancy to evaluate the safety and potential benefit on the outcome for the dam and the pups long-term.
5.4 Conclusions

Maternal high fat, high calorie diet consumption is likely never good for a pregnant woman or her offspring. Women should always strive toward maintaining the healthiest lifestyle that they can, not only while pregnant, but for their entire lives. However, in our mouse model, maternal high fat diet did not result in impaired glucose tolerance in offspring, and body weight was only affected in one offspring group. This suggests that the makeup of the diet is important far beyond what percentage of fat it contains. Finally, we must sometimes consider the relevance of any study outcome on a scale beyond a “significant p – value” and look more closely at study design and the comparisons that are being made.

Exercise has advantages for every human being and many animals as well. Exercise during pregnancy is an area of growing research. It is well supported that exercise during pregnancy improves outcomes for the mother – lower chance of gestational diabetes and preeclampsia, and an easier labor and delivery. In addition, promising studies show that maternal exercise can improve glucose tolerance, neuronal development, and cardiovascular parameters in offspring in animals. In rodents, however, the most common model of exercise is one of voluntary running, and in this document we explored the use of an alternate model with, arguably, more physiologic relevance. We showed promising outcomes in maternal parameters as well as improved glucose tolerance in young male offspring. This is exciting because even modest amounts of exercise were able to impart these advantageous effects.
One only has to go to any gym where people are exercising or drive past a retail store entirely devoted to selling supplements to appreciate the prevalence of dietary supplementation in the United States. Whether or not there is empirical evidence regarding the use of these readily available products, people are using them fairly ubiquitously. We are excited to show the potential utility of one such commercially available product in female mice. When combined with exercise, the BCAA supplement increased lean to fat mass ratio where exercise alone did not. This promising result leads to an excellent opportunity to supplement this product during pregnancy in rodents to determine its utility, or, at least, its safety. Many women who exercise and consume BCAAs regularly may cease consuming it during their pregnancy because its safety has not been evaluated.

5.5 Future Directions

The current studies were all carried out in female mice. There is, however, a growing body of literature regarding the impact of paternal influence. In chapter 2, males consumed the high fat diets while they were housed with the females for mating. Although this is only a brief window of high fat diet exposure for the male, it would be ideal to eliminate this exposure in future studies.

In addition, some of the diets used in chapter 2 were not sucrose matched. Future studies should employ only diets that are content matched. It was to our advantage, however, that we did employ purified control diets as opposed to chow diets for the
control animals. Ultimately, in the future, purified diets should be designed that are precisely content matched to the high-fat diets employed in any feeding study.

In chapter 3, the exercise intervention was always completed after lights-on. Mice, naturally nocturnal, run in the dark when given access to voluntary running wheels. Therefore, future studies should complete the intervention in the dark. Also, the pace of running was maintained throughout pregnancy and resumed after parturition. When given the choice, pregnant female mice decrease their run distance as pregnancy progresses, and maintain a very modest amount of activity during nursing. Future studies should mimic this natural pattern.

In chapter 4, the study was completed using voluntary running. This method of exercise, while commonly used, may not be the most physiologically relevant. This is because mice naturally run great distances for long durations when given the opportunity. Humans do not run with little break from sun-up to sunset. On the contrary, most humans may exercise for up to an hour a day, if at all. Current recommendations stand at 30 minutes of moderate intensity activity, most days of the week. Therefore, it would be ideal for future studies to employ a more modest amount of exercise in combination with BCAA supplement. The controlled exercise model would be a good choice. Alternately, the wheels could be locked and only unlocked for small durations throughout the week.

In conclusion, this dissertation has explored several important aspects of health behavior during pregnancy in mice. While consumption of high fat diet during pregnancy is never a good thing, we found minimal offspring consequences due to high fat diet. An
important message from this set of studies is that it is vital to pursue careful study design when using high fat feeding in mice. In addition, exercise during pregnancy is becoming a prominent area of interest. While voluntary exercise has been shown to improve offspring glucose tolerance, it is an imperfect intervention. The controlled exercise model used herein is a promising alternative. Third, the supplement industry in the United States is booming, and pregnant women are encouraged to consume rather high amounts of protein. BCAAs supplemented in female mice increased lean to fat mass ratio when combined with exercise. This exciting finding warrants further exploration of BCAAs as a pregnancy intervention. In summary, pregnancy provides a window of time during which both negative and positive behaviors may have dramatic impact on long-term health.
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      during murine pregnancy,” Graduate Center for Nutritional
      Sciences, Departmental Seminar Series
2013  “High fat diet, exercise, and branched chain amino acid
      supplementation in female mice,” Graduate Center for Nutritional
      Sciences, Departmental Seminar Series
2013  “Adult offspring of high fat fed dams are not different than
      controls,” Graduate Center for Nutritional Sciences, Departmental
      Seminar Series
2014 “A controlled exercise study saga,” Department of Pharmacology and Nutritional Sciences, Departmental Research Meeting
2014 “Impact of Positive and Negative Health Behaviors on Female Mice and/or their Offspring,” Defense Seminar

POSTER PRESENTATIONS
2008 Mitchell, K., and G. W. Gearner. “DNA Fingerprinting of Escherichia coli as a Tool to Track Host Sources of Watershed Fecal Contamination,” Celebration of Student Research Showcase. Morehead State University, Morehead, KY.
C57BL/6 Achilles tendon biomechanical properties,” Orthopaedic Research Society Annual Meeting, San Antonio, TX.


2014 **Platt, K.M.** and K.J. Pearson. “Branched-Chain Amino Acid Supplementation in Combination with Voluntary Running Improves Body Composition in Female C57BL/6 Mice.” South Eastern Conference Obesity Symposium, Atlanta, GA.


TEACHING
2014  Anatomy Teaching Certificate, University of Kentucky College of Medicine

Teaching Assistantship
ANA 209  Human Anatomy, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Fall 2013
IBS 610  Critical Readings in Integrated Biomedical Sciences, University of Kentucky College of Medicine, Fall 2013
ANA 611  Human Gross Anatomy, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Spring 2014
ANA 209  Human Anatomy, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Spring 2014

Instructor
BIO 137L  Anatomy and Physiology I Lab, Division of Natural Sciences, Bluegrass Community and Technical College, Summer 2013
BIO 137  Anatomy and Physiology I, Division of Natural Sciences, Bluegrass Community and Technical College, Fall 2013

Professional Development
2013  “Teaching Technologies Workshop,” Center for Excellence in Medical Education, College of Medicine Office of Medical Education, University of Kentucky
2013  “How Do I Handle This? Challenging Situations In and Out of the Classroom,” Center for the Enhancement of Learning and Teaching, University of Kentucky
2013  “Working with Distressed and Distressing Students,” Office of Faculty Enhancement, University of Kentucky
2013  “Making Lectures Engaging and Interactive,” Center for the Enhancement of Learning and Teaching, University of Kentucky
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<td>2014</td>
<td>“Defining and Cultivating Critical Thinking among your Students,”</td>
<td>Center for the Enhancement of Learning and Teaching, University of Kentucky</td>
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<td>2014</td>
<td>“Assertion-Evidence Practice: Rethinking Presentation Skills,”</td>
<td>Center for the Enhancement of Learning and Teaching, University of Kentucky</td>
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<td>2014</td>
<td>“Cheating: Curbing, Catching, and Consequences,”</td>
<td>Center for the Enhancement of Learning and Teaching, University of Kentucky</td>
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