Maternal and Paternal Obesity Differentially Impacts Body Weight and Glycemic Control in Male and Female Offspring

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Abstract

As the rate of obesity is increasing among children born to obese parents, our aim was to delineate how parental obesity promotes obesity risk in the offspring. A secondary aim was to determine if offspring born to obese parents would also be at risk of developing obesity-related metabolic dysfunction. Accordingly, the first project of this thesis work focused on maternal obesity and the prevalence of obesity and metabolic dysfunction in the offspring, which we assessed via recording of body weights, total adiposity, and overall glucose homeostasis. The second project of the thesis was explicitly focused on paternal obesity and how it may increase the risk of obesity and subsequent metabolic dysfunction in the developing offspring.

To address our aims, C57BL/6J female mice were either fed a high fat diet (HFD) (60% kcal from lard) or a low fat diet (LFD) (10% kcal from lard) for 5 weeks. After 5 weeks of HFD or LFD feeding, they were bred with a 12- week old C57BL/6J male. All offspring were weaned onto a LFD and kept until 14 weeks of age. Magnetic resonance imaging (MRI) and metabolic cage analyses were utilized to assess overall adiposity and in vivo energy metabolism, while glucose homeostasis was also monitored by glucose and insulin tolerance testing. In addition, body weights were recorded frequently to assess their risk for obesity. Our results demonstrate that the female offspring born to an obese dam had an increase in body weight at weaning (3 weeks of age), whereas at 14 weeks of age they had a reduction in body weight versus offspring born to lean dams. However, fat mass was not different at any age range between our various

groups of offspring. Last, measuring the expression of PGC1α in muscle, a key regulator of muscle energy metabolism, also revealed no differences between our various groups of offspring.

In our second study, C57BL/6J male mice consumed a HFD or LFD for 5 weeks prior to breeding with a C57BL/6J lean female. The offspring were weaned onto either a LFD or HFD. We performed magnetic resonance imaging (MRI) and metabolic cage analyses to assess adiposity and energy metabolism respectively. In addition, glucose and insulin tolerance tests were conducted to assess glucose homeostasis. In addition, body weights were recorded frequently to assess the risk for obesity. To our surprise, only the LFD offspring showed a significantly heavier body weights at the age of weaning in both male/ female offspring; however, the HFD offspring group revealed similar body weight curves, regardless if the male parent was lean or obese. Surprisingly, offspring whose male parent was obese showed no differences in glucose tolerance and body weight gain compared to offspring counterparts whose male parent was lean. Last, we measured *Ucp*$_2$ gene in muscle tissues of LFD offspring, a mitochondrial uncoupling protein that reduces ATP production and regulates thermogenesis. We observed a trend to higher *Ucp*$_2$ expression in male offspring in which the male parent was obese versus lean. This trend was reversed in the female offspring. Taken together, one of the main conclusions of our research is that both maternal and paternal obesity do not increase the risk for obesity in the offspring.

Preface

This thesis document is an original work by Hanin Aburasayn under the supervision of Dr. John Ussher. Most of the work had been conducted in the Ussher lab (2-055 Katz) in the Faculty of Pharmacy and Pharmaceutical Sciences. Some of the experiments were also conducted at various core facilities present at the University of Alberta.

Chapter 3 of this thesis document is a published work by Hanin Aburasayn, Rami Al Batran, Keshav Gopal, Malak Almutairi, Amina Eshreif, Farah Eaton and John Ussher entitled with " Female offspring born to obese and insulin resistant dams are not at increased risk for obesity and metabolic dysfunction during early development" in the ***Canadian Journal of Physiology and Pharmacology, 96(1), 97-102, Sept 2017,8***.

Dedication

This thesis is dedicated to the memory of my grandfather, Hussein Felemban, who was an excellent model for all the family. He always believed that education is the only way for any person to be a better version of her/himself.

This work is also dedicated in memory of King, Abdullah bin Abdulaziz, who supported many Saudis with a scholarship for the purpose of education. I have been honored to be one of those students.

To my best friend and supportive husband, Rayan Qara, who encouraged and supported me through my study period, who always believed in my abilities and skills, as I do believe in him.

To my two princesses, my daughters Lana and Leen Qara, who hold hands very tight and spread joy, hope and love everywhere through this journey.

To my father Mohammed Aburasayn, who made me a strong personality.

To my mother Samiayh Felemban for her emotional and financial support who remembered me always in her prayers.

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$-CH_3$	Methyl group
Akt	Serine/threonine-specific protein kinase
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
C57BL6	Wild type black mouse strain from Jackson laboratory
cDNA	Complementary deoxyribonucleic acid
C/EBPs	CCAAT enhancer binding proteins
c- fos	Proto- oncogene fos
CHST8	Carbohydrate sulfotransferase 8
CNS	Central nervous system
CO_2	Carbon dioxide
CS	Citrate synthase
CV	Caloric value
DNMT	DNA methyltransferase enzyme
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
Egr-1	Early growth response protein 1
EDTA	Anticoagulant agent

eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal–regulated kinase
FDA	Food and drug administration
FFA	Free fatty acid
FTO	Fat mass and obesity associated gene
GIT	Gastrointestinal tract
GTT	Glucose tolerance test
HDL	High density lipoproteins
HFD	high fat diet
hr	Hour
IGT	Impaired glucose tolerance
ITT	Insulin tolerance test
IL- 6	Interleukin 6
Jhdm2a	JmjC domain–containing histone demethylase 2A
Kcal/kg	Kilocalorie/ kilogram
KO	Knock-out mice
LCAD	Long chain fatty acyl CoA dehydrogenase
LFD	low fat diet
m^2	Meters squared
MCAD	Medium chain fatty acyl CoA dehydrogenase
MC4R	Melanocortin-4 receptor
mg/kg	Milligram/ kilogram
MRI	Magnetic resonance imaging

mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NAFLD	Non- alcoholic fatty liver disease
NEFA	Non-esterified free fatty acids
NFkB-65	Eukaryotic transcription factor nuclear factor-kappa B
Oral GTT	Oral glucose tolerance test
PARS	Predictive adaptive response
PCR	Polymerase chain reaction
Pdk4	Pyruvate dehydrogenase lipoamide kinase isozyme 4 gene
PGC1□	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Ppargc1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha gene
*Ppar*a	Peroxisome proliferator-activated receptor-alpha gene
PPAR-γ	peroxisome proliferator-activated receptor-γ
Ppia	Peptidyl-prolyl isomerase gene, which is known as cyclophilin
PTT	Pyruvate tolerance test
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
RXRa	Retinoid X receptor alpha
SEM	Standard error of the mean
SOD1	Superoxide dismutase
T2D	Type II diabetes

TNF-□	Tumor necrosis factor alpha
TZDs	Thiazolidinediones
U/kg	Unit per kilogram
UCP_1	uncoupling protein 1
UCP_2	uncoupling protein 2
UCP_3	uncoupling protein 3
VCO_2	Carbon dioxide production volume
VO_2	Oxygen consumption volume
WAT	White adipose tissue
WHO	World health organization
WT	Wild type mice

Chapter One

1. Relevant Background Information

1.1 The Obesity Epidemic:

Obesity is a serious medical condition that has been increasing dramatically worldwide these past few decades. This increase in obesity is not restricted to high-income countries, as it is also rising among low income and middle-income countries, due to changes in eating habits and sedentary lifestyles (Organization 2014). According to numerous research studies, obesity is a threat to every age category in adults, the elderly, and our children/adolescent population. In an epidemiology study by Ogden et al (2016), the obesity prevalence of US Children and adults was compared between two periods, 1988-1994 and 2003-2004, based on multiple surveys (Ogden et al. 2016). Their results showed a significant increase in obesity percentage in children aged 2 to 5 years with a rate of 7.2% in the 1988-1994 period, which increased to 13.9% in the 2003-2004 period. In addition, older children aged 6 to 11 years also had massive increases in obesity prevalence, increasing from 11.3% in 1988-1994 to 19.6% in 2007-2008 (Ogden et al. 2016). In addition, obesity percentage had changed in youths aged 12 to 19 years between 1988-1994 with 10.5%, while the percentage increased to 20.6% in 2013-2014 (Ogden et al. 2016). In addition, among adults who are suffering from obesity in the US, more than 60% of pregnant women are obese or overweight (Flegal et al. 2010). In addition, the Canadian obese population aged 20- 39 years shows a similar increasing pattern to US obesity prevalence rates. In the late 1980s to 2007-2009, the obesity percentage in Canadian men increased from 9.5% to 18.9%, whereas Canadian

women demonstrated an increase from 9.3% to 20.8% (Giroux 2006). This increase in obesity prevalence affects the world's population, as the World Health Organization (WHO) estimates that ~300 million women and ~200 million men are obese (Organization 2014). Furthermore, it is currently estimated that 42 million children are obese or overweight around the world (Organization 2014). Based on current research projections, it is estimated that there will be 60 millions obese children worldwide by 2020 (De Onis et al. 2010).

1.2 Complications of Obesity:

Based on previous statistics, the increased obesity prevalence is a serious medical issue threatening future generations due to obesity-related complications and obesity-related metabolic disorders. Importantly, obese children are at an increased risk for developing metabolic syndrome, insulin resistance, type 2 diabetes (T2D) and various cardiovascular diseases as significant comorbidities during adulthood (Dietz 1998; Furukawa et al. 2017; Sahoo et al. 2015). Moreover, metabolic syndrome is an adverse health issue that has been linked to an increasing prevalence of obesity (Furukawa et al. 2017; Reaven 1988). Obesity is also a well characterized risk factor for developing diabetes, and their rates of incidence increase in parallel to each other over time (Hannon et al. 2005; McKinlay and Marceau 2000). In Canada for instance, 1.5 - 20 out of 100 000 youth under the age of 18 are T2D individuals (Amed et al. 2010). Another example in the United States within the past few years, there was a high increase

in the prevalence of T2D by 33 %, which was associated with a corresponding increase in obesity prevalence (Catalano et al. 2003). Other chronic complications associated with obesity include hyperlipidemia, hypertension, and sleep apnea and orthopedic complications, which can negatively affect the patient's quality of life (Dietz 1998). Furthermore, a chronic complication that had been strongly linked to obesity is non- alcoholic fatty liver disease (NAFLD) which is now considered as the primary cause for other chronic liver diseases (Almeda-Valdes et al. 2014).

Along with previously mentioned complications, recent research papers in the field are linking obesity to inflammation markers (Bastard et al. 2006). For instance, several animal obese models have shown increased expression of tumor necrosis factor-alpha (TNF-α) in adipose tissues, which links obesity to inflammation as TNF-α is a well-characterized inflammatory cytokine that decreases insulin sensitivity in obesity (Hotamisligil et al. 1993; Jorge et al. 2018; Uysal et al. 1997). In addition, leptin is another example of an adipose tissue secreted hormone that has a vital role in energy metabolism and immune regulation. Human and mice studies have found that leptin-deficient models may alter immune responses, explicitly causing a suppressed immune response (Chandra 1980; Fernandes et al. 1978; Lord et al. 1998; Procaccini et al. 2017). It should be noted that leptin plays an important role in energy homeostasis since it regulates appetite and energy expenditure through acting on leptin receptors present in the hypothalamus (Li 2011). On the other hand, some studies have found links between leptin hyperregulation and obesity in the offspring. For

example, a study conducted by Lecoutre and colleagues demonstrated that male offspring rats born to dams that were fed a HFD before and during the pregnancy/lactation period, had heavier body weights at weaning than offspring born to lean rat dams, and higher circulating leptin levels. Furthermore, weight gain in the male offspring was monitored until three months of age, and resulted in glucose intolerance and hyperinsulinemia as they aged (Lecoutre et al. 2016).

1.3 Obesity Causes and Classification:

Obesity develops when there is a high amount of fat accumulated within body tissues due to positive energy balance, which is when the amount of consumed energy, is higher than the amount of expended energy (Silventoinen et al. 2010). As a result, an imbalance between energy consumption and energy expenditure can lead to obesity development (Sandholt et al. 2012). More specific definitions of obesity have been defined by Sun et al., where they define obesity as an accumulation of white adipose tissue (WAT) in obese individuals that will lead to metabolic disorders (Sun et al. 2011). Accumulated WAT mainly results from an increase in the number of adipocytes, which is known as hyperplasia, or increasing the size of the adipocytes, which is known as hypertrophy (Björntorp and Sjöström 1971).

According to WHO for obesity prevention recommendation's, energy balance in an individual is affected negatively by their eating behaviors, which often contain a high level of fat, specifically consumption of saturated fat or trans

fat more than unsaturated fats (Organization 2014). In addition, high amount of carbohydrate is another leading cause of imbalance the consumption energy along with the lack of exercise or physical activity. The overconsumption of carbohydrates and the lack of exercise both will decrease the expenditure energy, both leading to fat accumulation within the body (Organization 2014). Other leading causes are therapeutics agents such as diabetes medications, psychiatry medications, steroid hormones and contraceptives besides some different genetics, environmental and physiological causes (Wright and Aronne 2012). For instance, clozapine is an antipsychotic medicine that increases body weight gain in patients which is also associated with increasing levels of circulating triglycerides (Aronne and Segal 2003). In addition, diabetes medications such as insulin and thaiazolidinediones also cause weight gain as a common side effect for their users (McFarlane 2009). This weight gain is contributed to the improvement in glycemic control of the patients (McFarlane 2009).

The most common diagnostic tool to determine obesity is through measuring the height and weight of an individual, known as the body mass index (BMI). The formula for BMI is the weight in kilograms (kg) divided by height in meters squared (m^2) rounded to the nearest tenth (Flegal et al. 2010). For obesity classification based on BMI adults with BMI= 25-29.9 kg/m^2 score is considered overweight, while a score of BMI □30 kg/m^2 is considered obese (Pi-Sunyer et al. 1998). Further categorization by WHO had divided obesity to as (BMI= 30- □ 35 kg/m^2) as grade 1, (BMI= 35-<40 kg/m^2) as grade 2 and (BMI ≥40

kg/m^2) as grade 3 of obesity (Organization 1995). To be noted, higher grades of obesity are combined with the higher rate of mortality mainly as a result of cardiovascular diseases, diabetes and some types of cancers (Malnick and Knobler 2006; Orpana et al. 2010). The following table (Table 1- 1) is summarizing the classification of obesity based on BMI and WHO classification of obesity.

Table 1- 1:

Obesity Classification.

Classification	**BMI (kg/m^2)**
Normal weight	18.5- 24.9
Overweight	25-29.9
Obesity grade 1	30-<35
Obesity grade 2	35-<40
Obesity grade 3	≥40

The previous methods are indirect metrics to infer general obesity in people. There are also direct methods to more accurately measure fat content, but these methods are not commonly used in hospitals. These methods calculate the fat mass in the body from an individual's body weight and fat-free mass. Hydro densitometry, skinfold measurement, bioelectrical impedance analysis,

and dual-energy x-ray absorptiometry are examples of these direct measure methods (Hanley et al. 2010).

1.4 Obesity Prevention and Treatment:

There are ways to prevent fat accumulation and obesity complications such as lifestyle modifications or pharmacotherapy. However, if either of these fail, surgical methods such as gastric bypass may need to be considered.

Lifestyle modifications consist of diet modification to decrease caloric intake, improving physical activity through exercise. In addition, dietitian or psychiatric follow up through diet restriction or behavioral therapy are ways to improve the lifestyle of obese individuals (Wadden et al. 2013). Most obese people who maintain restricted lifestyle modifications can lose weight quickly, but it is easy to regain the lost weight if they fail to keep up with their lifestyle modifications (Wadden et al. 2011). For instance, reducing the daily calorie of food intake by 500-1000 kcal/day through lowering fat and carbohydrates intake is a diet modification to induce weight loss (Health 2000). Keeping regular exercise such as brisk walking for 30 minutes /day is an example of the physical activity that can help in reducing weight for obese individuals (Health 2000).

Nevertheless, when individuals fail to lose targeted weight through exercise and diet, and when obesity complications start to develop, a therapeutic agent can be used under medical supervision. For instance, obese individuals with BMI □ 30 who show signs of complications are recommended to undergo

pharmacotherapy (Patel 2015). For the therapeutic choices for preventing obesity and reducing body weight, there are two main classes of drugs (short-acting and long-acting therapies). The mechanism of action for the short-acting class is through suppressing the appetite by inhibiting the reuptake of noradrenaline in the central nervous system (Joo and Lee 2014). They include phentermine, diethylpropion, phendimetrazine, and benzphentamine, which are approved by the Food and Drug Administration (FDA) (Joo and Lee 2014; Patel 2015; Sweeting et al. 2015). On the other hand, long acting therapies comes as single medicine (orlistat, lorcaserin, and liraglutide) or combination medicine (naltrexone/bupropion and phentermine/topiramate) (Patel 2015; Sweeting et al. 2015). In some cases when obesity is severe and when previous methods of lifestyle modification and exercise are not successful, then some patients based on medical advice can choose bariatric surgery. Surgical intervention is considered the best option for preventing disease and providing noticeable weight loss, especially in severely obesity individuals (Aguilar-Olivos et al. 2016). Often a reversed of dyslipidemia, insulin resistance, adipokine levels and hepatic inflammation will be achieved following surgery (Sasaki et al. 2014).

Bariatric surgery is mainly divided into malabsorptive, restrictive procedures, or both. In malabsorptive operation, two-thirds of the distal stomach are removed then the proximal part of the stomach is attached directly to the ilium where the duodenum and jejunum are bypassed (Patel 2015). A well-known example of this type of procedure are the Roux-en-Y and resectional gastric bypass procedures (Colquitt et al. 2014). Conversely, the restrictive procedure

utilizes an inflatable silicone device that is inserted in the upper part of the stomach, while the size of the device can be controlled (Patel 2015). Another restrictive procedure is the sleeve gastrectomy where the surgeon makes a vertical incision for the purpose of reducing the size of the stomach by 25% less than its original size. (Figure 1- 1) shows a simple scheme illustrating the differences between the various types of bariatric procedures. The restrictive surgery may follow by other procedure after 6-12 months such as, gastric bypass if the weight loss target did not achieve by the sleeve procedure alone (Colquitt et al. 2014).

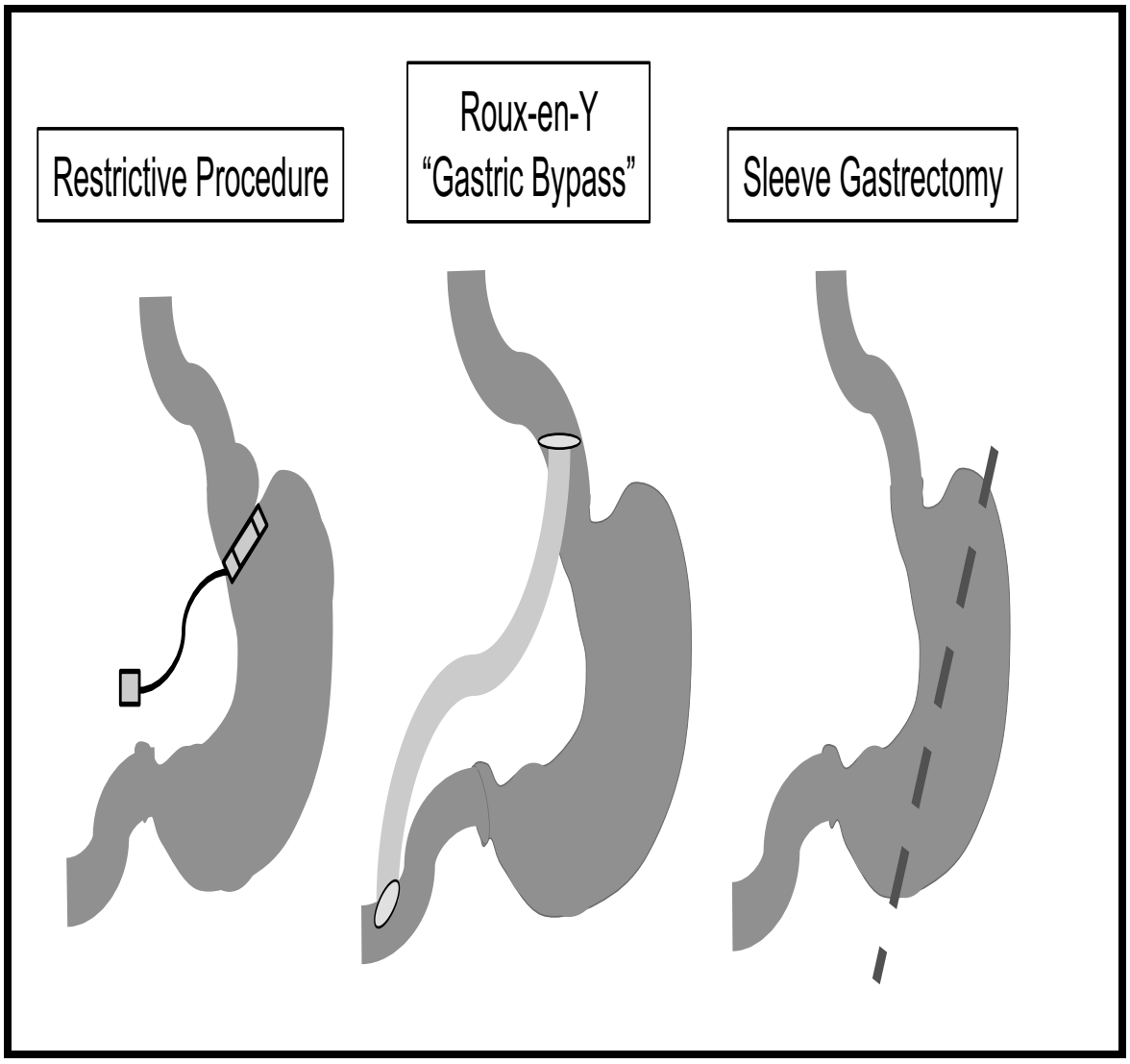

Figure 1- 1 Different types of Bariatric Surgeries. Adapted from (Vetter et al. 2012).

Currently, some promising studies are focusing on finding new pharmacological approaches to treat obesity, although none of these are yet clinically approved. Of those, targeting mitochondrial uncoupling proteins (UCP_s)

has received three patents as a target treatment for obesity (Jandacek and Woods 2004).

The over-expression of the mitochondrial uncoupling proteins such as, (UCP_1 UCP_2 and UCP_3) may protect individuals against obesity through producing heat as a result of uncoupling oxygen consumption from the production of energy (adenosine triphosphate (ATP)) (Figure 1- 2). The regulation of thermogenesis and reducing the mitochondrial toxicity are two suggested explanations of how UCP_s may protect against the risk of obesity (Nedergaard and Cannon 2003).

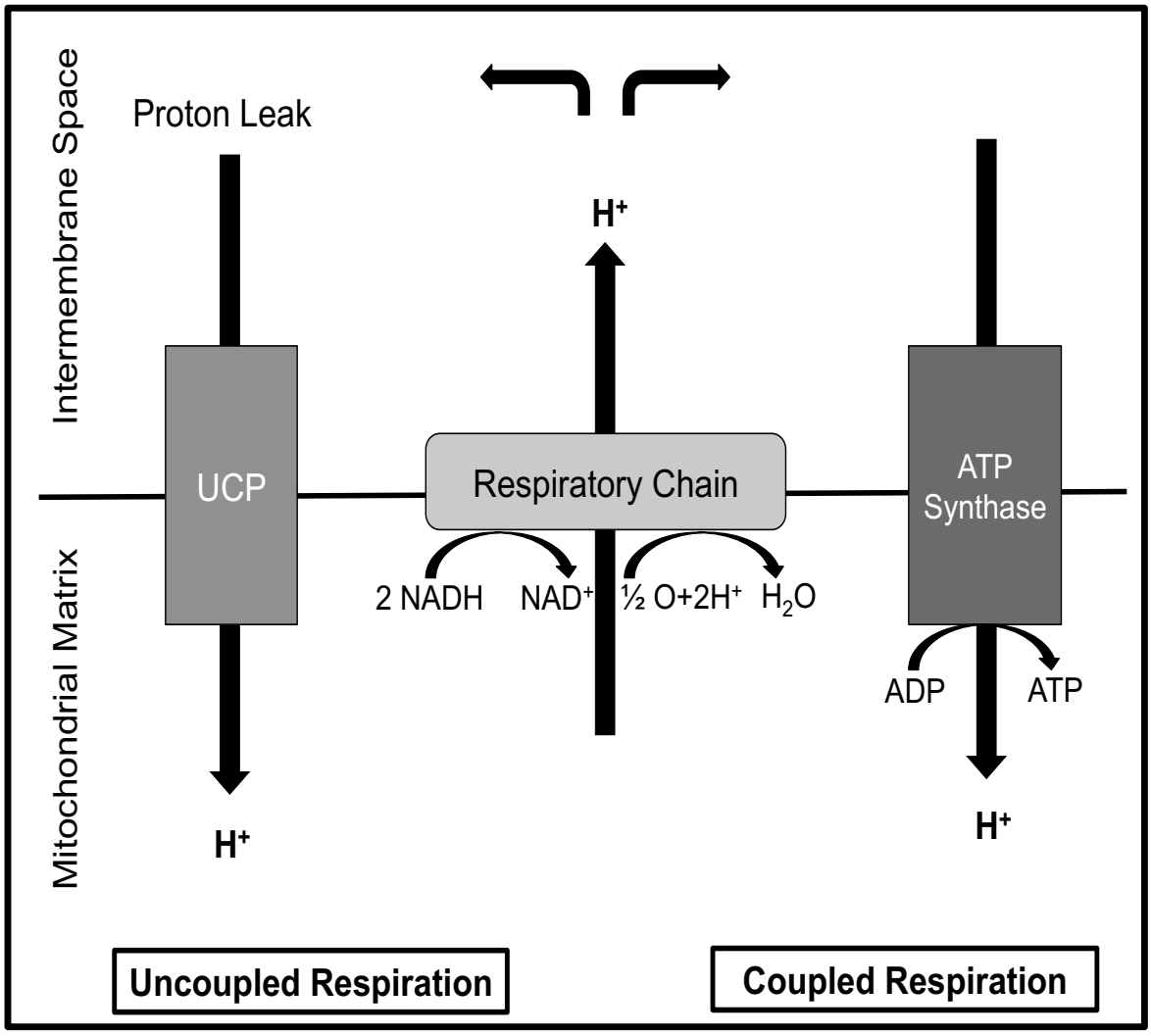

Figure 1- 2 Hydrogen that result from the respiratory chain reaction at the inner membrane of the mitochondria may produce ATP or may leak through UCPs which prevent ATP production. Reproduced from (Hirschberg et al. 2011).

For instance, UCP_1 is well known as thermogenin, which is a mitochondrial carrier expressed specifically in the Brown Adipose Tissue (BAT) (Nedergaard and Cannon 2003). In fact, some knock-out UCP_1 C57BL6 black mice (KO) have shown a remarkable reduction in food intake results when

comparing to wild-type mice (WT). Even though both groups were set up on HFD for 4- weeks, the KO had unexpected reduction in the food intake that was not expected from placing them on HFD, but what is really considering an interesting finding is that KO mice had shown a significant gain in body fat and increase in the total % of fat disposition comparing to WT (von Essen et al. 2017). Thus, these findings suggest that UCP_1 ablation is a factor that induces obesity and the storage of fat in the body, while the presence of UCP_1 may attenuate obesity via inducing thermogenesis in the setting of obesity (von Essen et al. 2017). It has also been demonstrated in transgenic mice overexpressing human UCP_3 in their skeletal muscle, that appetite is increased, but in contrast their body weights are reduced verses their WT littermates (Clapham et al. 2000). In addition, the UCP_3 overexpressed mice showed a reduction in fat mass which was associated with a better glucose clearance and a reduction in their circulating blood glucose, and insulin levels (Clapham et al. 2000).

Nonetheless, treating the obese or overweight population is a real challenge as treatment often takes place after severe obesity is already developed (Flegal et al. 2010), which will keep the obesity prevalence at a high rate. Although, pharmacological treatments are available to treat obesity consequences such as, diabetes, all studies that target obesity specifically have failed in finding a pharmacological treatment to cure obesity itself. As a consequence of the high rate of obesity, morbidities and mortalities of the affected population and their future generations will be negatively affected, which is a considerable burden on health systems costs for the present and the future

(Van Dijk et al. 2015). For these reasons, understanding how obesity and possible environmental and/ or genetic factors increase obesity development in subsequent generation is essential, which may help us prevent the coming generations from developing obesity and it's metabolic consequences.

1.5 The Link Between Parental Obesity & Obesity Risk in Adolescence:

WHO last estimation of obesity prevalence had revealed that there are ~ 300 million women and ~ 200 million men are obese worldwide. There are numbers of animal and human studies that have shown that children born to obese parents are likely to be heavier and at higher risk of obesity and obesity-related disorders than children born to lean parents (Catalano et al. 2003; McCurdy et al. 2009; Sen and Simmons 2010; Shankar et al. 2008). Maternal obesity complications such as stillbirth, macrosomia, and cesarean section are a threat to the health of both the mother and infant (Blackmore and Ozanne 2013). In addition, gestational diabetic mothers who have gestational diabetes as the main complication of obesity during pregnancy have been reported to give birth to heavier babies compared to nondiabetic mothers (Ehrenberg et al. 2004), in addition to having an increase in markers of cardiovascular disease like (e.g. BMI) (West et al. 2011). Of interest, paternal obesity, and not just maternal obesity within the family, will increase the risk of obesity during childhood/adolescence (Whitaker et al. 1997).

1.5.1 Maternal Obesity Studies:

Studies by (Knopp et al. 1985) have suggested that concentrations of triacylglycerol and free fatty acids (FFA) in pregnant women are positively linked to neonatal birth weight due to crossing the placenta in the last trimester of pregnancy. Another study reported similar results, where they observed links between obesity in pregnant women to heavier birth weight and skinfold measurements, in addition to higher serum FFA in neonates born to obese mothers compared to neonates born to lean mothers (Kliegman et al. 1984).In addition, an animal study found similar findings in pregnant sheep model with hyperinsulinemia resulting in elevated insulin in their fetus. That was associated with a decrease in FFA concentration and lipolysis process; on the other hand, it was associated with an increase in fat deposition of the fetus (Ogburn et al. 1989). Another animal study observed the effect of maternal obesity on offspring obesity through overfeeding female rats for 3 weeks with 220 kcal/kg per day, while lean females were fed 187 kcal/kg per day (Shankar et al. 2008). These females were subsequently used for breeding, and after breeding and subsequent cross-fostering to surrogate dams, offspring that were born to obese dams did not have differences in their body weights comparing to the offspring that were born to lean dams. However, body weight gain and body fat % in the

obese dams' offspring were markedly higher than the lean dams' offspring. Another study conducted on female Sprague Dawley rats who were either fed regular chow or western diet for 2 weeks, then mated to males rats and had their offspring weaned onto regular chow, revealed that the western diet increased offspring adiposity, which was accompanied by glucose intolerance (Sen and Simmons 2010). Moreover, a recent paper studied the effect of exercise on HFD-fed dams and the general health of their associated offspring (Stanford et al. 2017). C57BL/6 mice were used as a model for their study where they had four groups of females; 1) a HFD female exercised before and during pregnancy, 2) a HFD female exercised just during pregnancy, 3) a HFD female trained before pregnancy or, 4) a HFD female that was sedentary. The animals were then bred to lean males mice fed standard chow. The female offspring that were born to obese dams exhibited heavier body weights than offspring that were born to exercised mothers (both groups who trained pre and during pregnancy and who only trained in pregnancy period). In addition, their results had shown that HFD dams that were not exercised had female offspring with worsen glucose clearance compared to the control group at both 36 weeks and 52 weeks of age. Another interesting finding is that offspring born to exercised dams did not show impaired glucose tolerance, reduced insulin sensitivity, or increased adiposity. These exciting findings were associated with a better liver function profile in isolated liver cells, as the amount of both liver triglyceride and liver enzyme expression, those involved in glucose metabolism such as pyruvate dehydrogenase lipoamide kinase Isozyme 4 (PDK4) and citrate synthase (CS),

was enhanced in the offspring of exercised dams. These results suggest that exercise can completely reverse the negative effects of the HFD on the dam's developing offspring (Stanford et al. 2017).

Similarly, a study conducted by (McCurdy et al. 2009) had found that offspring born to HFD-fed female monkeys have three times more liver triglycerides, as well as increased liver oxidative stress and gluconeogenic enzymes, which might contribute to developing pediatric nonalcoholic fatty liver disease. Interestingly, switching the diet from HFD to a LFD during pregnancy reversed the level of hepatic steatosis and gluconeogenic enzyme expression (McCurdy et al. 2009).

1.5.2 Paternal Obesity Studies:

Current evidence in animal and human studies has also linked paternal obesity to increased risk for childhood obesity. Although there is less evidence to explain mechanisms for the influence of paternal obesity on childhood obesity risk versus that for maternal obesity, some studies have observed such a risk (Maffeis et al. 1998). For example, BMI of both mother and father was a predictor for their children BMI (Maffeis et al. 1998). A study conducted in Sprague-Dawley rats found that chronic consumption of HFD in males produced β-cell 'dysfunction' in the female rat offspring (Ng et al. 2010). Moreover, in the same study they found a positive correlation between HFD consumption in males and increased body weight, adiposity, glucose intolerance and insulin resistance in

female offspring comparing to control litters. Moreover, they utilized two groups of Sprague-Dawley male rats to conduct their research. One group was placed on a HFD while the other group was placed on a regular chow diet since the rats were 4 weeks of age until they reached 13 weeks. The model produced glucose intolerance and insulin resistance in animals, which was associated with heavier body weight, higher energy intake and higher cumulative energy intake in the obese male group. Then, obese males were bred to regular chow diet female mice. The resulting female offspring showed no changes in body weight or energy intake measurements. However, at 6 weeks and 12 weeks of age, the female offspring now showed higher circulating glucose levels and lower insulin secretion compared to controls. This data suggests that cell impairment in obese male offspring (Ng et al. 2010).

In addition, another study in C57BL6 mice fed either a HFD or regular chow for 10 weeks produced two further generations of offspring that demonstrated an increased obesity risk (Fullston et al. 2013). These results showed that obese C57BL6 male mice transmitted obesity and insulin resistance towards two subsequent offspring generations, despite the offspring being fed a regular chow diet throughout their lives since weaning (Fullston et al. 2013). These same investigators performed a follow-up study using the same obese male C57BL6 mouse model, but now fed the offspring either a HFD or a regular chow diet. They observed that the HFD male C57BL6 mice group produced offspring with the greatest body weights, adiposity, and levels of cholesterol,

triglyceride, and non-esterified free fatty acids (NEFA) compared to offspring born to male C57BL6 mice fed a regular chow diet (Fullston et al. 2015).

1.6 Theories Explaining Developmental Obesity in Children:

David Barker and colleagues produced one of the first theories to explain increased risk for childhood obesity, through observing a group of men since they were infants until they became 64 years old. Their body weights were recorded, and medical measurements, including circulating glucose levels, were recorded (Barker et al. 1989). Their theory referred to as the “fetal origins hypothesis” was the beginning of understanding that there is an inverse relationship between birth weight and mortality resulting from ischemic heart disease (Barker et al. 1989). Afterword, Barker and colleagues performed further investigations on the fetal origins hypothesis. Their studies revealed similar inverse observations associated with fetal birth weight and developing metabolic syndromes such as, impaired glucose tolerance (IGT) and T2D (Hales et al. 1991). Those results led to another hypothesis entitled the ‘thrifty phenotype hypothesis’, which suggested maternal unhealthy nutrition during pregnancy may result in fetus malnutrition in order to accommodate and survive the post-birth environment (Hales et al. 1991). Previous theories have also led to the concept of “developmental programming”, which is a broad concept that describes the neonatal regulation of body systems and cell metabolism pathways under the effect of the mother’s systemic risk factors during conception. Those risk factors include malnutrition,

the environment, and the mother or fetus genome factors, that result in modifications to body composition or metabolic syndrome of the fetus during their postneonatal lives and their future generations (Armitage et al. 2004; Barker 2004). The latest evolution of the “fetal origins” hypothesis that has been evolved in recent years is the concept termed the “Developmental Origins of Health and Disease (DOHaD)” which is also called “Developmental programming” or “Conditioning” (Barker 2004; Blackmore and Ozanne 2013). This concept, in particular, is referring to the etiology beyond individual’s diseases, and is not restricted to only preconception conditions and prenatal life. In addition, developmental programming is linked to the time pre- and during conception or fertilization of oocytes (Blackmore and Ozanne 2013), where those periods can be affected with overnutrition or undernutrition and environmental reasons of the parents that enhance their generations prevalence to diseases in their adulthood (Blackmore and Ozanne 2013). The DOHaD hypothesis considers the parent’s overnutrition or undernutrition as a temporary environmental trigger that may cause some changes in the fetus during developmental stage which will eventually cause long-lasting changes, such as chronic diseases that may last to their adult lives (Van Dijk et al. 2015). This concept suggests that as an adaptive mechanism of the fetus or offspring to adjust with intrauterine circumstances, which will lead to everlasting changes and long-term programming to compensate potential different environmental condition after birth (Gluckman et al. 2008). Different environments between neonatal life and adulthood life, which needs adaptive changes known as predictive adaptive response (PARS) that

may negatively lead to develop a disorder in the adulthood or inheritance changes that can pass from generations to others (Gluckman et al. 2008). To merely explain PARS, when individuals adapt well to the postneonatal environment as predicted from the parents' environment, they are considered adapters, but individuals who fail to adjust with the new environment may develop a disorder as a maladaptive mechanism (Uauy et al. 2011).

It should be noted that the DOHaD concept may categorize the previous mentioned high percentage of obese mothers during pregnancy as a risk factor that may influence obesity among their children (Catalano 2003; Shankar et al. 2008). Many mechanisms are involved in causing childhood obesity, and it is difficult to name one mechanism causing this phenomenon due to the complexity of obesity, overnutrition and their complications such as gestational diabetes (Drake and Reynolds 2010). Those mechanisms include inflammation, oxidative stress, lipotoxicity, affected adipocytes, hypercholesteremia, insulin signaling defects, renin-angiotensin system defects and epigenetics (Blackmore and Ozanne 2013). Most of those mechanisms are well-established and known, such as inflammation during pregnancy, which can result in impaired heart contraction of offspring and is accompanied by a high level of stearoyl-CoA desaturase, which is important for fat metabolism/synthesis, leading to mitochondrial damage (Dong et al. 2013). The same study reported phosphorylation of serine/threonine-specific protein kinase (Akt) and adenosine monophosphate-activated protein kinase (AMPK) with damage to insulin signaling (Dong et al. 2013). Some in vitro studies have also linked between the inflammation marker TNF-□ and affected

heart contractions (Friedrichs et al. 2002; Oral et al. 1997). Moreover, mice studies have revealed a high level of lipid in the hearts of mice that were born to obese dams fed a HFD (Turdi et al. 2011). In addition, adipocyte development in neonates and fetuses can be affected by a mother's obesity, leading to changes in the number and function of the neonatal adipocytes (Lecoutre and Breton 2014; Lukaszewski et al. 2013) primarily that adipocyte stem cells are plastic and sensitive to the mother's nutrition during neonatal age and fetus development (Tang and Lane 2012). For example, human adipocyte development is active in their early childhood, which will be a major factor in determining their fat mass when they are adults (Spalding et al. 2008). Insulin is another example that has been suggested as an expected mechanism that can cause childhood obesity. In a study on mice dams that were set on HFD during pregnancy and lactation, their circulating insulin levels were high which result in ventricular hypertrophy in their born offsprings. Due to suggested increase in insulin signaling activation proteins like Akt, extracellular signal-regulated kinase (ERK) and mechanistic target of rapamycin (mTOR) within those offspring (Fernandez-Twinn et al. 2012). In addition, rat offspring that were affected by obesity showed high levels of norepinephrine and renin in their renal tissues (Samuelsson et al. 2010).

1.7 Epigenetics

An attractive candidate that may contribute to the DOHaD concept and the increased risk for offspring obesity due to parental obesity involves alterations in

epigenetics. The word “epi-genetics” means the gene’s information “ above” the DNA sequence (Friso and Choi 2014). It encompasses the reversible heritable molecular process that change gene expression patterns without affecting the DNA sequence (Heerboth et al. 2014). This process results from the interaction of the genome of the organism with the surrounding environment through their lifespan period that will lead to gene function modifications, which may change the gene transcription (Gluckman et al. 2009; Heerboth et al. 2014). Most importantly is that epigenetic changes are heritable, which means they can be passed from parental cells to the daughter cells (Heerboth et al. 2014). There are some principal epigenetics modifications illustrated in (Figure 1- 3) that may change gene function/expression and be passed on to the next generation, which includes DNA methylation and histone methylation, acetylation of histone, and chromatin modifications (Ptak and Petronis 2008).

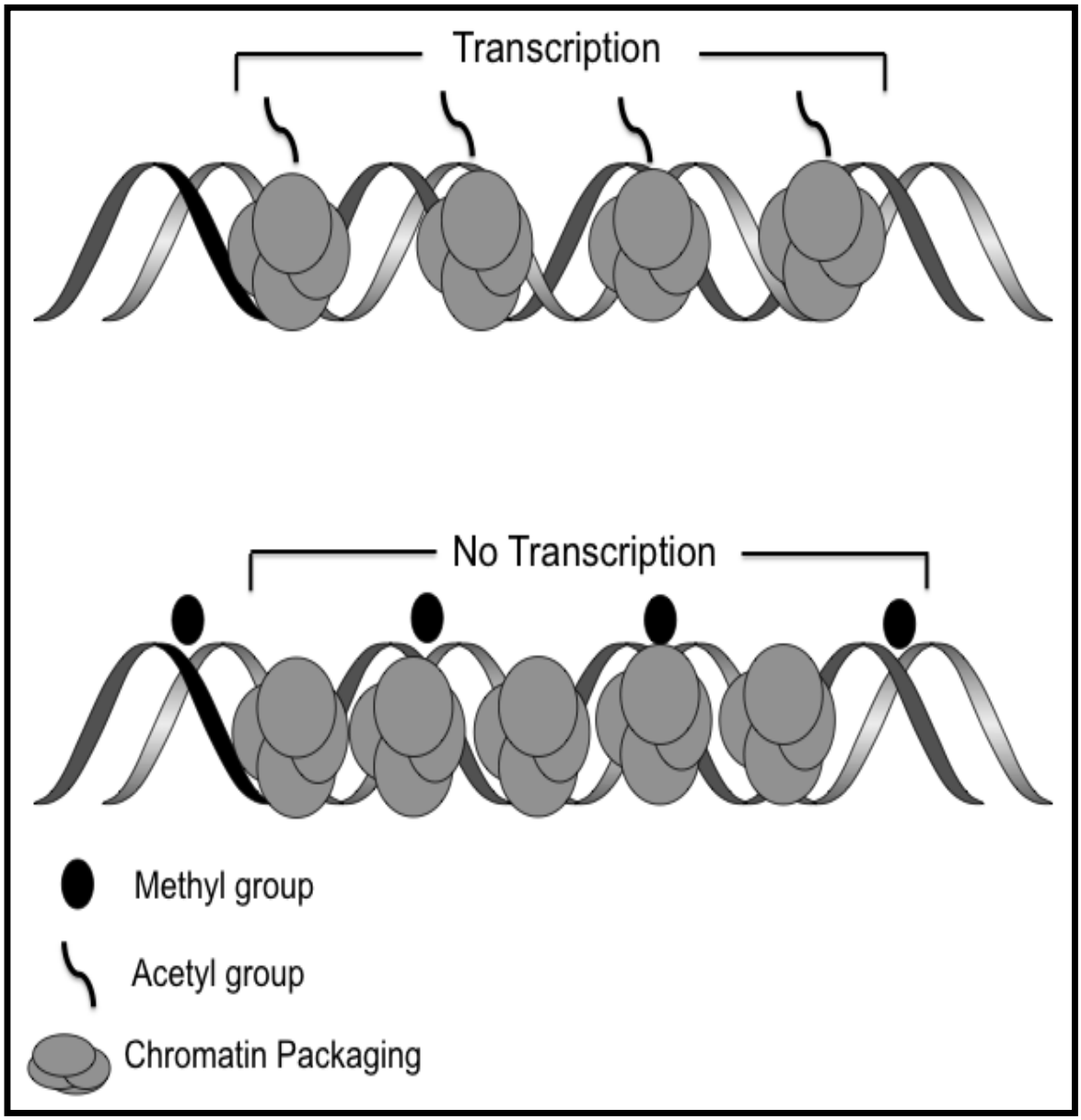

Figure 1- 3 Types of Epigenetics Modifications. Transcription of DNA is possible when there is no methyl group above the DNA sequence, acetyl is attached to DNA and chromatin packaging is not condensed as illustrated in the upper part of the figure. At the lower part of the figure, transcription is not allowed as the methyl group is added to DNA, acetyl is not attached and the chromatin package is condensed.

Regarding epigenetic alterations, the DNA sequence is packaged in two portions (histone 2A, histone 2B, histone 3 and histone 4) of a protein known as histone, and those histone proteins are packed via the 146 base pairs sequence (Jenuwein and Allis 2001). Attached to the tails of the histones, there are short amino acids sequences, which are the targeted site of epigenetic histone modifications, thus leading to changes in gene expression (Friso and Choi 2014).

While methylation mainly occurs on the DNA sequence, histone modifications mainly modify the histone proteins by methylation, acetylation, phosphorylation, ubiquitination and biotinylation, which will affect chromatin and subsequent gene expression (Margueron and Reinberg 2010). At the tail region of the histone, there are lysine and arginine residues, which may undergo methylation or acetylation. To be noted, histone acetylation is regulated by the actions of histone acetyltransferases, while histone deacetylation is regulated by the action of histone deacetylases. Histone acetyltransferases adds an acetyl group to the lysine moiety of the histone tail, resulting in loosening of the chromatin packaging the DNA. On the other hand, histone deacetylases removes an acetyl group from the lysine moiety, which will result in more condensed chromatin, thus causing the silencing of DNA transcription (Hunter 2015). Another principle epigenetic modification is chromatin modification, which affects the configuration of chromatin either by condensing or de condensing the chromatin (Friso and Choi 2014).

Many studies have shown an involvement of epigenetic modifications in the development of chronic disorders such as cardiovascular diseases, neurological diseases, metabolic disorders, and different types of cancer (Ptak and Petronis 2008). Although epigenetics regulation is not believed to directly cause T2D or obesity, epigenetic modifications in gene expression are still considered a potential risk factor leading to these disorders (Wren and Garner 2005). An example of a study confirming that epigenetic changes are risk factors for diabetes disease is a study conducted by (Pinney et al. 2011) where they observed a relation between the delayed growth of the fetus and methylation of DNA for pancreatic factor during adulthood. Another study has also observed links between epigenetics and diabetes, where changes in histone methylation have been linked to temporary hyperglycemia, which resulted in changes in the transcription of the transcription factor nuclear factor-kappa B (NFkB-65), which is related to diabetes development (Brasacchio et al. 2009).

Furthermore, it has been demonstrated that epigenetics is linked to diabetes; there are also research papers linked epigenetic to obesity. Of these studies, a study confirmed that a negative relation between methylation of some genes (endothelial nitric oxide synthase (eNOS), retinoid X receptor alpha (RXRa), and superoxide dismutase (SOD1)) and adiposity at childhood period (Godfrey et al. 2011). In additon, other have observed histone demethylation of some genes is related to obesity, whereby JmjC domain-containing histone demethylase 2A (Jhdm2a) deficient mice become obese mice and demonstrate a significant reduction in the expression of key genes regulating lipid metabolism

(Tateishi et al. 2009). For example, Jhdm2a deficient mice showed down-regulation of *Ucp*$_2$ and peroxisome proliferator-activated receptor alpha (*Ppara*) genes in their skeletal muscles. Moreover, enzymes involved in fatty acid □-oxidation such as medium chain fatty acyl CoA dehydrogenase (MCAD) and long chain fatty acyl CoA dehydrogenase (LCAD) were also down-regulated in the Jhdm2a KO mice skeletal muscles. Studies in Wistar rats that were born to obese dams were heavier with increased adiposity (at both 12 days and 21 days of age) versus their counterpart offspring that were born to lean dams. Of interest, as these rats aged to 9 months, the rats born to obese dams also demonstrated higher circulating leptin levels and leptin mRNA levels in their adipose tissue (Lecoutre et al. 2017). Interestingly, extracted DNA from the adipose tissue was subjected to bioinformatics analysis and revealed a number of different epigenetics modifications to certain regions of the CpG promoter of the leptin gene, which was associated with increasing leptin levels that may have been passed from the HFD-fed dams and affected offspring development. The epigenetic modifications they observed were a low level of DNA methylation and inactivation of histone in some regions and DNA hydroxymethylation and activation of histone in other regions. Their study finding, which showed a high level of leptin expression in adipose tissue, is related to the DOHaD theory which linked between epigenetic involvement and parents obesity (Lecoutre et al. 2017).

Due to the reversible and heritable nature of epigenetics, it is a promising field to study, as it may represent a possible cause and treatment target of many

chronic diseases that are affected by multiple factors including environmental and nutritional factors (Friso and Choi 2014). Because epigenetics is a reversible process, ameliorating and targeting those specific epigenetic changes a targeted goal for potential disease therapeutics to be achieved (Heerboth et al. 2014). Some agents that modify epigenetics are already being used in the field as the FDA has approved a number of drugs to treat various types of cancers (Mann et al. 2007). For instance, histone acetylase inhibitors are class of epigenetic drugs that have been used to treat some types of cancer (Dekker and Haisma 2009). Another class of epigenetic medications is the methylation inhibitor such as azacytidine which also had been used to treat cancer (Bird 2002).

1.7.1 DNA Methylation:

The most well studied epigenetics modification that can be inherited from parents to their children is DNA methylation (Figure 1- 4). Early studies on DNA methylation had merely implicated DNA methylation in the promoter and enhancer region of the gene as a repressor for gene expression (Jones 2012).

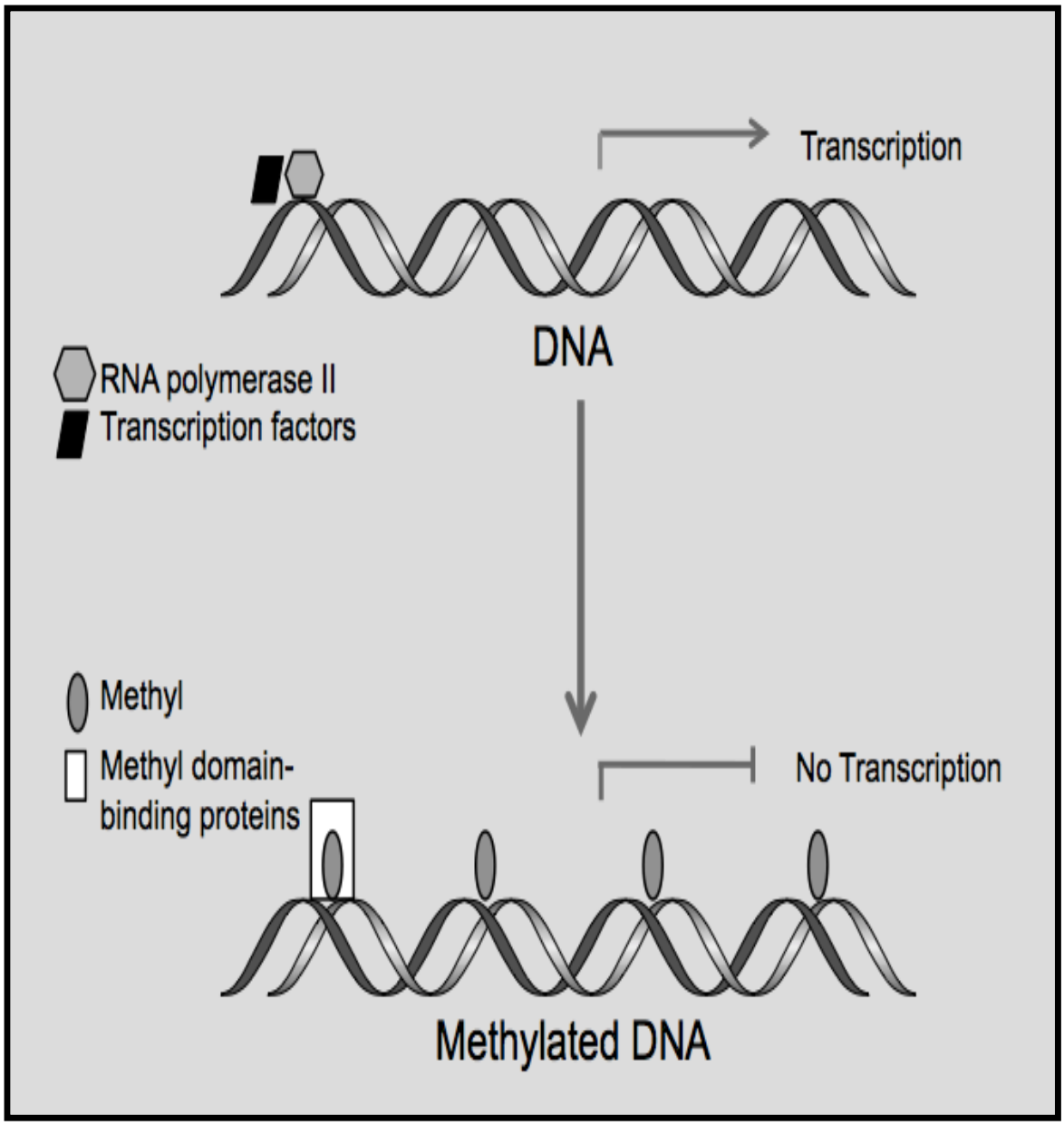

Figure 1- 4 DNA methylation is one type of epigenetic modification that may lead to gene silencing if methyl group had bind to the DNA sequence.

More specifically, DNA methylation is a reversible process that occurs throughout the lifespan; for example, in the development stage, fetus cells will inherit a certain level of DNA methylation from their parents (Heerboth et al. 2014). Following, a massive amount of DNA demethylation occurs during the fertilization stage, which is followed by excessive de novo DNA methylation during the development stage (Li 2002). Opposite to methylation of DNA, the demethylation in the promoter region of the DNA sequence will allow RNA polymerase II and transcription factors to be attached, so transcription of the gene can proceed, whereas methylation of DNA will prevent and inhibit the attachment and binding of the transcription factors and RNA polymerase to the promoter region, causing gene silencing (Heerboth et al. 2014).

Simply, DNA methylation can be explained as adding a methyl or hydroxymethyl group to the un-methylated DNA sequence daughter strand of the new DNA replicate without affecting the actual sequence arrangement of the DNA that will happen exactly on the 5^{th} position of the cytosine–phosphate–guanine in CpG dinucleotide position in order to form 5 methylcytosine or hydroxymethylcytosine (Bird 1986; Li 2002). The methyl group ($-CH_3$) will be added to un-methylated DNA by the action of specific enzymes called DNA methyltransferase (DNMT) enzymes that are responsible for maintaining DNA methylation at a certain level (Bird 2002). Once DNA methylation by DNMT enzymes at the CpG promoter region takes place, the transcription of the gene will be prevented and hence gene expression has been silenced (Heerboth et al. 2014). DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L are different types of

DNMT enzymes that mediate DNA methylation within our cells. Their classification depends on their function, DNMT responsible for maintenance of DNA methylation level are DNMT1, while DNMT3a, DNMT3b and DNMT3L are de novo DNMTs (Bird 2002). To be noted any changes in the functions of the DNMTs enzymes will lead to changes in DNA methylation patterns, which may affect histone translation and histone tail modifications, causing additional epigenetic changes, resulting in varying gene expression of certain genes. The opposite process of DNA methylation is the DNA de-methylation, which is also a vital process during the development of an embryo and various differentiation stages (Friso and Choi 2014).

1.7.2 DNA Methylation in Obesity:

There are a number of studies that have demonstrated that obesity impacts DNA methylation. Of interest, clinical studies in humans have also demonstrated that epigenetic changes may impact the next generation by studying alterations in DNA methylations levels. Donkin et al. collected sperm from obese and lean human males and found a number of DNA methylation changes for genes that are critical regulators of appetite such as; brain-derived neurotrophic factor (BDNF), melanocortin-4 receptor (MC4R), and genes responsible for fat metabolism such as fat mass and obesity associated (FTO), carbohydrate sulfotransferase 8 (CHST8). This raises the intriguing possibility that epigenetic modifications in appetite can be transferred to the developing

child and may modify the central nervous system (CNS), appetite, and whole-body metabolism in a manner that may predispose to obesity. Moreover, Donkin et al. demonstrated that spermatozoa DNA methylation changes induced by obesity could be normalized 1-year following gastric bypass surgery.

Furthermore, obesity during pregnancy can indeed transmit DNA methylation changes towards the developing offspring. For example, Laker's Lab found that DNA methylation is significantly higher at weaning in skeletal muscle tissue of C57BL/6 offspring mice that were born to sedentary mothers fed a HFD during pregnancy/lactation versus offspring that were born to either sedentary mothers fed a normal diet, or exercised mothers fed a HFD during pregnancy/lactation. These changes in the DNA methylation were observed in the promoter (-260) region of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1□) (Laker et al. 2014). In fact, glucose tolerance and insulin tolerance were worse in 9 month-old offspring that were born to sedentary HFD dams versus offspring that were born to either sedentary mothers fed a normal diet, or exercised dams fed a HFD during pregnancy/lactation (Laker et al. 2014). Similarly, a study conducted by Howie et al. (2009) showed that offspring that were born to Virgin Wistar Rat dams who have been on HFD throughout life (including pregnancy/lactation) are heavier compared to offspring born to dams fed a regular chow through their lives. In addition, offspring that were born to an obese dam fed a HFD during pregnancy and lactation period were also heavier than offspring born to dam fed a regular chow through their lives. Their findings suggested that HFD restriction during lactation and

pregnancy is a factor itself that may induce offspring obesity (Howie et al. 2009). Some studies have also been highlighted that epigenetics is a contributor for obesity development. One of those studies, a study that found DNA methylation changes in genes that regulate energy metabolism in HFD rats (Boqué et al. 2013). In this study Wistar rats were placed on a regular chow diet, a HFD, or a HFD plus apple polyphenols. Their findings showed that the DNA expression of PGC1 □ in the adipocytes tissue was decreased in the HFD group relative to the control group as DNA methylation of the gene is suggested to be increased while it was significantly increased after the apple polyphenol diet added to the HFD suggesting a reverse programming mechanism in this group. In addition, their findings showed that leptin was hyperregulated in the adipocytes of HFD group, while it showed a significantly lower expression in the apple polyphenols group (Boqué et al. 2013).

It has also been demonstrated that dams that were provided excessive calories via liquid diet supplementation for 3 weeks produced offspring exhibiting DNA methylation changes in a number of pro-adipogenic factors in WAT such as, peroxisome proliferator-activated receptor-γ (PPAR-γ), and CCAAT enhancer binding proteins (C/EBPs) (Borengasser et al. 2013).

A study by Lillycrop and colleagues (2008) used Wistar Rats as a model to investigate the prenatal effect of restricted dietary protein. The dam's diet was modified with various amounts of folic acid in different three groups of rats (control, restricted protein with 1 mg/ kg, and limited diet with 5 mg / kg). Their analysis of liver's of rat of the female's offsprings tissues showed a significant

reduction in methylation of PPAR in the group with the higher amount of folic acid, Compared to control group this reduction was linked to the loss in at CpG dinucleotide. On the other hand, the offspring that were born to dams supplemented with high folic acid did not show a consequence difference in the DNA methylation of the same gene. The reason of their results is that folic acid considered a methyl group donor group that is essential for DNA methylation (Lillycrop et al. 2008).

Obesity in the father may also elicit epigenetic alterations in the developing offspring. For example, a study conducted by (de Castro Barbosa et al. 2016) in which male Wistar rats were provided either a LFD or HFD for 12 weeks following which the male rats were bred to a female Wistar rat fed a LFD. Two generations of offspring had their spermatozoa extracted and analyzed for DNA methylation status by gene sequencing and gene ontology. Furthermore, the two generations of offspring were either fed a LFD or HFD for 12 weeks. They observed that the first generation of female offspring whose male parent was obese rat and fed a LFD were not heavier than female offspring fed a LFD whose male parent was lean rat. Furthermore, smaller cells were observed using pancreatic tomography in the offspring whose male parent was obese rat. In addition, the second generation of female offspring whose male parent was obese and fed a HFD were heavier than the female offspring fed a HFD whose male parent was lean rat, which was also associated with a worsening of glucose tolerance. These data suggest that there is a strong relationship between paternal obesity and the transition of unique DNA methylation signatures in the

developing offspring that can affect multiple generations. Confirming their suggestion, sperm was collected and purified from male rats and first generation male offspring to study DNA methylation using a special DNA methylation capture method, followed by gene ontology. Of interest, this study demonstrated that the majority of changing DNA methylation patterns involved genes that regulate lipid and glucose metabolism, as well as insulin signaling (de Castro Barbosa et al. 2016).

1.7.3 Obesity, Inflammation and Epigenetics:

Previously mentioned studies have observed a link between obesity and inflammation (Bastard et al. 2006). Specifically, the pro- inflammatory markers TNF-□ and Interleukin 6 (IL- 6) show increased levels of expression in WAT of obese model animals and severely obese humans (Jorge et al. 2018; Uysal et al. 1997). This high level of inflammation molecules will activate the NFkB-65 signaling pathway that may contribute to diabetes development and reduced insulin sensitivity (Brasacchio et al. 2009). Of interest, the NFkB-65 transcription factors are also subject to epigenetics regulation such as DNA methylation and histone acetylation, which would affect the transcription of key inflammatory genes such as the primary response genes (proto- oncogene fos (*c- fos*) and early growth response protein 1 (*Egr-1*)) (Bayarsaihan 2011; Medzhitov and Horng 2009). In addition, the pro- inflammatory markers TNF-□ and IL- 6 are

regulated by changes in methylation or acetylation via DNMT and histone acetyltransferases / deacetylases (Shanmugam and Sethi 2013).

1.8 Rationale, hypotheses and objectives:

1.8.1 Rationale:

Obesity is a serious health concern to the future generation and the prevalence of obesity continues to rise as most of our pharmacological and/or preventative measures have had limited success. Most of the research in the medical field focusing on obesity is about preventing obesity before it happens, or treating obesity complications after it has already developed (Flegal et al. 2010). Moreover, current pharmacological treatments for decreasing appetite or treating obesity complications all have side effects affecting the quality of life of the affected obese individuals (Patel 2015). In addition, some of the medical solutions for obesity complications are inconvenient such as bariatric surgery.

Other investigations are focused on finding new therapeutic agents targeting key factors that may upregulate metabolism, such as UCPs (Clapham et al. 2000; von Essen et al. 2017). However, these studies are still promising and need years of research to be conducted on animals and humans before reaching a possible treatment for obesity. Within those coming years the obesity will even affect so many millions around the world, especially, children who are going to grow as obese adults with chronic diseases threatening their lives. On the other hand, there is an important area of research that is neglected and needs to seek more attention where the main focus of this kind of studies is to

understand how obesity develops. As it is a well known that obese parents are likely to produce offspring at increased risk of obesity, understanding how obese parents considered risk factors for their offspring obesity is important and may help in minimize the obesity prevalence and save years of next generation's lives.

Therefore, our goal is to confirm whether paternal or maternal obesity do indeed increase the risk for obesity in the developing offspring, and if so, is this due to changes in epigenetics in key genes regulating energy metabolism.

1.8.2 Hypotheses:

1. Offspring that are born to an obese parent (either father or mother) will be at risk of developing early-onset obesity and obesity-related metabolic dysfunction due to the influence of epigenetics that passes the risk of obesity from one generation to the next.
2. Offspring that were born to an obese parent will have epigenetic alterations in critical genes implicated in the control of energy metabolism such as a targeted gene PGC1 .
3. PGC1 mRNA expression will be decreased in skeletal muscle of offspring that are born to HFD dams as a result of increased DNA methylation due to epigenetic inheritance.

1.8.3 Objectives:

1. Establish a model of parental obesity for assessing the consequences on offspring metabolic health.
2. Examine if offspring that are born to either an obese dam or male mouse are predisposed to early onset obesity and metabolic dysfunction (e.g. glucose intolerant and insulin resistant).
3. Determine if the potential metabolic dysfunction in offspring that were born to obese dams is associated with altered expression of *Ppargc1a*.
4. Determine if the potential metabolic dysfunction in offspring whose male parent was obese is associated with altered expression of Ucp_2 and Ucp_3.

Chapter Two

2. METHODOLOGY

2.1 Methods:

2.2.1 Animal Model Experiments:

2.2.1.1 Animal Model:

All animal experiments were carried out using protocols approved by the Animal Research Ethics Board at the University of Alberta (UofA). Mice were housed under a 12-hours light/dark cycle in the UofA Health Sciences Laboratory Animal Services facility with free access to a diet containing either 10% kcal (low-fat diet, (LFD)) or 60% kcal (high-fat diet, (HFD)) from lard (Research Diets) and water. All mice model used were C57BL/6J from Jackson.

2.2.1.2 Parental Obesity Model:

To establish obese and lean parents models, 8-week old C57BL/6J female and male mice were fed either a LFD (10% kcal from lard, Research Diets D12450) or a HFD (60% kcal from lard, Research Diets D12492) for 5 weeks and subsequently were used for mating experiments.

Both genders of obese and lean mice were bred to an age-matched C57BL/6J male/female mouse fed a LFD. For our maternal obesity studies, once we confirmed the presence of a vaginal plug, males were removed and the female dams remained on their current diet. There were two main groups, the obese dam group and the obese male group of offspring.

2.2.1.3 Offspring Handling:

After offspring birth, identical litter size was chosen in each group of bred mice with a maximum size of 5 per breeding. For example, if the HFD dams had 5 offspring while the LFD dams had 6 offspring, then 1 offspring was euthanized in order to keep an identical number of offspring being nursed by each dam.

2.2.1.4 Cross-Fostering:

Fostering was done where some offspring had surrogate dams to feed them during the 3 weeks (18 days) of age period instead their biological dams. Only the offspring born to the HFD dams were cross- fostered to LFD supplemented surrogate dams, while the offspring born to lean dams assigned to be feed by their biological dams.

2.2.1.5 Offspring Groups:

All offspring born to either obese dams or obese males were weaned at 3-weeks of age and provided free access to a LFD. However, one group of offspring born to obese male mouse was assigned to a HFD to see the effect of HFD in inducing obesity in the offspring.

2.2.2 In vivo Experiments:

2.2.2.1 Glucose Tolerance Test:

Oral glucose tolerance test (Oral GTT) and Intraperitoneal glucose tolerance tests were performed in fasted mice. Mice fasted for an overnight period (almost 15 hours fast) with free access to drinking water. Mice were given glucose oral or intraperitoneal using the doses of 2 g/kg. Before the injection, blood glucose reading was measured from mice tails at the 0- time point as a baseline reading and then after the injection in 15-, 30-, 60-, 90- and 120-minutes time points post the glucose injection. Tail bleeds used for blood glucose measuring by Contour next blood glucose monitoring system by Bayer.

2.2.2.2 Insulin Tolerance Test:

Mice have fasted for 6 hours, following which they were administered an insulin intraperitoneal injection with a dose of 0.7 U/kg or 0.3 U/kg (Novolin (biosynthetic human insulin), Novo Nordisk). Then, blood glucose readings were measured from tail-snip bleeds at 0-, 15-, 30-, 90- and 120- minutes after insulin administration. The blood glucose was measuring as previously described. In addition, a syringe of glucose was always prepared in each insulin tolerance test for emergency purposes if hypoglycemia happened during the insulin test then 100 μl glucose was given as an intraperitoneal injection to hypoglycemic mouse and the mouse was immediately excluded from the test along with breaking the fast by providing free access food.

2.2.2.3 Pyruvate Tolerance Test:

The test was conducted in mice fasted overnight with free access to a drinking water. Then, mice were subsequently injected with pyruvate (2 g/kg) by the intraperitoneal route, and glucose readings were recorded at 0-, 15-, 30-, 90- and 120- minutes after the pyruvate injection.

2.2.3 Indirect Calorimetry:

2.2.3.1 Metabolic Assessment:

Weaned offspring underwent Magnetic Resonance Imaging (MRI) by using the instrument EchoMRI -4in1/700 body composition analyzer at different times of their ages (4- weeks, 8- weeks and 14- weeks) to assess adiposity, explicitly measures lean/fat mass. In addition, indirect calorimetry by Oxymax comprehensive lab animal monitoring system (Columbus Instruments) was used to determine in vivo energy metabolism. The mice were held in a single cage each with free access to water and specific food. After 24 hours passed in the individual cages, mice were taking out to their regular pens and data were extracted for analysis.

Indirect calorimetry measures energy expenditure from the respiratory gas exchange (oxygen and carbon dioxide), the oxygen consumption (VO_2) and the

carbon dioxide production (VCO_2) can be calculated through using the following equations (Even et al. 1994).

Equation 2- 1: Equation to measure the VO_2:

$$VO_2 = (V_I \times F_I O_2) - (V_E \times F_E O_2)$$

V_I and V_E are the volumes of air inspired and expired

$F_I O_2$ and $F_E O_2$ are the fractions of O_2 in the inspired and expired air

Equation 2- 2: Equation to measure the VCO_2:

$$VCO_2 = (V_E \times F_E CO_2) - (VI \times F_I CO_2)$$

V_I and V_E are the volumes of air inspired and expired

$F_I CO_2$ and $F_E CO_2$ are the fractions of CO_2 in the inspired and expired air.

After calculating the oxygen consumption and the carbon dioxide production, the respiratory exchange ratio (RER) was calculated as the ratio between oxygen consumption and carbon dioxide release. It should be noted RER is a ratio that does not have a unit.

Equation 2- 3: Equation to measure the RER:

$$RER = VCO_2 / VO_2$$

Heat also can be estimated through the output data of the indirect calorimetry through RER that determine the caloric value (CV). Then heat can be evaluated based on the V and the VO_2.

Equation 2- 4: Equation to measure the Heat:

$$Heat = CV \times VO_2.$$

2.2.4 Euthanasia:

After all planned animal experiments were finished, animals were euthanized at 15- weeks of age, using euthasol reagent as euthanizing solution with 100 μl for lean mouse and 150 μl for obese mice respectively.

2.2.5 Tissue Extraction:

The following tissues (heart, soleus muscle, gastrocnemius muscle, liver and fat) were collected from each mouse in 2 ml Eppendorf clean tubes each for future tissue analysis. However, the heart and gastrocnemius tissues were collected in aluminum foils. Then, all the collected tissues were immediately frozen in liquid nitrogen, followed by storage all the frozen samples in -80 °C freezers until the day of their assigned experiment. In addition, blood samples were collected from our mice model using cardiac puncture technique into clean Eppendorf tubes that contained anticoagulant agent (EDTA) that was followed by centrifuging blood samples at 3000 rpm for 10 minutes to collect plasma

samples. Frozen tissues specifically liver and muscle tissues were used for polymerase chain reaction (PCR) analysis experiments to detect certain gene expressions, while other tissues (heart, blood and fat) were continued to be stored at -80 °C frizzier for future study plan analysis.

2.2.6 In vitro Experiments:

2.2.5.1 Tissue Preparation:

Frozen tissue (liver and muscle) was powdered with liquid nitrogen in a cold mortar. Then, a weighted portion of the powdered sample was used for the real-time PCR experiment.

2.2.5.2 Real-Time PCR:

To perform the real-time PCR, RNA extraction and cDNA preparation were two main steps in the experiment. The two steps were performed by using Ussher lab's protocols to run the experiment.

2.2.5.3 RNA Isolation

TRiazol reagent (1 ml) was added for homogenizing the liver and muscle tissues according to the lab protocol guidelines. Then, samples were frozen in dry ice immediately followed this step. After that, samples were either frozen at -80 °C or started thawing. The samples were kept at room temperature for 5

minutes. On the thawed samples, 1 ml of chloroform was added, and the samples were mixed vigorously using the vortex machine. Then, the samples were left for 5-10 minutes at room temperature. Samples were centrifuged for 15 minutes at 12,000 xg at 4 °C. In the followed step, samples transferred to new autoclaved 1.5 mL Eppendorf tubes. Then, 0.5 mL of ice-cold isopropanol was added, and samples were left for 5-10 minutes at room temperature. Samples were placed in the centrifuge again for 10 minutes at 12,000 xg at 4 °C, the pellet of the RNA can be recognized after this step of centrifuging. Without disturbing the pellet, isopropanol was either poured out into a waste container or evaporated by vacuum air. Samples were then washed twice with 1 mL of 75% ice-cold ethanol and centrifuged again for 5 minutes at 7,500 xg at 4 °C. After last wash of alcohol was removed from the samples, the tubes were opened to let the RNA pellet air-dry for 10 to 20 minutes. Then, RNAse/DNAse free water was added to each tube for re-suspending the pellet. Finally, RNA was quantified using a Nanodrop 2000 spectrophotometer to have the RNA concentrations that was used in the next step of the PCR experiment.

2.2.5.4 cDNA Preparation:

The synthesis of the first strand cDNA was performed from RNA concentration achieved from the last step. In brief, 2 μg of RNA of each sample was added to a mixture of RNase free water and 10x DNAse buffer to a volume of 10 μl. Then, ice-cold mixture of EDTA and Hexa random primer was added to each sample. This reaction was kept at 70° C for 12 minutes, followed by

keeping the sample in ice for 2 minutes. The following reaction was the addition of a mixture of (5x buffer, DTT, dNTP mix) to each sample then the reaction was kept at 25 ° C for 5 minutes. The final reaction was to add the SuperScript III synthesis system (Invitrogen, Carlsbad, CA) as the reverse transcriptase to each well before the whole plate was kept in 70 ° C for 75 minutes.

2.2.5.5 Real-time PCR Quantification:

Using a real-time PCR machine (Bio-Rad Laboratories Inc.), quantitative analysis of targeted mRNA expression was conducted in 96 well optical reaction plates. 480 μl reaction mixture was used, which contained 48 μl yellow water and 48 μl primer to a blend of total cDNA plus blue water mixture. After optical adhesive cover sealed the plate, the plate was centrifuged at 22 ° C for 2 minutes, then followed by PCR running. To be noted, primers used for the below-listed genes.

2.2.5.6 Real-time PCR Data Analysis:

The relative gene expression was used to measure the real-time PCR data. The $2^{-\Delta\Delta Ct}$ method was used in the analysis as it was described in Applied Biosystems and as explained by Livak and Schmittgen (Livak and Schmittgen 2001). All genes data were analyzed as fold change in gene expression normalized to the reference peptidyl-prolyl isomerase gene (*Ppia*) as our housekeeping internal control gene, which is also known as cyclophilin.

2.2.5.7 Genes Used in the PCR Experiment:

Ppargc1a:

Peroxisome proliferator-activated receptor gamma coactivator1-alpha.

Pdk4:

Pyruvate dehydrogenase lipoamide kinase isozyme 4.

Ppara:

Peroxisome proliferator-activated receptor alpha.

Ucp:

Mitochondrial Uncoupling Proteins (*Ucp*$_2$ and *Ucp*$_3$).

Table 2- 1:

TAQ man Primer Table that Used in the PCR:

TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA).

Gene	**TAQ man Primer**
Ppargc1a	Mm01208835-m1
Ppara	Mm00440939-m1
Pdk4	Mm01166879-m1
Ppia	Mm02342430-g1

Table 2- 2:

Designed Primer Table that Used in the PCR:

Sybr green Primers

Gene	Forward Primer	Reverse Primer
Ucp$_1$	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
Ucp$_2$	ATGGTTGGTTTCAAGGCCACA	CGGTATCCAGAGGGAAAGTGAT
Ucp$_3$	CCGATTTCAAGCCATGATACGC	CCTGGCGATGGTTCTGTAGG

2.2 Statistical Analysis:

All data values were expressed as mean± Standard Error Mean (SEM). Statistical analysis tests such as two-tailed Student's *t*-test and two-way analysis of variance (ANOVA) were used to see the significance of difference that was followed by Bonferroni post-hoc analysis using the statistical program Graph Pad Prism the 6th version. The level of significance in all the statistical analyses was sit as p value < 0.05.

Chapter Three

3. Female Offspring Born To Obese And Insulin Resistant Dams Are Not At Increased Risk For Obesity And Metabolic Dysfunction During Early Development

This work has been published by Hanin Aburasayn, Rami Al Batran, Keshav Gopal, Malak Almutairi, Amina Eshreif, Farah Eaton and John Ussher with the title " Female offspring born to obese and insulin resistant dams are not at increased risk for obesity and metabolic dysfunction during early development" in the ***Canadian Journal of Physiology and Pharmacology, 96 (1), 97- 102, Sept 2017,8.***

Abstract:

The percentage of women who are obese at the time of conception/pregnancy is increasing, with animal and human studies demonstrating that offspring born to obese dams/mothers are at increased risk for obesity and the metabolic syndrome. Our goal was to confirm in an experimental model of metabolic syndrome in the dam, whether the offspring would be at increased risk of obesity. Conversely, we observed that male offspring born to dams with metabolic syndrome had no alterations in their body weight profiles, whereas female offspring born to dams with metabolic syndrome were heavier at weaning, but exhibited no perturbations in energy metabolism. Moreover, they gained weight at a reduced rate versus female offspring born to healthy dams, and thus weighed less at study completion. Hence, our findings suggest that factors other than increased adiposity/insulin resistance during pregnancy are responsible for the increased risk of obesity in children born to obese mothers.

Key Words: Obesity, pregnancy, high fat diet, offspring, glucose homeostasis, metabolic syndrome

3.1 Introduction:

Currently in Canada there are over 2 million children who are overweight/obese, and the majority of these children will grow up to become adults who are also obese and at increased risk for developing metabolic syndrome (Morrison et al. 2007). Of interest, the concept of "developmental programming" suggests that an individual's risk for developing disease throughout life is intricately connected to the environment of the womb that individual was exposed to (Blackmore and Ozanne 2013). This idea was first put forward by David Barker and colleagues and termed the "fetal origins hypothesis" to explain the inverse relationship between birth weight and mortality resulting from ischemic heart disease (Barker et al. 1989). Over the years the "fetal origins hypothesis" has continued to evolve, with a more recent evolution of this initial concept termed the "Developmental Origins of Health and Disease (DOHaD)". The DOHaD hypothesis postulates that the risk for disease susceptibility in a developing individual is not simply confined to prenatal and early postnatal development, but is actually influenced by a more broader developmental window that includes the period from pre-conception to fertilization of the oocyte (Blackmore and Ozanne 2013; Pereira et al. 2014).

With regards to childhood obesity, as there are now an increasing percentage of pregnancies in overweight/obese women at conception (Flegal et al. 2012), the DOHaD hypothesis would suggest that this may be a significant factor accounting for the increased rates of obesity in our pediatric population (Catalano 2003; Shankar et al. 2008). Indeed, a number of studies have

demonstrated that offspring born to obese dams are at increased risk for obesity and obesity-related metabolic dysfunction, with many studies implicating a key role for epigenetic alterations in mediating this phenomenon (Borengasser et al. 2013; Laker et al. 2014). For example, Howie and colleagues demonstrated that offspring born to dams supplemented with a High-fat Diet (HFD) prior to and during pregnancy/lactation are more susceptible to obesity, regardless if they are weaned onto a low-fat diet (LFD) or HFD (Howie et al. 2009). Moreover, it has been demonstrated that C57BL/6J mice fed a HFD prior to and during pregnancy/lactation produce offspring at increased risk of glucose intolerance, which was associated with reduced peroxisome proliferator-activated receptor gamma coactivator 1- alpha (PGC1α) mRNA expression as a result of increased PGC1α gene methylation (Laker et al. 2014) .Nevertheless, there is inconsistency among studies exploring maternal obesity's actions on offspring development, as some studies have observed no impact of maternal obesity on offspring body weight (Buckley et al. 2005) (Holemans et al. 2004).

Therefore, our objective was to develop an animal model of obesity and related metabolic dysfunction in the pregnant dam to study whether the DOHaD hypothesis would be relevant to and impact the developing offspring. If this proved to be correct, our secondary objective was to assess whether such findings may be due to altered expression of critical genes regulating energy metabolism.

3.2 Methods:

3.2.1 *Animal Care*

All animals received care according to the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee. 8-week-old C57BL/6J (Jackson Laboratory) females were either fed a low-fat diet (LFD, 10% kcal from lard, Research Diets D12450J) or high-fat diet (HFD, 60% kcal from lard, Research Diets D12492) for 5 weeks. After 5 weeks of LFD/HFD supplementation, females were mated to a 12-week-old male C57BL/6J mouse supplemented with a LFD. Upon confirmation of a vaginal plug, the male breeder was removed and female dams remained on their respective diet throughout gestation. Following birth, pups born to the dam supplemented with a LFD remained with the dam throughout the 21-day nursing/lactation period, whereas offspring born to the dam supplemented with a HFD were cross-fostered to a surrogate dam supplemented with a LFD. This was carried out to ensure that potential body weight differences would not be due to offspring being nursed by an insulin resistant dam receiving their nutrition primarily from a HFD. A total of 4 dams were used for each experimental group (4 surrogate dams to foster pups from the 4 dams supplemented with a HFD, and 4 dams supplemented with a LFD). For each individual breeding, it was ensured that litter sizes between the surrogate dam and the LFD dam were identical at 3 days post-birth, with a maximum litter size of 5. Hence, if one dam was nursing 5 pups at 3 days post-birth and another dam was nursing 4 pups, 1 pup was randomly euthanized from the dam nursing 5 pups, such that both dams nursed an identical number of pups

(4 pups) from 3 days post-birth until weaning. At 21-days of age all offspring were weaned into separate cages and supplemented with a LFD until euthanization at 14-weeks of age.

3.2.2 *Magnetic Resonance Imaging (MRI) Body Composition Analysis*

All female dams and offspring underwent assessment of body composition via quantitative nuclear magnetic resonance relaxometry to quantify total lean/fat mass utilizing an EchoMRI-4in1/700 body composition analyzer.

3.2.3 *Glycemic Assessments*

We assessed glucose homeostasis in female offspring as previously described (Ussher et al. 2014). In brief, oral and intraperitoneal glucose tolerance were assessed either in female dams fed a LFD or HFD for 5-weeks, or in female offspring following an overnight fast at 10- and 11-weeks of age, respectively, using a glucose dose of 2 g/kg body weight. Intraperitoneal insulin tolerance was assessed either in female dams fed a LFD or HFD for 5-weeks, or in female offspring following a 6 hr fast at 12-weeks of age, using an insulin dose of 0.3 U/kg body weight (Novolin (biosynthetic human insulin), Novo Nordisk).

3.2.4 *Indirect Calorimetry*

In vivo metabolic assessment via indirect calorimetry was performed in female offspring at 8-weeks of age using an Oxymax comprehensive lab animal

monitoring system (Columbus Instruments) as previously described (Ussher et al. 2016).

3.2.5 *Statistical Analysis*

All values are presented as means ± standard error of the mean (SEM). Significant differences were determined by the use of an unpaired, two-tailed Student's t-test, or a two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis.

3.3 Results:

3.3.1 *5-weeks of high fat feeding leads to a marked increase in adiposity and impairs glucose homeostasis in female dams.*

8-week-old female C57BL/6J mice were fed either a LFD or HFD for 5 weeks prior to conception with a lean 12-week-old male C57BL/6J mice fed a LFD. 5-weeks of HFD supplementation lead to significant increases in body weight in female mice, and this was associated with a significant increase in total fat mass and percent body fat (Figure 3- 1 and Figure 3- 2 A). This increase in adiposity was associated with impaired glucose homeostasis in female mice fed the HFD, as they exhibited both worse glucose and insulin tolerance versus their female counterparts fed a LFD (Figure 3- 2 B, C).

3.3.2 *Female offspring born to dams supplemented with a HFD are heavier at weaning but exhibit reductions in weight gain throughout early development.*

Male offspring born to dams fed a HFD for 5 weeks prior to conception had similar body weights to their counterparts born to dams fed a LFD, while also gaining weight at a similar rate (Figure 3- 3). Conversely, female offspring born to dams fed a HFD for 5 weeks prior to conception were significantly heavier at weaning than their counterparts born to dams fed a LFD (Figure 3- 4 A). This difference in body weight quickly dissipated by 6- to 8-weeks of age, as female offspring born to dams fed a LFD gained body weight at an accelerated pace (Figure 3- 4 B). At study completion when the female offspring were 14-weeks of age, those born to a dam fed a HFD actually weighed less than those born to a dam fed a LFD (Figure 3- 4 A). These differences in body weight at weaning and at study completion in female offspring were not associated with changes in total fat mass and percent body fat (Figure 3- 4 C and Figure 3- 5).

3.3.3 *Glucose homeostasis and in vivo energy metabolism are normal in female offspring born to dams supplemented with a HFD.*

Because the male offspring demonstrated similar body weight curves throughout their early development (Figure 3- 3), we focused the rest of our studies on the female offspring. When all female offspring were 10- and 11-weeks of age, they underwent oral and intraperitoneal glucose tolerance tests, respectively, and we observed no differences in glucose tolerance between both

groups of offspring (Figure 3- 6 A, B). In addition, the female offspring born to dams fed a HFD exhibited no abnormalities in insulin sensitivity at 12-weeks of age in response to an insulin tolerance test (Figure 3- 6 C). During assessment of indirect calorimetry, we observed no differences in whole body oxygen consumption rates normalized to lean body mass in female offspring born to dams fed a HFD (Figure 3- 7 A), while substrate preference also appeared similar as respiratory exchange ratios were comparable between groups (Figure 3- 7 B). The reduced body weight gain in female offspring born to dams fed a HFD is not due to changes in animal ambulatory activity (Figure 3- 7 C), but may involve changes in appetite, as 24-hr food intake demonstrated a trend to a mild reduction (Figure 3- 7 D).

3.4 Discussion:

In this study we observed that female dams fed a HFD for 5 weeks prior to conception and during pregnancy, produced female offspring that exhibited no adverse alterations in glucose homeostasis throughout their juvenile and young adult development. In support of the DOHaD ideology, these female offspring were heavier at weaning, but unexpectedly gained less weight than their counterparts born to dams fed a LFD.

The principle concepts of DOHaD suggest that the risk for disease susceptibility in a developing individual is actually influenced by a more broader developmental window that includes the period from pre-conception to oocyte

fertilization, versus just the periods of prenatal and postnatal development (Blackmore and Ozanne 2013). As such, we hypothesized that dams fed a HFD prior to conception would produce offspring at increased risk for metabolic syndrome and early onset obesity. To our surprise, female offspring born to dams fed a HFD exhibited no abnormalities in glucose homeostasis. We initially surmised that reasons for this unexpected finding could be due to our experimental model of HFD supplementation in the dam not inducing any overt metabolic dysfunction or adiposity, but assessment of total adiposity in our dams fed a HFD prior to conception revealed robust increases in total fat mass, as well as significant impairments in glucose and insulin tolerance.

Taken together, it remains unclear why we did not observe changes in glucose homeostasis in our offspring born to dams fed a HFD prior to conception, and our findings are in contrast to previous studies indicating that offspring born to obese dams are at increased risk for both elevated weight gain and metabolic dysfunction. A study by Howie and colleagues demonstrated that both male and female rat offspring gained weight at increased rates regardless if they were supplemented with a standard chow diet or HFD at weaning if born to dams either fed a HFD (45% kcal from lard, Research Diets) throughout life, pregnancy, and lactation, or only throughout lactation and pregnancy (Howie et al. 2009). Conversely, studies from Laker and colleagues utilizing a pregnancy model in which female C57BL/6 dams were fed a HFD (60% kcal from lard, Research Diets) for 6 weeks prior to conception and during gestation, demonstrated no differences in offspring body weight over a 1-year period versus

offspring born to dams fed a standard chow diet (Laker et al. 2014). Nevertheless, 9-month old offspring born to dams fed a HFD exhibited impairments in glucose and insulin tolerance versus offspring born to dams fed a LFD (Laker et al. 2014). The impairment in glucose homeostasis in the offspring born to dams fed a HFD was attributed to epigenetic changes in PGC1α, a key regulator of mitochondrial function, as PGC1α methylation was increased and subsequent PGC1α mRNA expression decreased in skeletal muscles of these offspring. However, our measurements of soleus PGC1α mRNA expression indicate similar levels at weaning in our female offspring born to dams fed a HFD, and in 14-week-old female offspring born to dams fed a HFD, versus their female counterpart offspring born to dams fed a LFD (Figure 3- 8), which may explain why glucose homeostasis was unaltered in our studies. Conversely, it is possible that if we allowed our female offspring to age to 9- months, we may have also observed impaired glucose homeostasis in the offspring born to dams fed a HFD, supporting previous findings (Laker et al. 2014).

Of importance, a number of studies support the notion that epigenetic inheritance may be a critical feature of the DOHaD ideology and the risk for disease susceptibility in a developing individual, including pediatric obesity and metabolic syndrome. For example, dams made obese via liquid enriched diet supplementation (40% excess calories) for 3 weeks led to robust alterations in offspring DNA methylation of pro-adipogenic factors (e.g. CCAAT enhancer binding protein β) in WAT stromal vascular cells (Borengasser et al. 2013). To ensure any potential epigenetic alterations in this study were likely acquired via

inheritance from the dam fed a HFD and exposure to obesity like intrauterine environment, all offspring born to the dam fed a HFD were cross-fostered to a surrogate lean dam. Conversely, the pregnancy models used by Howie and colleagues or Laker and colleagues had the dams remain on a HFD during the nursing/lactation period. This differentiating factor could influence epigenetic mechanisms controlling body weight gain in the offspring during nursing, potentially explaining the early onset obesity in offspring weaned to a LFD and born to dams fed a HFD observed by Howie and colleagues (Howie et al. 2009), or the impaired glucose tolerance in offspring born to dams fed a HFD observed by Laker and colleagues (Laker et al. 2014) . Hence, this may explain why we did not see an increased risk for early onset obesity or metabolic syndrome in our offspring born to dams fed a HFD for 5 weeks prior to conception, as we also cross-fostered our pups to a surrogate dam fed a LFD immediately upon birth. Alternatively, our findings do have a significant limitation in that we did not foster pups born to dams fed a LFD, and it is possible that our observations in female offspring are the result of the stress of fostering, and not the offspring being conceived by a dam fed a HFD and the subsequent intrauterine exposure to an obese metabolic environment. Furthermore, it is possible that maternal care from a foster mother is not as strong as that from the genetic mother, which could also contribute to our observations in the female offspring. However, we would thus expect to have seen a similar body weight pattern in our male offspring, but body weights in male offspring born to dams fed a HFD and fostered to a dam fed a LFD were identical at all time points when compared to male offspring born to

dams fed a LFD without fostering. Nonetheless, this remains a very important lingering question that we plan to address in our future studies.

In spite of this limitation, our results are unique as our female offspring born to dams fed a HFD were actually heavier at weaning than their counterparts born to dams fed a LFD, but gained weight at a reduced pace that they actually weighed less once they reached 14-weeks of age. On the other hand, male offspring born to HFD supplemented dams in our study had normal body weights at weaning and gained weight at similar rates to their male offspring counterparts born to LFD supplemented dams. It remains unclear as to why we observed this unique sex-dependent body weight profile in our offspring versus those observed in previously aforementioned studies, though our results in male offspring are consistent with findings in male rat offspring born to HFD supplemented dams by Buckley and colleagues (Buckley et al. 2005). Of interest, our findings are consistent with the set-point theory of body weight regulation (Farias et al. 2011) and it is possible that the reduced rate of body weight gain in female offspring born to a dam supplemented with a HFD could be a centrally regulated defense mechanism resulting from an elevated weaning body weight and intrauterine exposure to an obese environment. As such, it would be interesting to have continually monitored glucose homeostasis in our offspring as they aged even further, and whether this could be negated by weaning the female offspring onto a HFD, though that is beyond the scope of this specific study. It is worth noting though if this set-point theory of body weight regulation is correct, it would have also been anticipated to occur in the male offspring, which we did not observe in

our study.

Another potential factor is that we utilized a sucrose-matched low-fat control diet for our control dams, whereas most studies simply utilize standard rodent chow. One of the most common standard chow diets employed in animal facilities is the 2018S diet of Teklad, and the micronutrient compositions of these standard chow diets can be vastly different from the micronutrient composition of the Research Diets LFD/HFD utilized in our study. As such, we have observed differences with regards to glucose tolerance and insulin tolerance when comparing 2018S versus Research Diets LFD (unpublished data). This leads to the question of whether obesity itself, or the macronutrient composition of the HFD, is responsible for the offspring phenotypes observed in previous studies, or is it potential differences in micronutrient composition between the Research Diets HFD and the standard chow diet the dam is exposed to that is contributing to the offspring phenotype.

In summary, our findings did not reproduce those of previously published papers supporting the DOHaD ideology that obesity and metabolic dysfunction in the pregnant dam lead to increased risk for early onset obesity and metabolic dysfunction in the developing offspring. Whether this is due to differences in micronutrient composition in the diets we utilized versus previous studies, or because we cross-fostered our pups during nursing/lactation to a lean dam fed a LFD, remains to be determined. Future studies characterizing molecular factors that lead to increased body weight at weaning, but reduce the rate of body weight gain in female offspring born to dams fed a HFD are critical.

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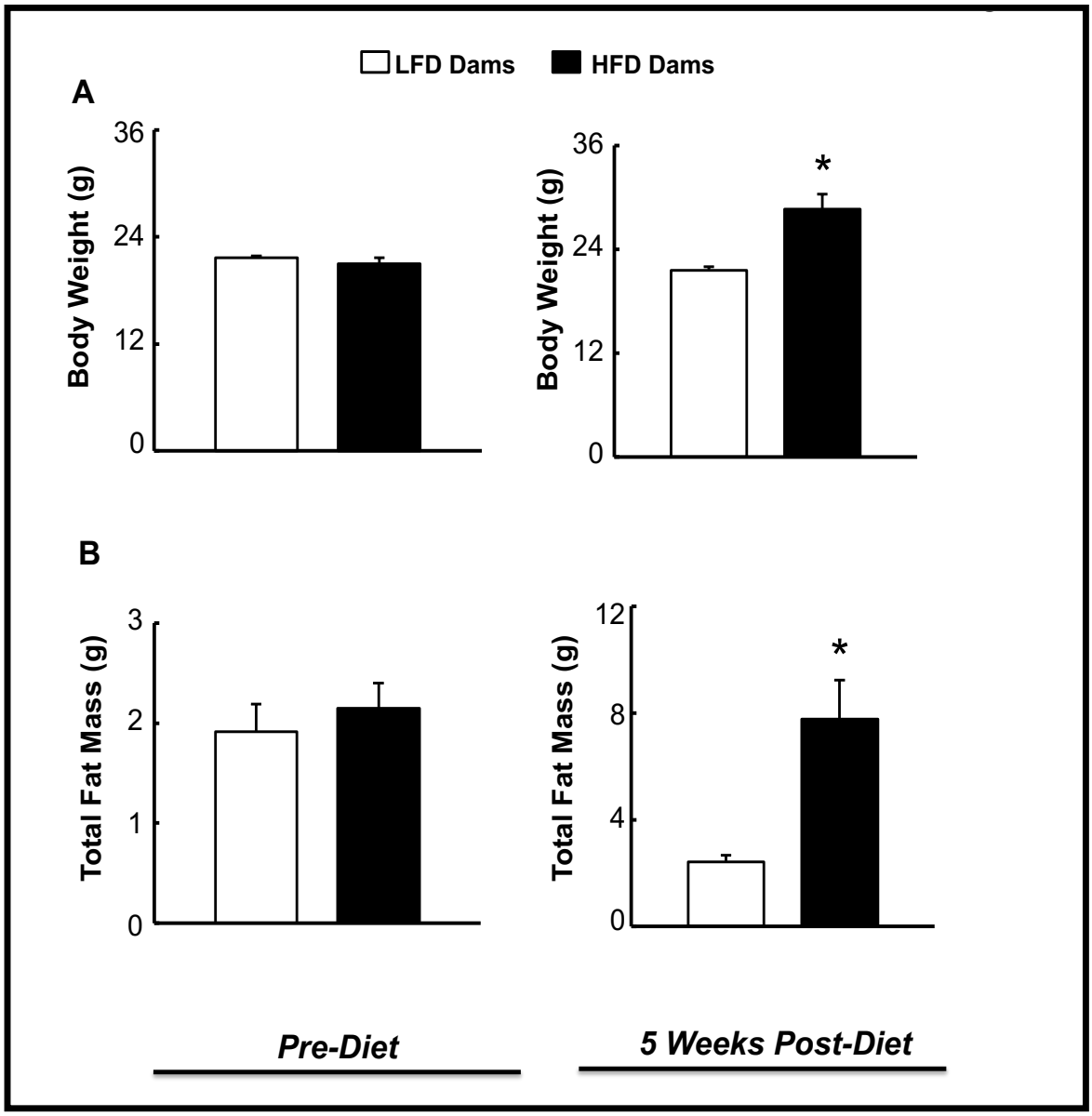

Figure 3- 1 Five weeks of HFD supplementation increases adiposity and precipitates metabolic dysfunction in female C57BL/6J mice fed a LFD or HFD for 5 weeks. (A) Body masses in female C57BL/6J mice (n = 6). (B) Total fat mass in female C57BL/6J mice (n = 6). Values represent mean ± SEM. Differences were determined by the use of an unpaired, two-tailed Student's t test, or two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P < 0.05.

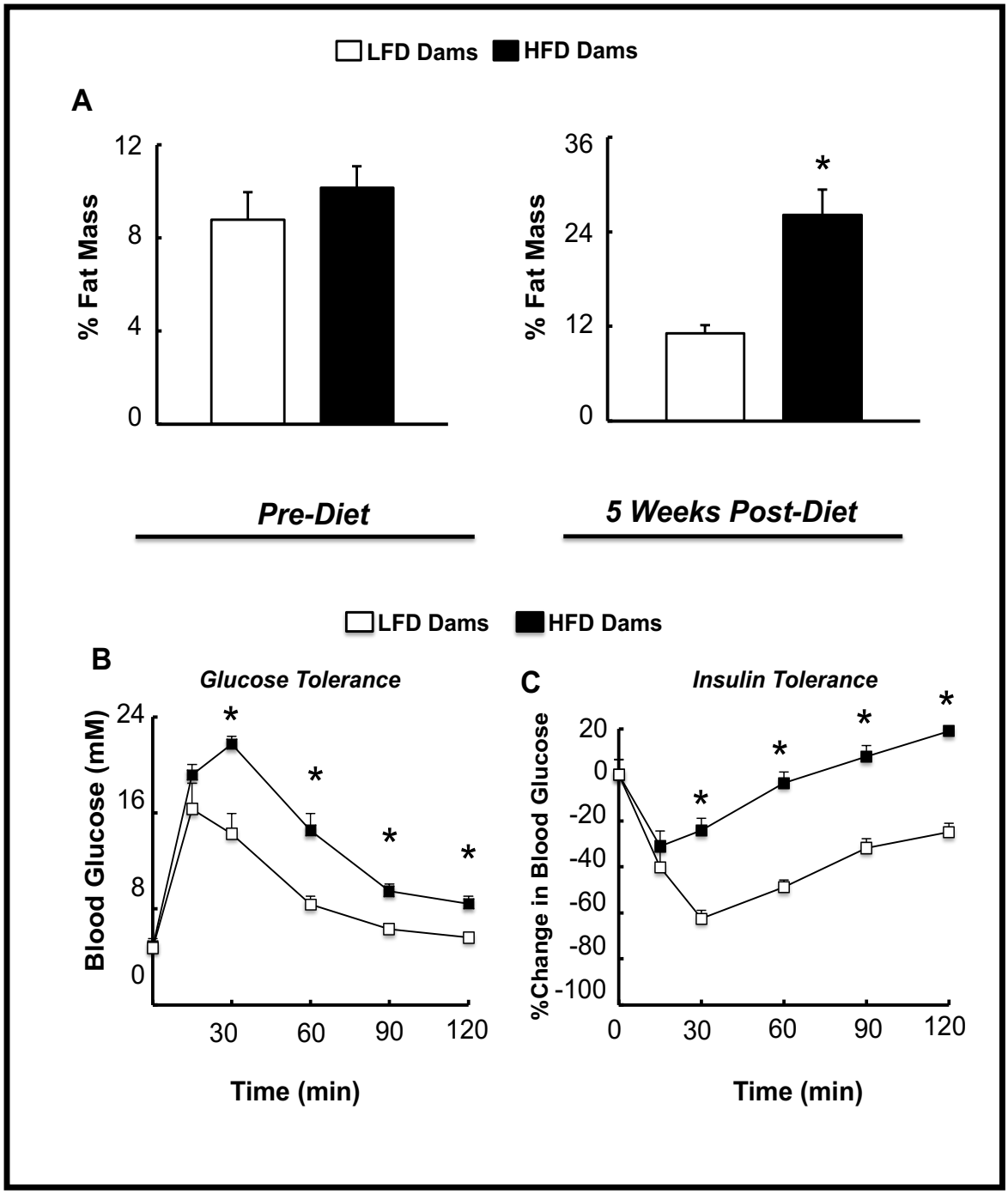

Figure 3- 2 (A) Fat mass as a percentage of total body mass (n = 6). (B) Glucose tolerance in female C57BL/6J mice (n = 6). (C) Insulin tolerance in female C57BL/6J mice (n = 4). Values represent mean ± SEM. Differences were determined by the use of an unpaired, two-tailed Student's t test, or two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P < 0.05.

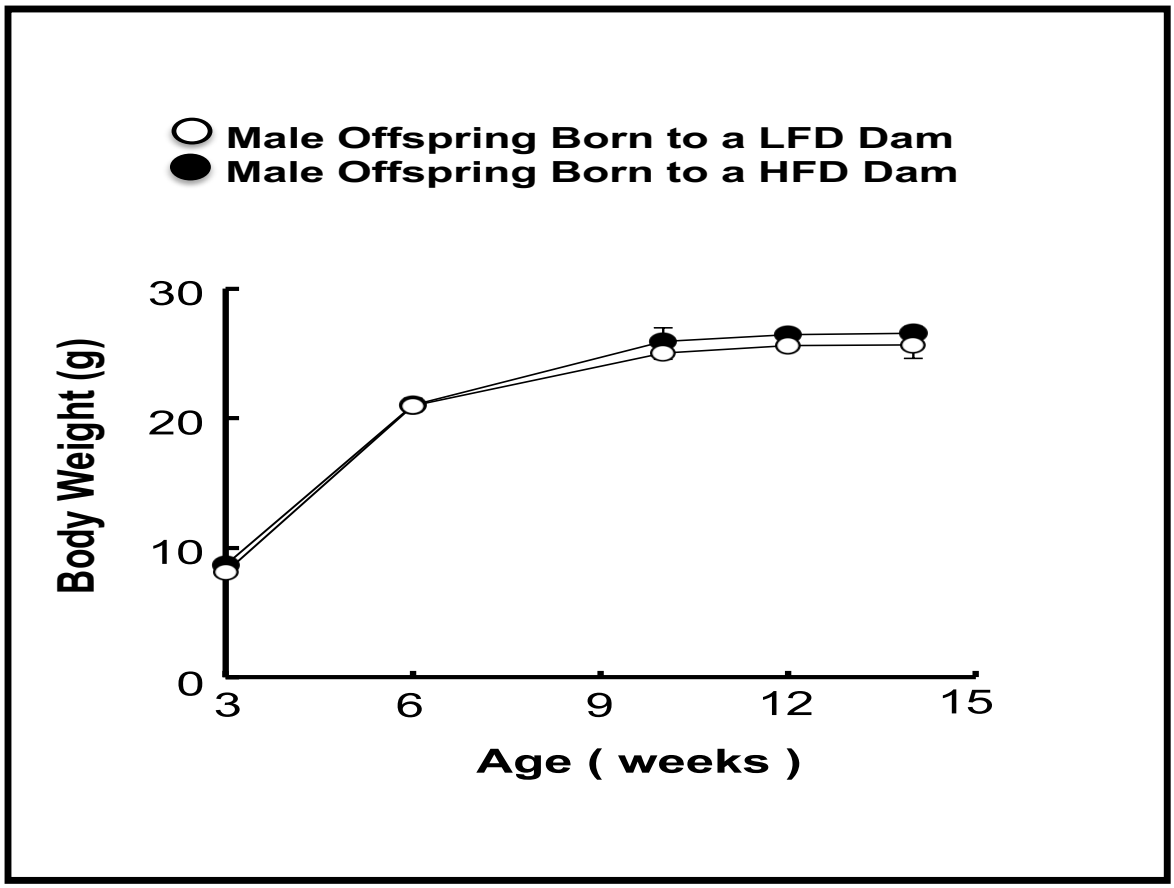

Figure 3- 3 Body masses from weaning until 14 weeks of age in male offspring born to C57BL/6J dams fed a LFD or HFD for 5 weeks (n = 3, 4). *P < 0.05.

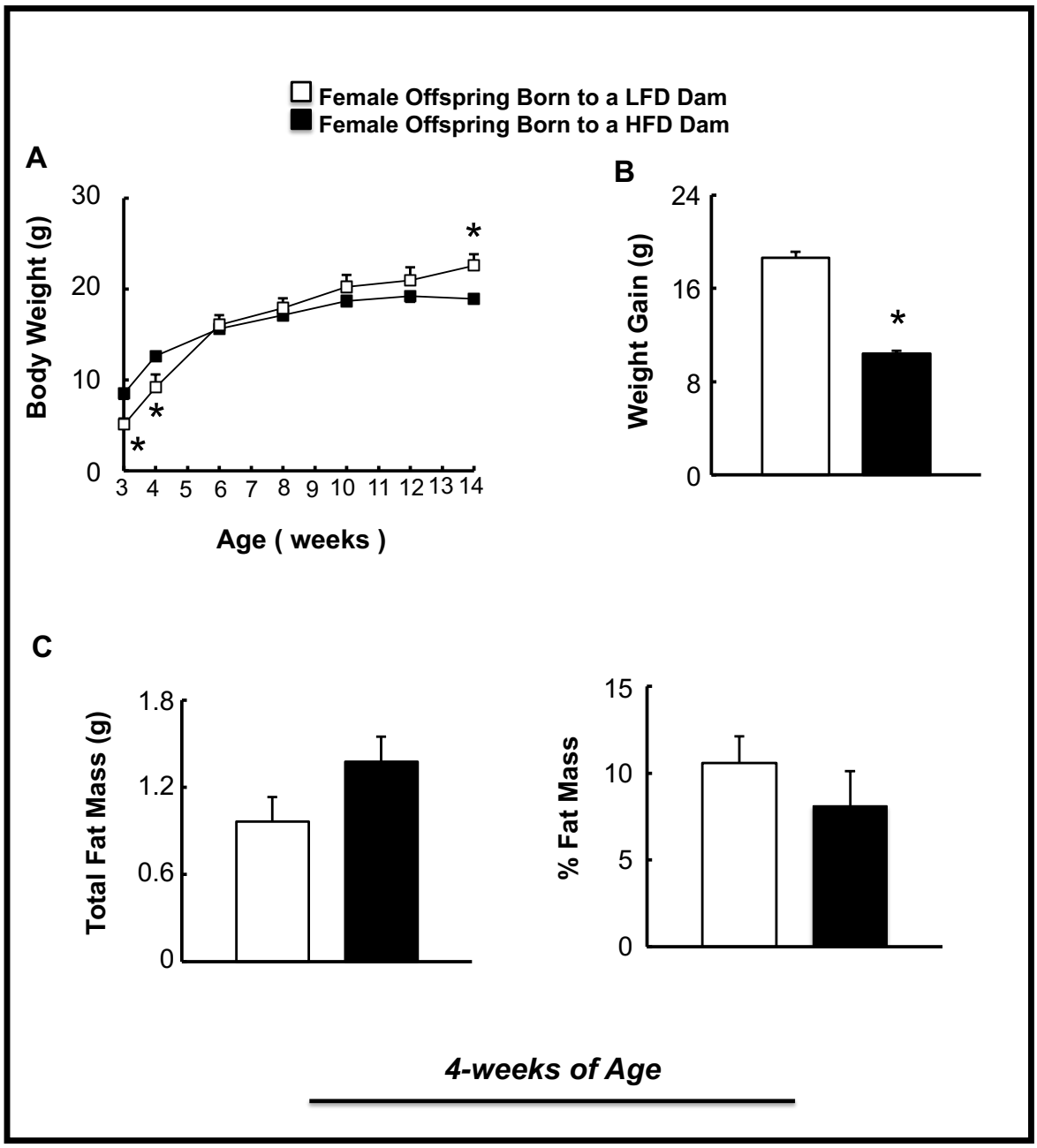

Figure 3- 4 Body masses and adiposity in female offspring born to C57BL/6J dams fed a LFD or HFD for 5 weeks. (A) Body masses from weaning until 14 weeks of age. (B) Total body weight gain over an 11-week period. Total fat mass and % fat mass in (C) 4-week-old. *P < 0.05.

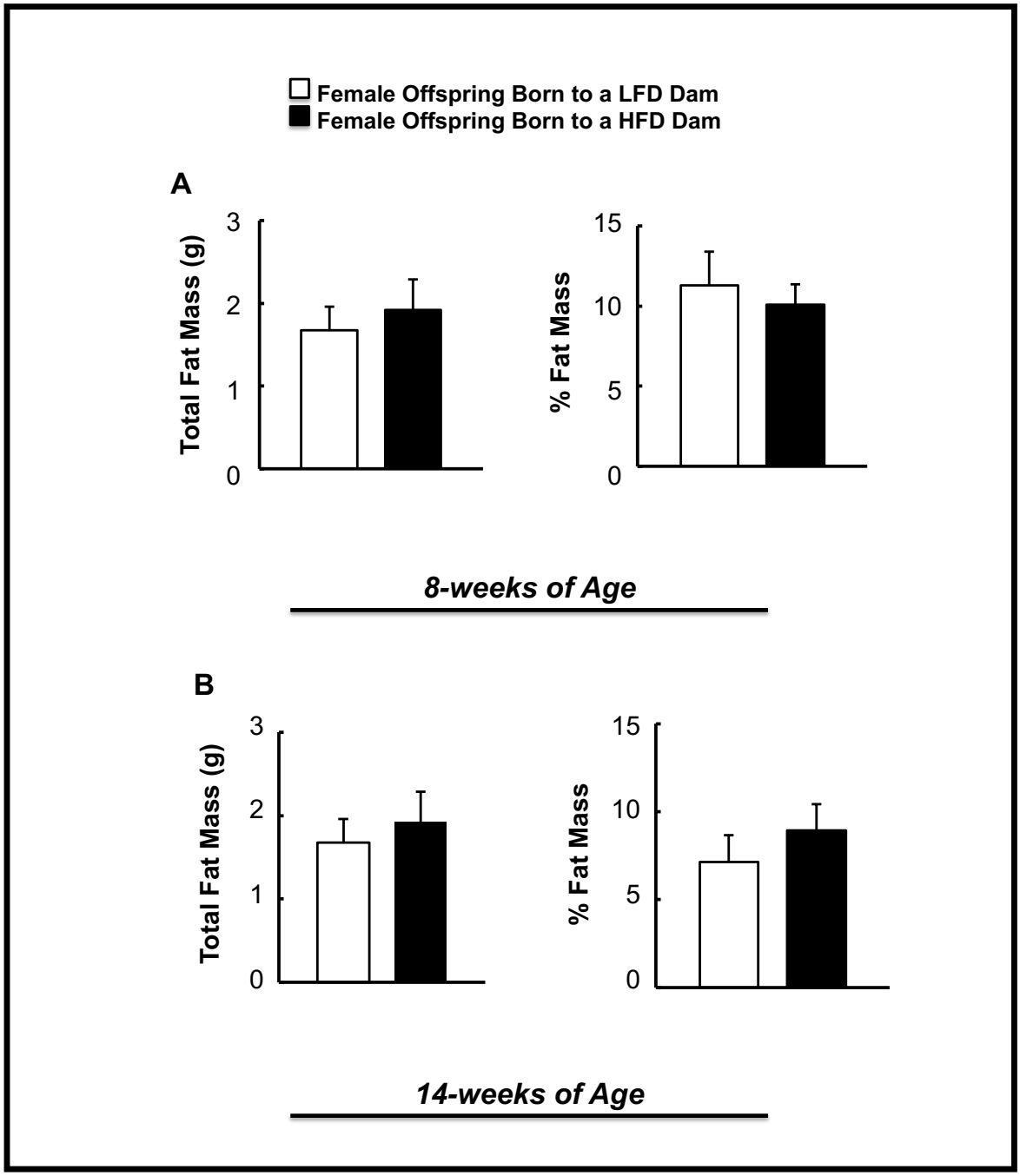

Figure 3- 5 Total fat mass and % fat mass in (A) 8-week-old, and (B) 14-week-old female offspring. Values represent mean ± SEM (n = 6). Differences were determined by the use of an unpaired, two-tailed Student's t test, or a two-way ANOVA, followed by a Bonferroni post-hoc analysis. *P < 0.05.

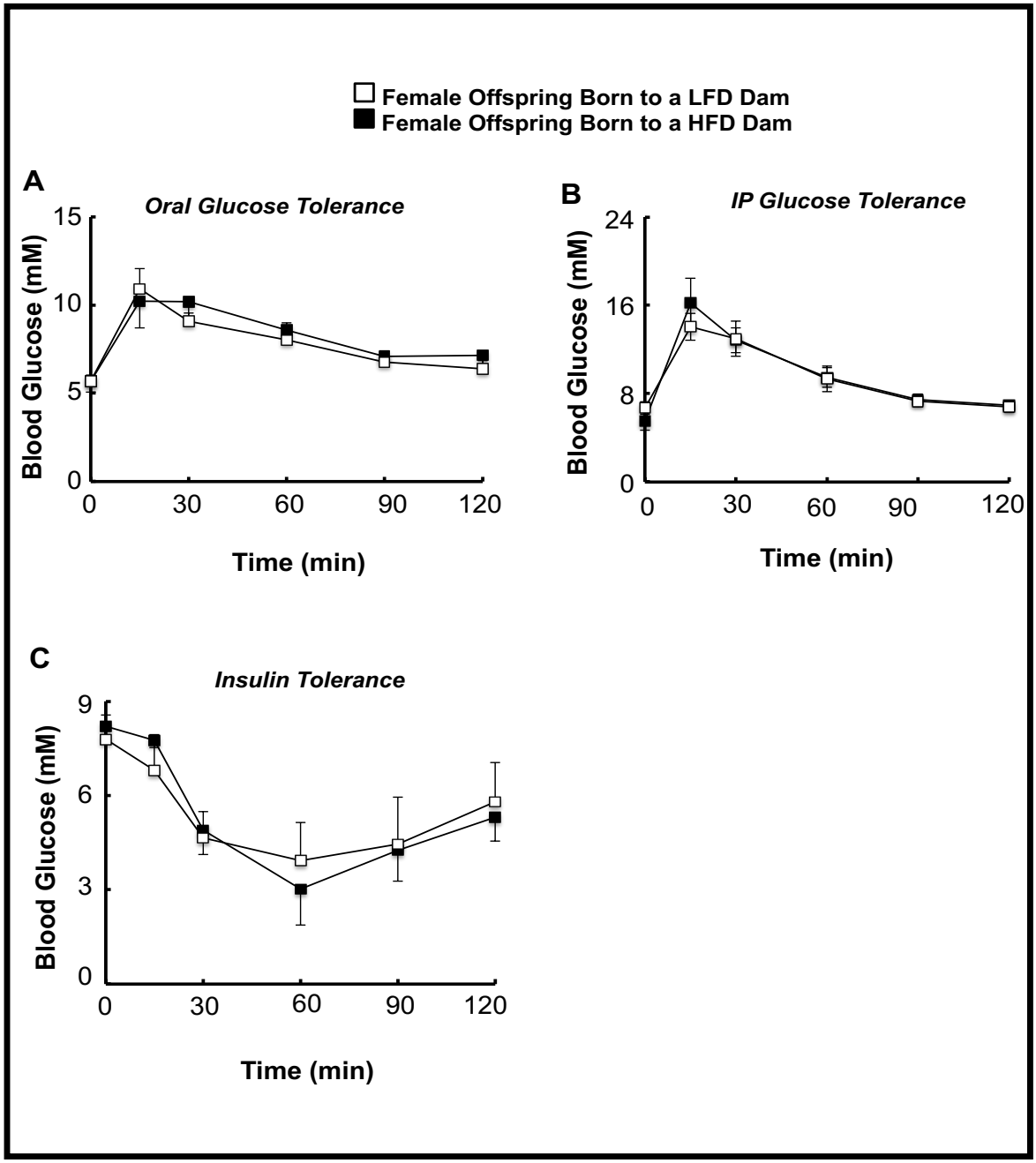

Figure 3- 6 Glucose homeostasis and in vivo metabolism in female offspring fed a LFD or HFD for 5 weeks. (A) Oral glucose tolerance in 10-week-old female offspring (n = 5). (B) Intraperitoneal (IP) glucose tolerance in 11-week-old female offspring (n = 5, 6). (C) IP insulin tolerance in 12-week-old female offspring (n = 5, 6). *P < 0.05.

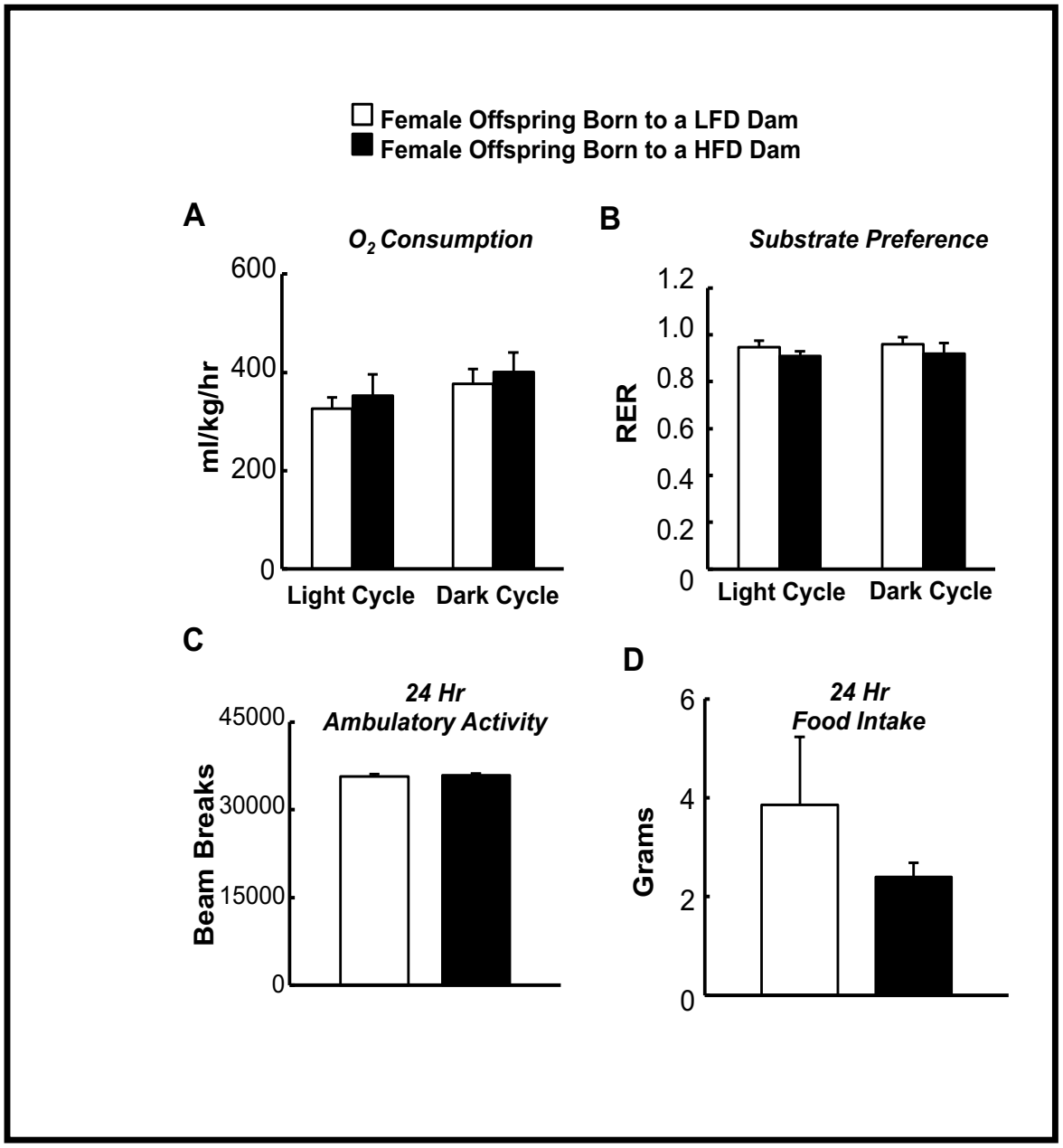

Figure 3- 7 (A) Oxygen consumption in 8-week-old female offspring (n = 3). (B) Respiratory exchange ratio (RER) in 8-week-old female offspring (n = 3). (C) Ambulatory activity in 8-week-old female offspring (n = 3). (D) Food intake (24 h) in 8-week-old female offspring (n = 3). *P < 0.05.

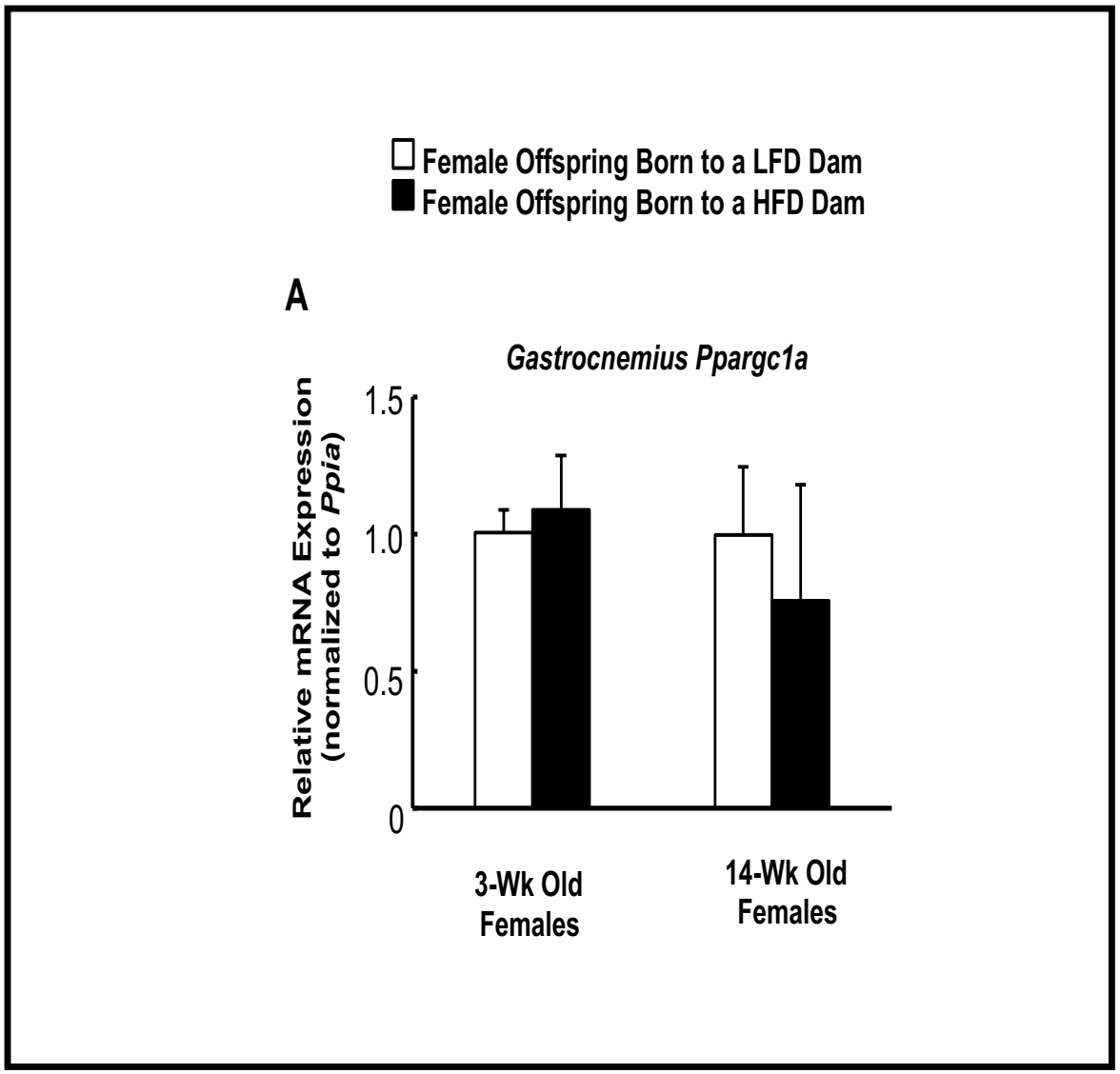

Figure 3- 8 *Ppargc1a* mRNA expression in gastrocnemius muscles from 3-week-old or14-week-old female offspring (n = 3, 4). Values represent mean ± SEM. Differences were determined by the use of an unpaired, two-tailed Student's t test, or a one-way ANOVA, followed by a Bonferroni post-hoc analysis. *P < 0.05.

Chapter Four

4. Paternal Obesity Differentially Affects Body Weight and Glycemic Control in Male and Female Offspring

This work is a manuscript in preparation by Hanin Aburasayn, Malak Almutairi, Keshav Gopal, Rami Al Batran, Amina Eshreif, Farah Eaton and John Ussher with the title “ Paternal Obesity Differentially Affects Body Weight and Glycemic Control in Male and Female Offspring ”.

Abstract:

Childhood obesity is a serious problem that continues to increase at an alarming rate. Although there are many studies explaining maternal obesity as a risk factor, paternal obesity has received less attention. Our aim was to study the effect of paternal obesity on C57BL/6J mice offspring development. To establish the offspring model, we fed male C57BL/6J mice a LFD or HFD, and then bred the lean or obese male to a lean female mouse. Our results showed that offspring whose male parent was obese were significantly heavier at weaning compared to their counterparts whose male parent was lean. Of interest, female offspring whose male parent was obese and weaned onto LFD demonstrated a significantly heavier body weight as they aged versus their offspring counterparts whose male parent was lean, while indirect calorimetry revealed that they also consumed less oxygen despite being more active. In addition, we did not observe any significant changes in their energy metabolism profiles via indirect calorimetry. Therefore, our observations suggest that paternal obesity does not increase the risk for obesity development in the offspring.

Key Words: Obesity, obese male, high fat diet, offspring, glucose homeostasis, metabolic syndrome**.**

4.1 Introduction:

Obesity prevalence is increasing at a rapid rate in developed and developing countries. Furthermore, childhood obesity prevalence is also increasing, and it has been estimated that there are 42 million children around the world that are affected by obesity and struggling with overweight issues. This number is predicted to reach 60 million by 2020 (De Onis et al. 2010). Taking into consideration that these obese children will be at risk of developing insulin resistance, T2D, cardiovascular diseases and metabolic syndrome in their adulthood, it is important we address the obesity issue in our childhood population. Although many epidemiological studies have focused on post-conception risk factors of childhood obesity, there are quite a few studies that have focused on parental obesity as a risk factor that may affect the developmental period of their children (Blackmore and Ozanne 2013). The developmental affect period means the paternal or maternal obesity during the conception or pregnancy which had been explained since the 80s as the fetal origins hypothesis by David Barker (Barker et al. 1989). This fetal origin hypothesis has since evolved, and a more modern version of it is known as the Developmental Origins of Health and Disease (DOHaD) hypothesis (Blackmore and Ozanne 2013). The DOHaD hypothesis has suggested that parental obesity is a major risk factor for childhood obesity; however, the majority of studies investigating this concept have focused on maternal obesity (Maffeis et al. 1998).

Because of the lack of research work focusing on paternal obesity and its effect on offspring development, this work aimed to address this question. In support of the concept that parental obesity may increase risk for offspring obesity, a study conducted on obese male rats (Sprague-Dawley) demonstrated that males who consumed a HFD had offspring with heavier body weights, more fat mass and worsened glucose homeostasis, compared to offspring whose male parent was lean (Ng et al. 2010). In addition, a previous study had shown that HFD consumption in obese C57BL6 male mice results in offspring predisposed to early onset obesity. Their results demonstrated that offspring whose male parent was obese, had heavier body weights, higher adiposity rates, higher levels of cholesterol and triglyceride compared to offspring whose male parent was lean (Fullston et al. 2013).

As we are interested in understanding the causes of childhood obesity and how it links to parental obesity, our previous work focused on the effect of maternal obesity on mice offspring development (Aburasayn et al. 2017). Our work demonstrated that maternal obesity does not increase the risk of obesity in the developing offspring. Therefore, our objective was to investigate the role of paternal obesity on body weight and the risk of developing metabolic syndrome in the developing offspring.

4.2 Methods:

4.2.1 *Animal Studies*

All animal experiments were carried out using protocols approved by the Canadian Council on Animal Care and the University of Alberta Health Sciences Animal Welfare Committee. Mice were housed under a 12-hr light/dark cycle in the UofA animal facility with free access to an assigned diet and water. Two groups of 8-week-old C57BL/6J (Jackson Laboratory) males were either supplied with a (LFD, 10% kcal from lard, Research Diets D12450J) or (HFD, 60% kcal from lard, Research Diets D12492) for 5 weeks. Male mice were subsequently bred with a lean 12- week old C57BL/6J female fed a LFD. Vaginal plug test was done to confirm pregnancy, and then males were removed from the cages. All offspring were weaned at 3-weeks of age onto separate cages and provided free access to either a LFD or HFD.

4.2.2 *Body Composition Analysis*

Mice underwent quantitative nuclear MRI through an EchoMRI-4in1/700 body composition analyzer to assess their body composition and calculate their total lean/fat mass as previously described (Aburasayn et al. 2017).

4.2.3 *Glucose Tolerance Test*

All offspring at age 10 and 11 weeks were assigned for Oral glucose tolerance and intraperitoneal glucose tolerance tests, respectively. Overnight fasted mice had given glucose orally or intraperitoneally with a dose of 2-g/kg glucose. Blood glucose reading was taken from mice tails at the 0-time point and then after the injection at 15-, 30-, 60-, 90- and 120 - minutes time points post the glucose injection. Tail-snip bleeds were used for blood glucose measuring by Contour next blood glucose monitoring system by Bayer.

4.2.4 *Insulin Tolerance Test*

At the age of 12 weeks, fasted offspring mice had intraperitoneal insulin injection with the dose of 0.7 U/kg or 0.3 U/kg (Novolin (biosynthetic human insulin), Novo Nordisk). Then, blood glucose readings were taken from mice tails at 0-, 15-, 30-, 90- and 120- minutes after insulin administration.

4.2.5 *Pyruvate Tolerance Test*

The pyruvate tolerance test (PTT) was performed in fasted mice at the age of 13 weeks. Pyruvate was intraperitoneally injected with a dose of 2 g/kg with glucose readings taken at 0-, 15-, 30-, 90- and 120- minutes following the pyruvate injection from mice tail blood.

4.2.6 *In vivo metabolic assessment*

Indirect calorimetry was used for metabolic assessment in the offspring at 4 and 14 weeks of age by Oxymax metabolic cages (Columbus Instruments) as previously described (Ussher et al. 2016).

4.2.7 *Real-Time PCR Analysis*

First-strand cDNA was synthesized from total RNA using the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was processed with the Real-time PCR machine (Bio-Rad Laboratories Inc.) using Sybr green Gene Expression Assays. Relative mRNA transcript levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using *Ppia* as our housekeeping internal control gene.

4.2.8 *Statistical Analysis*

All data are calculated as mean ± SEM. The statistical test; unpaired, two-tailed Student's t-test, or two-way ANOVA test were used to measure the Significant differences which were followed by a Bonferroni post-hoc analysis.

4.3 Results:

4.3.1 *Progression of body weight in mice offspring born to lean or obese males*

Male and female offspring fed a LFD whose male parent was obese exhibited significantly heavier body weights when compared to their counterparts whose male parent was lean at weaning (3 weeks) (Figure 4- 1 A, B). However, as the offspring aged, this difference in body weight disappeared (Figure 4- 1 A, B). However, the female offspring born to obese male mice showed a significant increase in their body weight gain once again at 11 weeks of age compared to their counterparts born to lean males (Figure 4- 1 B). On the other hand, the offspring that were provided a HFD at weaning and whose male parent was obese demonstrated similar body weights versus offspring whose male parent was lean for the entire study duration. (Figure 4- 1C, D).

4.3.2 *Assessment of offspring adiposity did not reveal differences between offspring born to obese or lean male mice*

Assessment of adiposity for the LFD offspring at 4 and 14 weeks of age did not show any differences between offspring born to either an obese or lean male mouse (Figure 4- 2). However, LFD female offspring born to obese males demonstrated a trend to higher fat % compared to offspring born to lean males at 4 weeks, but this trend disappeared as they aged. Similarly, the HFD offspring showed a trend to higher fat mass in the female offspring whose male parent was

obese at 4- and 14- weeks of age (Figure 4- 3).

4.3.3 *Sex- specific differences in glucose homeostasis in the offspring*

LFD and HFD offspring underwent intraperitoneal and oral glucose tolerance testing at 10 and 11 weeks of age to assess their glucose homeostasis. Unexpectedly, the glucose tolerance of all offspring groups (the LFD and the HFD) whose male parent was obese was not significantly different from offspring whose male parent was lean. However, the female offspring fed a HFD or a LFD whose male parent was obese demonstrated a trend to improved glucose tolerance compared to the control group (Figures 4- 4 A- D). Conversely, male offspring whose male parent was obese mouse exhibited a trend to worse glucose clearance than their offspring counterparts whose male parent was lean (Figures 4- 4 A- D).

Insulin tolerance tests in 12- week old offspring revealed a trend to worse glucose clearance in both the LFD; HFD offspring groups whose male parent was obese (Figure 4- 5 A-D). The following week, PTTs were then performed in 13- week old offspring and demonstrated increases in circulating glucose levels in males fed a LFD whose male parent was obese (Figure 4- 6 A). However, females fed a LFD whose male parent was obese showed similar circulating glucose levels to their counterparts whose male parent was lean (Figure 4- 6 B).

4.3.4 *Assessment of in vivo energy metabolism via indirect calorimetry*

Assessment of indirect calorimetry was performed in offspring at 8 weeks of age; we observed no differences in whole-body oxygen consumption rates for offspring whose male parent was obese and fed a HFD (Figure 4- 7 A-D). Although there were no changes in oxygen consumption, we noticed that there was a trend of reduction in oxygen consumption for LFD female offspring whose male parent was obese. This finding was accompanied by a significant increase in their ambulatory activity, especially during the dark cycle when the animals are awake and active (Figure 4- 8 A-D).

4.3.5 *Sex- specific differences in Ucp_2 and Ucp_3 gene expression in muscle tissue from offspring born to obese versus lean male mice*

The expression of both mitochondrial proteins (*Ucp_2*, *Ucp_3*) had been measured in the soleus muscle of both female and male offspring whose male parent was obese fed a LFD and their counterparts whose male parent was lean. We observed an impressive finding in different sex set of offspring. Both *Ucp_2* and *Ucp_3* expression revealed a trend to a lower expression in females fed a LFD whose male parent was obese compared to control offspring whose male parent was lean (Figure 4- 9). However, *Ucp*s gene expressions showed a trend to a higher expression in males fed a LFD whose male parent was obese compared to their counterparts whose male parent was lean (Figure 4- 9).

4.4 Discussion:

We placed C57BL/6J male mice on either a HFD or a LFD for 5 weeks before breeding with lean female mice to study the effect of paternal obesity on offspring development. Consistent with the DOHaD hypothesis, our LFD supplemented offspring had heavier body weights at weaning; however, their body weight curves afterward had reflected similar body weights regardless if their male parent was obese or lean.

Furthermore, based on our previous work during maternal obesity, we had reached a remarkable speculation that the micronutrient composition of the food consumed by the offspring was a critical factor affecting the results (Aburasayn et al. 2017). We had placed our offspring on either a LFD or a HFD, as we wanted to test the effects of parental obesity on offspring development under both conditions. We assumed that in the HFD offspring we might observe a different body weight curve than the LFD group. We expected that HFD offspring whose male parent was obese to have a significantly heavier body weight than those whose male parent was lean. However, we were surprised that the HFD group did not show the trend we expected. To our surprise, both female and male offspring of the LFD group demonstrated a notably heavier body weight gain at the weaning age, and unexpectedly the HFD offspring exhibited insignificant body weight gain results at the weaning time. Moreover, evidence for possible negative metabolic results arose from a study in Sprague-Dawley male rats supplemented with HFD and bred to a lean dam; the model produced glucose

intolerance and insulin resistance female offspring that were exhibited similar body weights profile (Ng et al. 2010). Therefore, we focused on testing the metabolic health of the offspring.

In fact, the glucose and insulin tolerance tests did not reveal any abnormalities in the glucose/ insulin tolerance for the offspring whose male parent was obese, which was not expected in our offspring model especially the group that was supplemented with HFD. Of interest, our results opposed to other results in the field of paternal obesity that used same model of C57BL6 black mice in their research. Although our results regarding the glucose and insulin tolerance are opposite to others in the field, other studies also reported negative results in further generations of mice (Fullston et al. 2013). Hence it seems likely that if we kept our obese and lean male breeders for additional litters, these offspring might show similar results more consistent with previous findings in the field. Conversely, our offspring, which are considered the first generation of our obese male breeders, did not show glucose homeostasis abnormalities. One main difference that may explain this difference is that we fed our male mice a HFD for 5 weeks, whereas Fullston and colleagues fed their animals HFD for 10 weeks (Fullston et al. 2013).

Of interest, we observed a trend to improved glucose levels only in female offspring when compared to their counterparts born to a lean male, which recapitulates observations that demonstrated a significant improvement in glucose tolerance only in the female offspring (Ng et al. 2010). Although we do not yet know the reason for this trend, we observed regarding improved glucose

tolerance in the female offspring, sex/ gender differences maybe involved. Sex hormones are central gender differences between different sexes. For instance, the higher percentage of estrogen in females could be a factor affecting our results. Gender differences may be an influential risk factor for offspring development, as the DOHaD hypothesis is a broad concept that suggested not only parental risk factors may influence offspring obesity, but also offspring gender may lead to the development of chronic disease in those offspring born to obese parents (Barker 2004).

Along with the trend of improved glucose clearance, additional observations suggested sex-specific differences, including findings of improved pyruvate tolerance in LFD fed male offspring whose male parent was obese. However, the age of 13 weeks is at the beginning of the mice adulthood period, which means our male offspring may have a protective mechanism when they were children, but that protective mechanism may be no longer active, as they are adults. Interestingly, the PCR findings for the Ucp_2 and Ucp_3 gene expression of muscle tissue in both female and male offspring demonstrated a unique trend in different gender. Those findings may explain some of our metabolic indirect calorimetric results of the LFD offspring whose male parent was obese, where female offspring exhibited more ambulatory activity with lower oxygen consumption which suggesting a more efficient muscle in our female offspring model. The *Ucp*s results in our models are unique and to be further investigated as the increase and the decrease of *Ucps* may play a role in body weight gain and preventing obesity (Clapham et al. 2000; von Essen et al. 2017).

We also had remarked that most of the previous studies had used the regular chow as a control diet to the experimental HFD model, which may explain the differences between previously conducted work and our current results. To our knowledge, there is a difference in the micronutrient composition between LFD and regular chow that may affect the results.

On the whole, our results contrast with those of previous studies consistent with the DOHaD theory suggesting that paternal obesity leads to offspring obesity and metabolic dysfunction during development.

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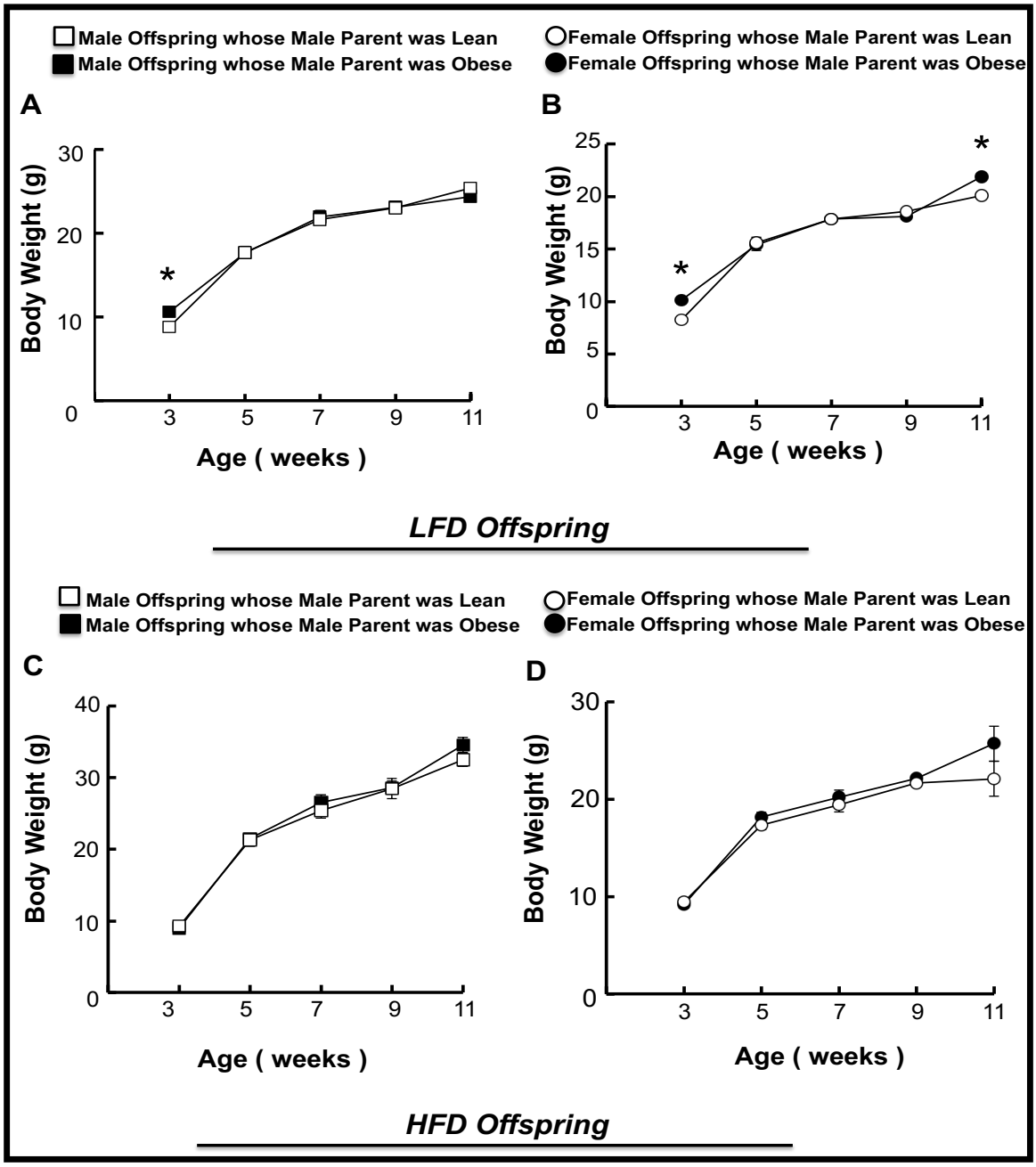

Figure 4- 1 Body weights measurement from weaning until 14 weeks of age in male and female offspring (n =11). (A and B) Body weight Curves for male and females (LFD) offspring and (C and D) Body weight Curves for the male and female (HFD) offspring. *P < 0.05.

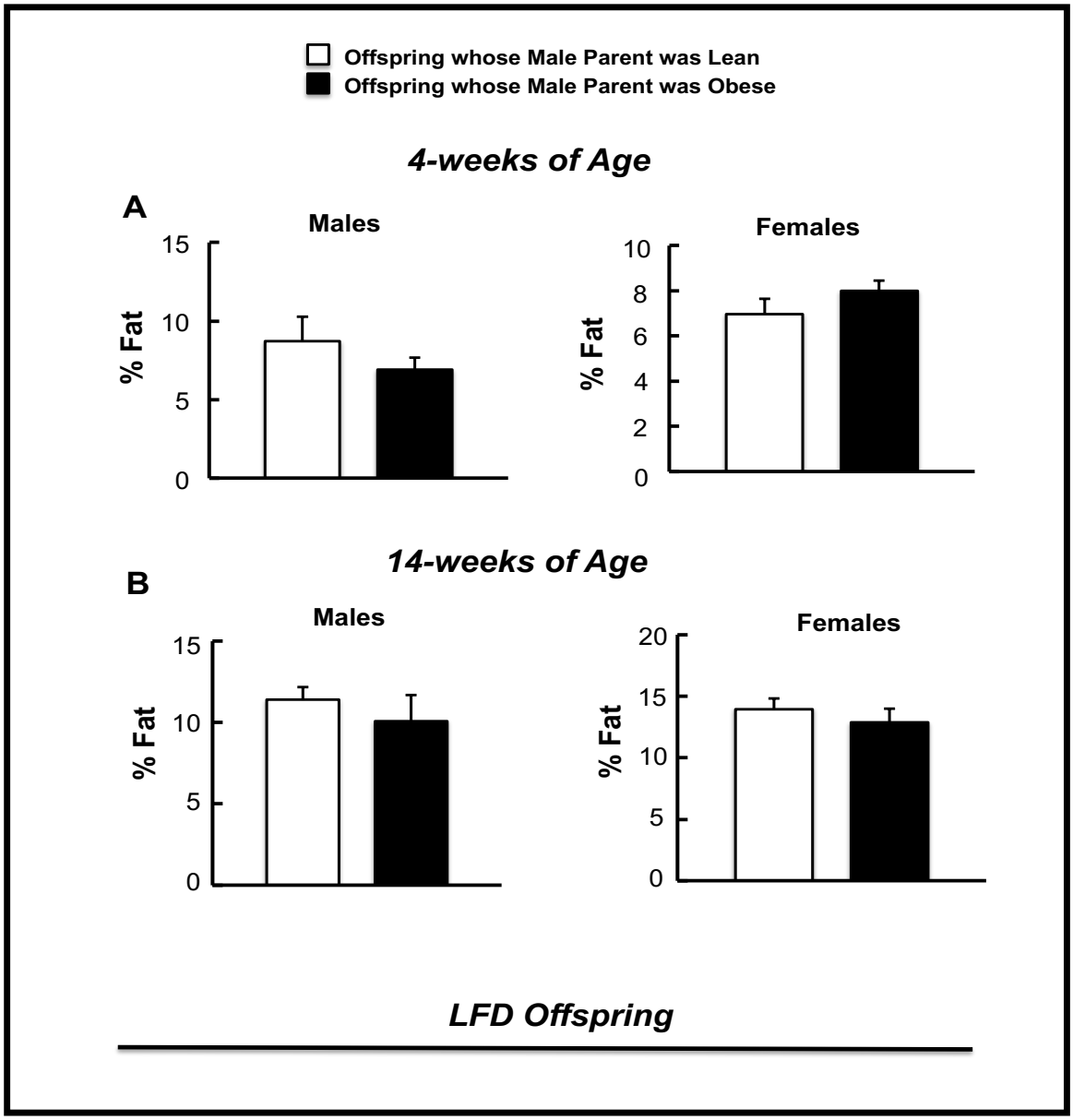

Figure 4- 2 Adiposity in male and female offspring whose male parent was obese and fed a LFD. (A) Fat mass of LFD offspring at 4 weeks (males n=7, females n=9). (B) Fat mass for LFD offspring at 14 weeks of age (n = 6). *P < 0.05.

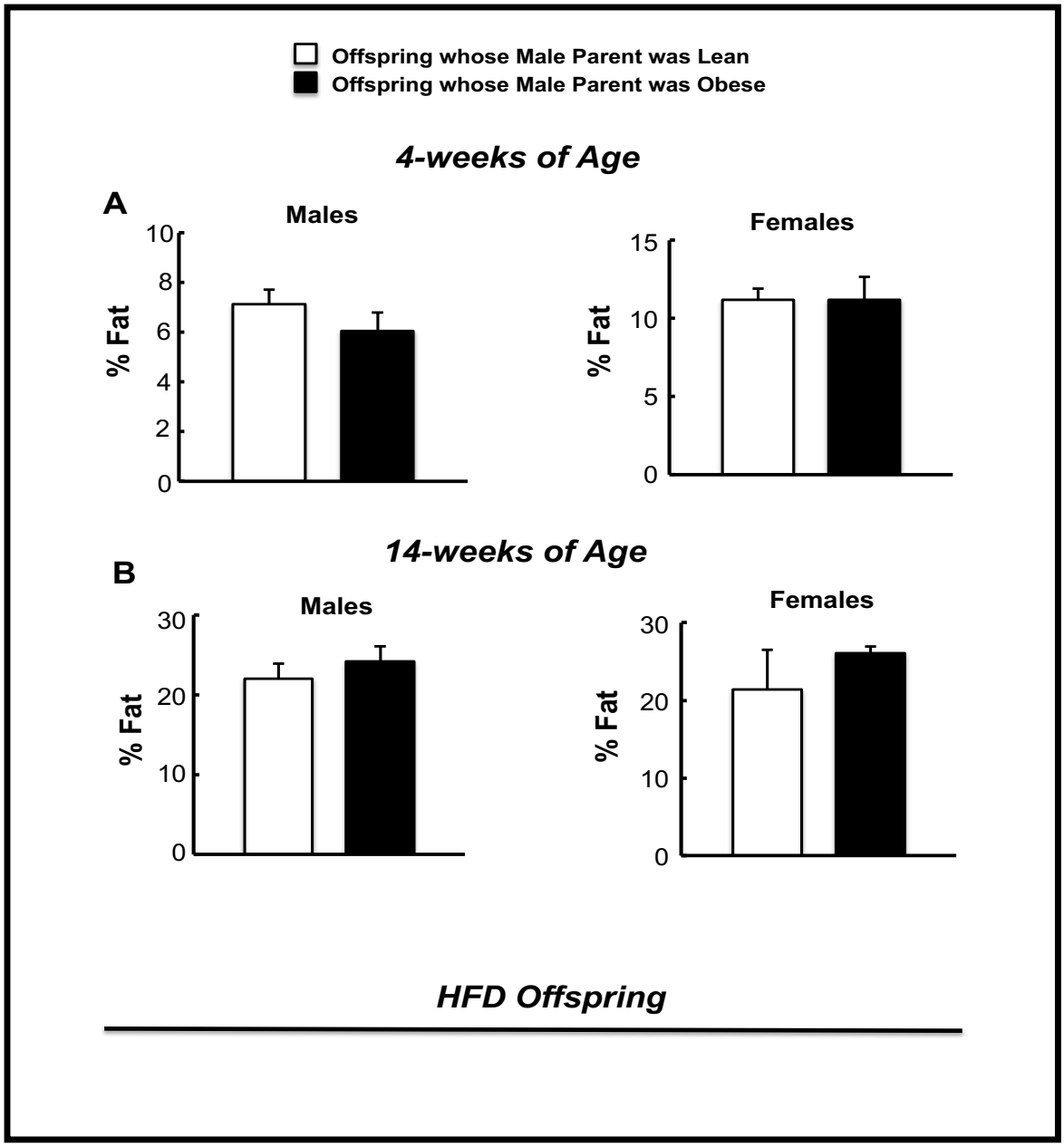

Figure 4- 3 Adiposity in male and female offspring whose male parent was obese and fed a HFD (A) Fat mass of HFD offspring at 4 weeks (males n=7,8, females n=3,6). (B) Fat mass for HFD offspring at 14 weeks of age (males n=7,8, females n=3,6). *P < 0.05.

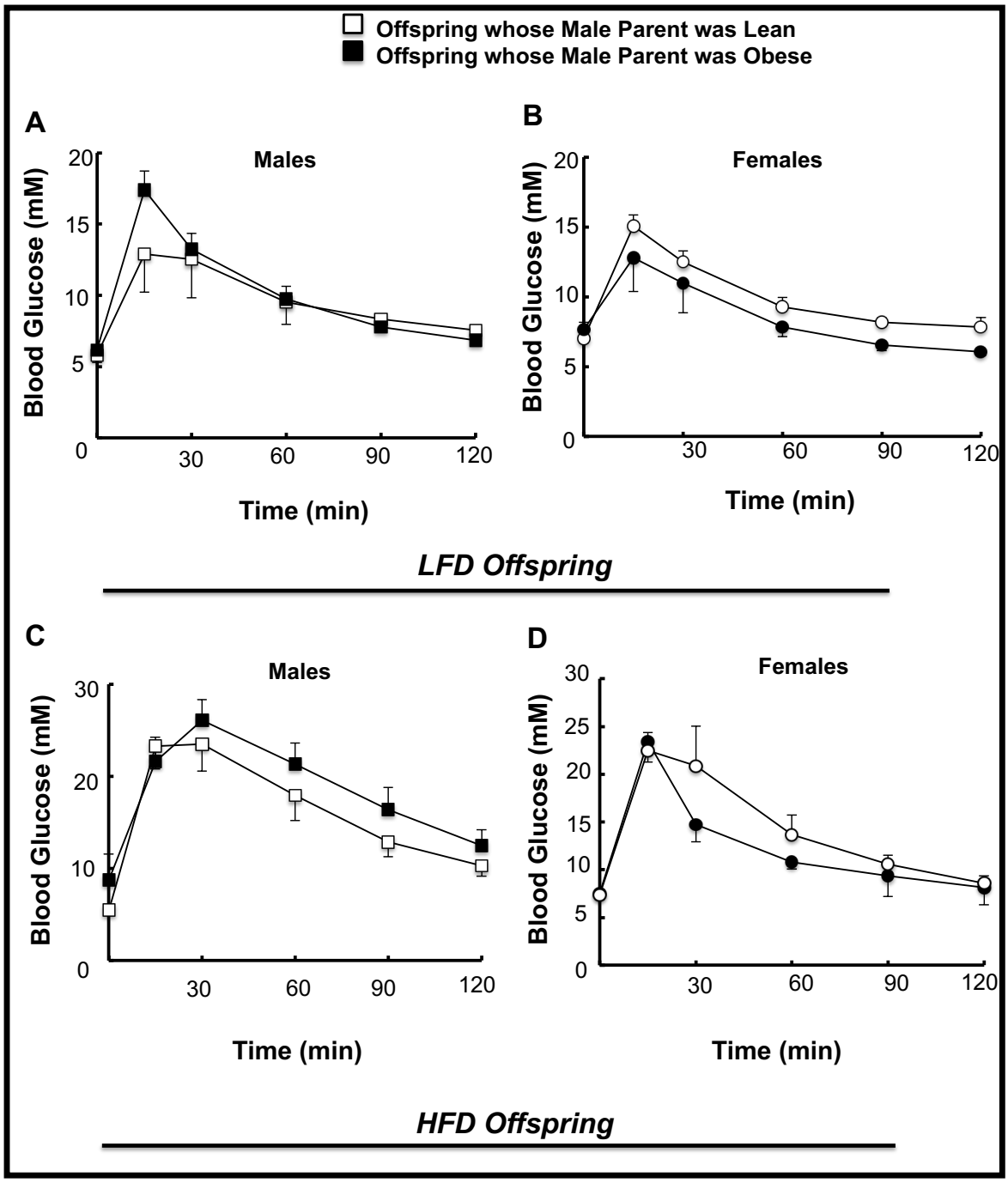

Figure 4- 4 Offspring Intraperitoneal Glucose tolerance test results at 11 weeks of age for male and female offspring whose male parent was obese and fed a LFD or HFD. (A and B) glucose tolerance test in the LFD group (n=5). (C and D) glucose tolerance test in the HFD group (males n=7,8, females n=3,6). *P < 0.05.

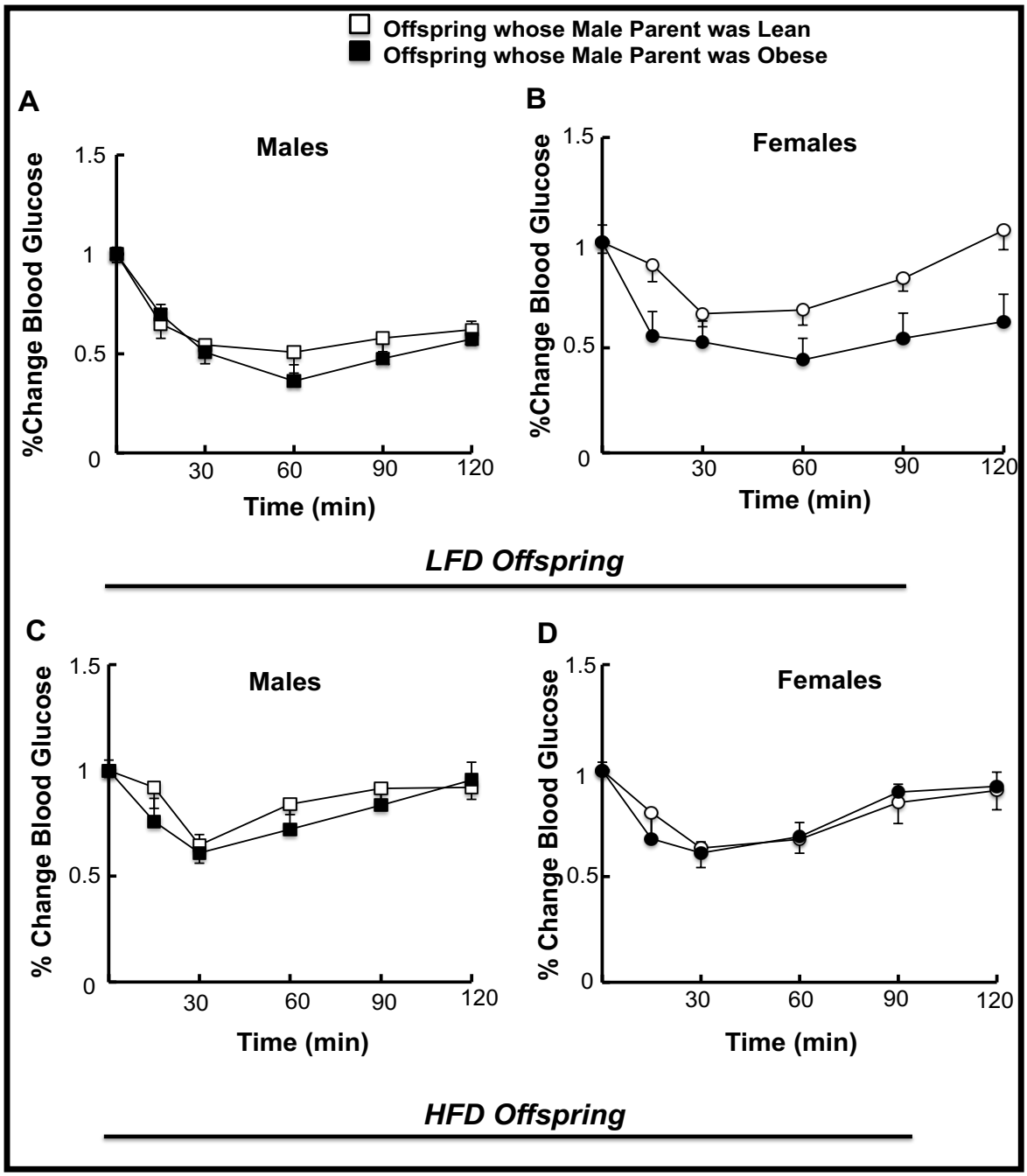

Figure 4- 5 Insulin tolerance test results of offspring whose male parent was obese at 12 weeks of age. (A and B) the ITT results of the LFD group (males n=6, females n=8,6). (C and D) the ITT results for the HFD offspring (males n=7,6, females n=3,6). *P < 0.05.

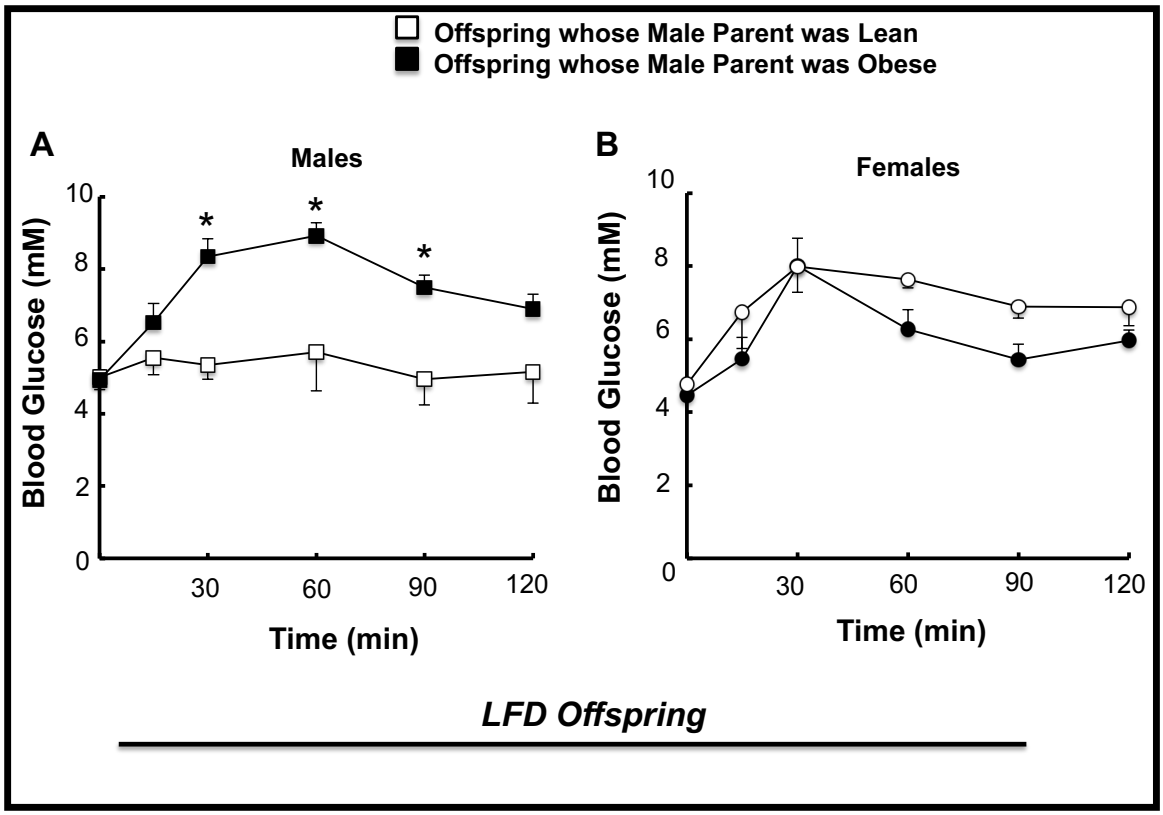

Figure 4- 6 The Pyruvate tolerance test done in 13 weeks of age for the LFD offspring whose male parent was obese and their counterparts whose male parent was lean (A) the male offspring results (B) the female offspring results (males n=6,7, females n=6). *P < 0.05.

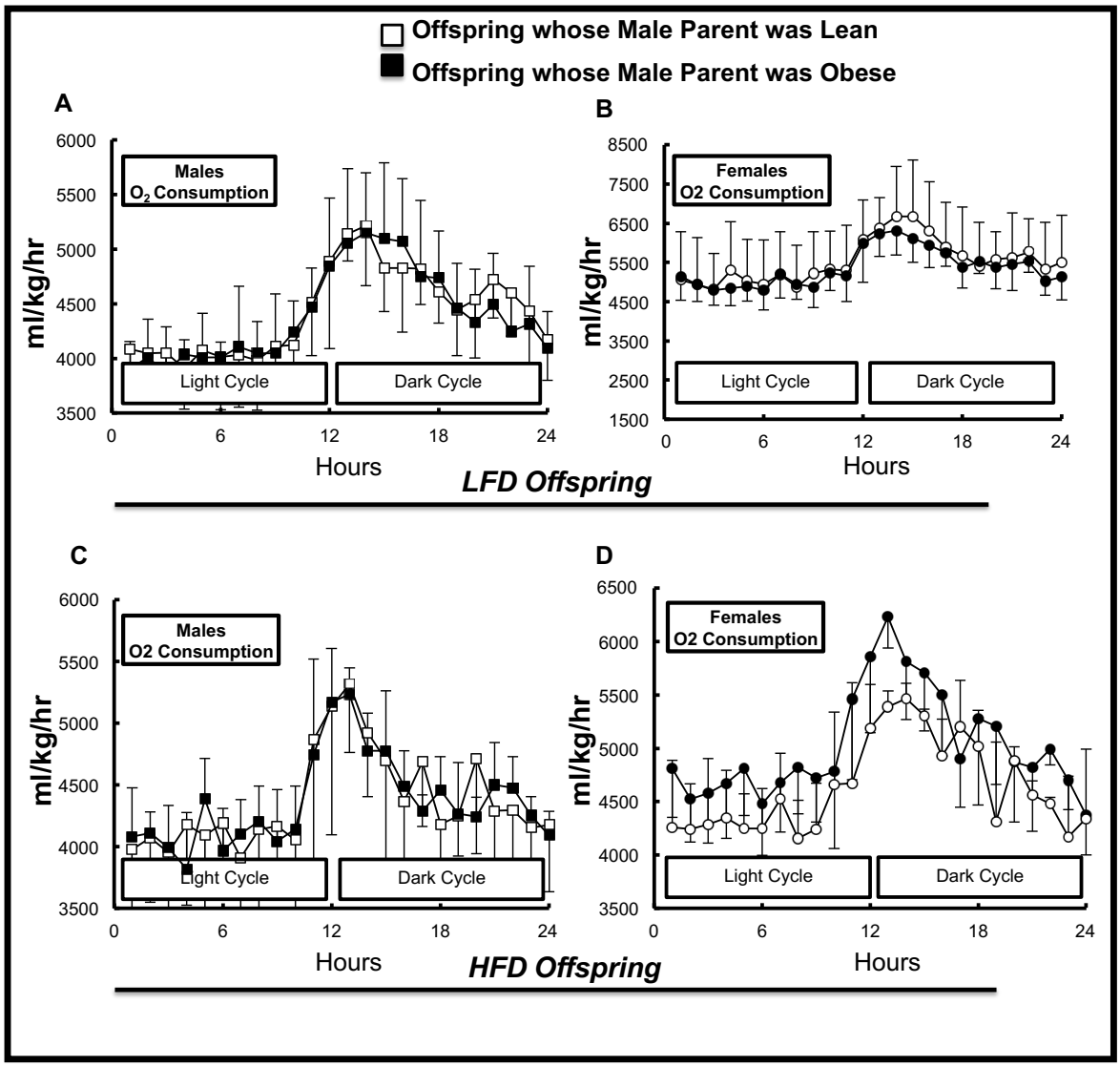

Figure 4- 7 O_2 consumption results of offspring whose male parent was obese. (A and B) showing results of the LFD group (n=6). (C and D) results for the HFD offspring (males n=4,5, females n=3,6). *P < 0.05.

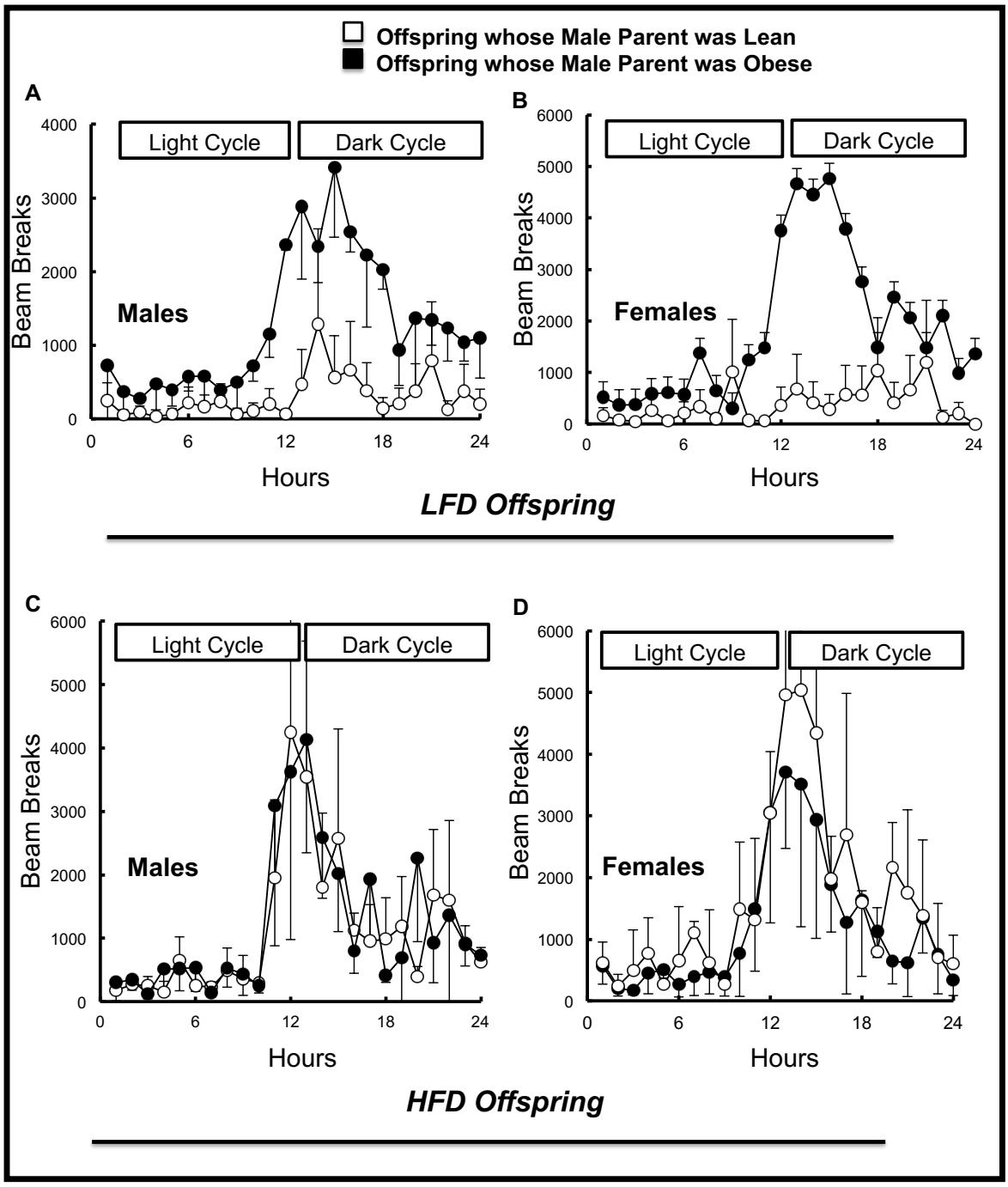

Figure 4- 8 Ambulatory Activity results of offspring whose male parent was obese. (A and B) showing results of the LFD group (n=6). (C and D) results for the HFD offspring (males n=4,5, females n=3,6). *P < 0.05.

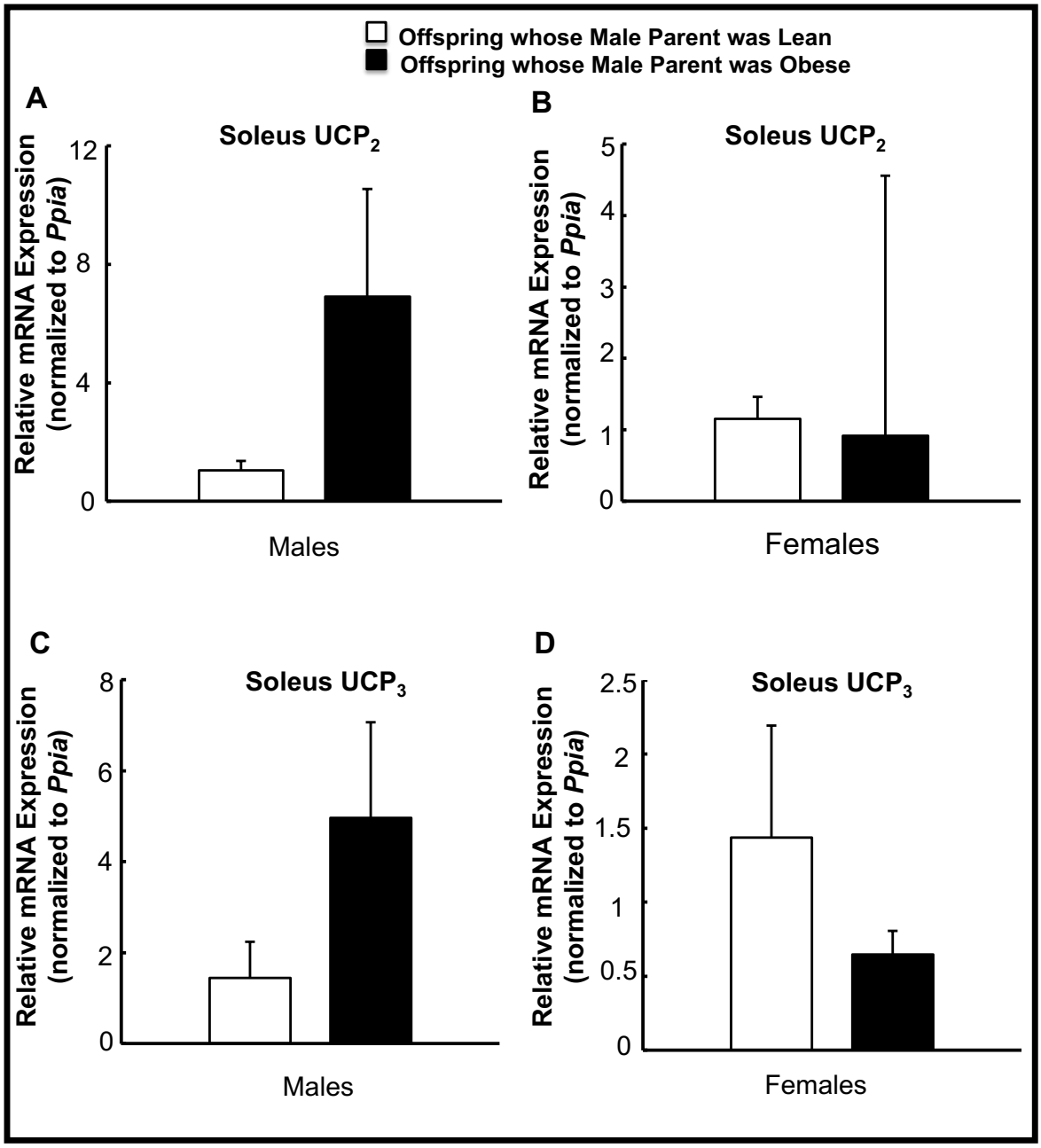

Figure 4- 9 mRNA expressions of *Ucp2* and *Ucp3* in soleus tissue of LFD offspring group. (A and B) showing results of the *Ucp2* (Males n=3, Females n=4,3). (C and D) *Ucp3* results (n=3). *P < 0.05.

Chapter Five

5. Discussion

5.1 Discussion:

Previous research exploring how parental obesity increases the risk for obesity in the developing offspring has primarily focused on maternal obesity, but recent studies have demonstrated that paternal obesity also plays a role. Thus, our research was focused on looking at both maternal and paternal obesity as independent risk factors for obesity development in the offspring. For these reasons, our primary hypothesis was that offspring born to an obese dam or offspring whose male parent was obese would have significantly heavier body weights through out their lives. In addition, we hypothesized that these offspring would show signs of early obesity- related metabolic dysfunction, which maybe due to inheriting a specific molecular epigenetic signature in key genes regulating energy metabolism that would be inherited by the offspring. Indeed, this is supported by previous work (Fullston et al. 2013; Howie et al. 2009; Laker et al. 2014). To our surprise, our findings did not reproduce the previous experiments of others in the field. Our primary conclusion that directly contrasts previous work is that parental obesity does not increase the risk for obesity in the developing offspring. Furthermore, most of our glucose and insulin tolerance tests failed to match our hypothesis. Although we established our parental obese model carefully, we observed negative results in the offspring that we did not expect. For instance, in both projects we used a HFD to induce obesity and insulin resistance/glucose intolerance in either the female dams or male mice before breeding. The body weights of the offspring that were born to obese dams and

whose male parent was obese did not show striking differences as seen in previously seen by others (Howie et al. 2009; Laker et al. 2014). However, during weaning we did see significant body weight differences in the female offspring born to the obese dam, and in both sexes whose male parent was obese. Even though we had a group of offspring on HFD, the obese male's offspring body weight was not heavier even during the weaning period. Our offspring were placed on different types of food because we aimed to test whether placing the offspring on LFD or on HFD that induces their obesity. We wanted to determine if there was an increased risk of body weight gain and metabolic dysfunction in the offspring that were born to obese dams or whose male parent was obese.

Of importance, the LFD we utilized as our control diet is the appropriate control diet to match our HFD since they are from the same manufacturer. However, most previous studies do not employ the proper control diet and instead use their animal facility standard chow, which may greatly differs in the micronutrient composition. For instance, many studies often use a regular chow diet (2018S) from Teklad, which is used in most animal facilities as a regular supplemented chow, for their control group (Laker et al. 2014). While in our study we had the LFD from lard (Research Diets) supplemented for our control groups. The micronutrient composition has some differences between the Teklad standard chow and the Research diets LFD that may cause the difference in our results. According to (Warden and Fisler 2008), there are two different main compositions between the regular chow and the LFD, which are the phytoestrogen and the sucrose compositions. The phytoestrogen micronutrient is

found in high amount in regular chow, whereas they are nonexistent in the Research diets formulations. Oppositely to the phytoestrogen in the regular chow, the sucrose which is considered as a carbohydrate source that is found in the special diets formulas, but not in the ordinary chow. Phytoestrogen in diet compositions is believed to affect appetite, motor activity, fat mass and the metabolism of lipids (Torre-Villalvazo et al. 2008) (Lephart et al. 2004). Thus, differences in micronutrient composition may account for differences in our offspring phenotypes versus that reported by others in the field.

For the increased body weight observed during weaning, in our female offspring born to obese dams, this could be due to the cross-fostering that we performed by having only the obese dam offspring receive nursing from a surrogate lean dam during lactation. That may stress the offspring causing unexpected effects on body weight or a foster mother may not nurture the pups the same as the natural mother. The reason we cross-fostered was to study the epigenetic modifications that may be transmitted from the dams to their offspring. Our unexpected results could also be explained by the set-point theory that directly suggests that if offspring had intrauterine lives from their obese dam, this might induce a defense mechanism during their postnatal lives to prevent the risk of obesity (Farias et al. 2011). In addition, Barker and colleges had explained this defensive mechanism in their ‘thrifty phenotype hypothesis’ where fetuses born to unhealthy mothers may become not obese as a way of accommodation (Hales et al. 1991). However, our maternal obesity study (Aburasayn et al. 2017) demonstrated a major drawback, which is cross-fostering was not conducted to

the offspring that were born to LFD dam by an obese dam. To address this issue, a new breeding group was set up, where we had an obese surrogate dam to the offspring born to lean dam and vice versa for the lean surrogate dam. Then, body weights were recorded to see the rate of body weight gain and compare it directly to offspring that were fostered with lean dams (Figure 5- 1). This data demonstrated that the offspring born to obese dams showed similar body weights to their counterparts born to lean dams. Consistently, our work in chapter 3 (Figure 3- 3) also shows that when we only cross- fostered the obese dam offspring with a lean dam, there were no differences in body weight. All these bodyweight profiles we observed with the cross-fostering in offspring are suggesting sex-related differences, especially in our female offspring. However, we only used male offspring for this new cross- fostering study and it would be important to do females in the future as well.

For that reason, we speculate if females that were born to lean dams were also cross-fostered with an obese dam, their body weights will be similar to the female offspring in chapter 3 (Figure 3- 4 A), where only females born to HFD dam were cross- fostered with a lean dam.

Along with female offspring born to HFD distinctive pattern, the paternal obesity project results also demonstrated a similar trend. Even though we had a group of offspring supplemented with a HFD, their body weight, and metabolic profile results did not reflect an increased risk of developing obesity. However, the profile of the HFD group unexpectedly exhibited a normalized body weight gaining pattern between the groups, but the LFD group showed a significant

body weight gain at the weaning period in both genders which suggested sex-related differences. In addition, our glucose homeostasis assessments showed a unique trend in female offspring, but not in the male offspring whose male parent was obese, where the female offspring have a trend to lower circulating glucose levels when they compared to female offspring whose male parent was lean. It remains unclear as to what accounts for the sex-related differences we observed in the developing offspring, but it has been well established that males and females have both hormonal and metabolic differences that affect their development in all stages of life.

Moreover, the prevalence of certain diseases such as metabolic syndrome, diabetes and obesity are known to exhibit different effects on gender (Glümer et al. 2003). More specifically, some studies have reported that glucose intolerance has a higher incidence in females than males (Glümer et al. 2003; van Genugten et al. 2006). Moreover, it has been reviewed by (Mauvais-Jarvis 2015) that females and males have different ways of regulating and maintaining the metabolism equilibrium. Most of the results from our maternal and paternal studies resulted in trends in one gender that we did not observe in the other. Our results are thus consistent with the “sexome” theory proposed by Art Arnold, which postulates that each gender has unique effects on gene and molecular regulation, which in consequence will produce a gender-specific phenotype which may differentially affect multiple aspects of whole-body energy metabolism (Arnold and Lusis 2012).

One major difference between previous studies and our studies is the age of the offspring as most of the studies keep their animal models for months after they born such as five months and 12 months (Howie et al. 2009; Laker et al. 2014; Ng et al. 2010). Importantly, significant differences in glucose tolerance or the body weight gaining in those studies are observed at adult ages or further ages than our study. More interestingly, before the animals aged in the previous studies, their results showed similar results as our study that mainly had similar body weights in offspring born to obese parents and their counterparts with similar glucose tolerance between the groups as well. In our studies, we observed the offspring model born to either an obese dam or obese male for a maximum period of 4 months, and it is possible if we let our animals age for a longer period, we may have observed differences in metabolism, glucose tolerance and weight gain. Our reason to keep the animals for only 4 months and not more was to study the progression of these disorders during childhood period. Taken together, our findings and previous findings suggest that offspring that were born to obese parents may not develop obesity and metabolic syndrome during adolescence, however, they may start to gain weight and have metabolic disorders when they become adults regardless of their daily diet.

Overall, our study aimed to confirm whether either maternal or paternal obesity truly increases the risk for obesity and metabolic dysfunction in the developing offspring. In contrast to many previous studies in the literature, our preclinical findings using experimental mouse models do not support that parental obesity is a major risk modifier for obesity in children, though we did

observe unique sex-related differences in both male and female offspring. We propose that a major potential factor accounting for our contrasting findings is that the majority of previous studies have not used the appropriate control diet for their experimental high fat diet that is used to produce parental obesity for generating offspring to study development.

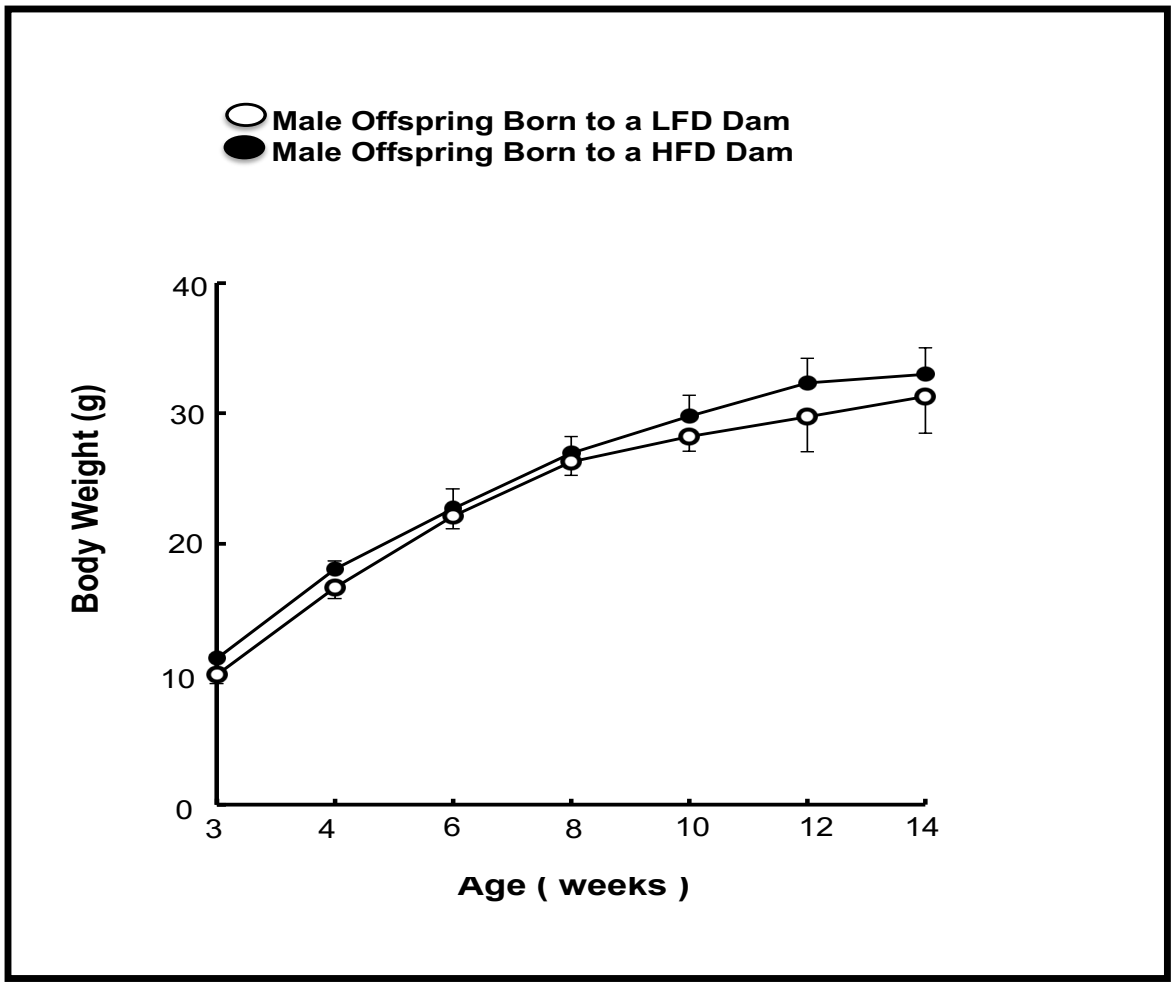

Figure 5- 1 Body masses from weaning until 14 weeks of age in male offspring born to C57BL/6J dams fed a LFD or HFD for 5 weeks (n = 3, 4). Both group of offspring were cross-fostered with opposite diets dams than their genetic mothers. *P < 0.05.

Chapter Six

6. Summary and Future Directions

6.1 Summary:

Obesity is a serious health concern that is affecting many societies in every country. Correspondingly when the rate of obesity is increasing, the risk of other chronic disorders is also rises, including metabolic syndrome, T2D, and cardiovascular diseases. It is not only adults that are affected by obesity, but our childhood population is also showing increased prevalence rates of obesity. Thus, a future generation of adults is likely to be suffering from chronic disorders, which will produce a huge burden on the healthcare system. Therefore, the field has invested a huge effort in trying to find ways to pharmacological treat or prevent obesity, yet the vast majority of those studies have found limited success. Recent studies have focused on understanding how parental obesity may affect offspring development and the risk for obesity, as that may be a novel approach to prevent obesity risk in future generations. One proposal that may contribute to increasing the risk of obesity in children includes epigenetic alterations, and it is possible that preventing epigenetic inheritance may help in reducing the risk for pediatric obesity.

Over the past few years, understanding the mechanism of epigenetics has been an area of focus for many researchers who are considering new treatment approaches for treating chronic diseases. Specifically targeting the gene expression or reversing the epigenetic modifications occurring during disease development through drugs is now a promising field of discovery. In addition, the reversible nature of epigenetics could be suitable for suggested future treatment

through targeting certain genes. In fact, approved drugs targeting epigenetics modifications already in existence and available for use are known as the new generation of epigenetics drug discovery (Hunter 2015). The research medical field is suggesting epigenetics therapeutic agents as a possible treatment to develop within the next few years. These agents may prevent or reverse specific disorders, especially if the diseases are targeted during the disease's development (Heerboth et al. 2014). Therefore, this thesis work provides an analysis of numerous aspects that may lead to obesity in offspring born to an obese male or dam. The diet and gender of offspring are two factors requiring exploration through our work.

The first project of our study aimed at studying the effect of maternal obesity on offspring by exploring how this may increase the prevalence of offspring obesity. To address our study's objective, we developed obese C57BL/6J female mice by placing them for 5 weeks on HFD. We produced a validating model of maternal obesity and observed glucose intolerant obese animals. After establishing the obese model, this model was bred with a C57BL/6J lean male to study the prevalence of obesity in their offspring. Data from offspring suggested that maternal obesity did not lead to risk of obesity in offspring according to body weight and circulating glucose level. However, we observed a significant increase in the body weight of female offspring born to an obese dam at the age of weaning (3 weeks) and a significant decrease in the body weight at 14 weeks of age. Therefore, tissue analysis of the gastrocnemius muscle tissue was conducted for female offspring born to an obese dam and

their counterparts to discover the difference in *Ppargc1a* mRNA expression between offspring aged 3 weeks and 14 weeks. The real-time PCR results did not show any differences in the expression of *Ppargc1a* in gastrocnemius tissue at 3 weeks and 14 weeks respectively. All our results concluded that there are risk factors other than maternal obesity that may lead to offspring obesity, as our offspring model did not show any signs of obesity or metabolic disorder.

The second project of our study explored the effect of the father's obesity on the next generation. To achieve this goal, males of C57BL/6J mice were fed a HFD for 5 weeks to develop a weight gain in those mice in comparison to the control mice that were fed a LFD for the same period of time. Afterward, breeding with LFD female was confirmed by a plug test. For this project, two groups of offspring were placed on either a LFD or a HFD for their entire lives. Similar to our previous maternal study, we did not observe what we had hypothesized in these offspring. Our findings revealed that the LFD offspring group whose male parent was obese had a significant heavier body weight only at the time of weaning for both genders. However, HFD offspring did not show any significant differences in body weight. Moreover, no differences were observed in fat mass analysis and in vivo analysis of energy metabolism for the offspring. For the glucose tolerance test, even though no significant differences were recorded, trends of lower glucose clearance in female offspring whose male parent was obese were observed. However, in the LFD offspring group, a significant increase in the circulating glucose level of male offspring born to obese male was found at 13 weeks of age when the pyruvate tolerance test was analyzed. Finally,

measuring the mRNA relative expression for *Ucp*$_2$ and *Ucp*$_3$ genes in the LFD offspring group showed an increase trend, particularly in *Ucp* expression in soleus tissue of males whose male parent was obese compared to offspring whose male parent was lean. However, the *Ucp*$_3$ measurement did not result in the same observations.

The findings of the previous two projects in this study showed that either a trend or a difference in observations between genders; however, the reason of this trend is unknown yet. This study also revealed that there are factors other than inheriting obesity from parents that may lead to obesity in offspring during the period of childhood. This may explain why other researchers have found different results for another period of ages. In summary, my thesis work refutes the notion that parental obesity transmits a signal during development that predisposes the offspring to obesity, and it is likely that other factors, such as diet and physical activity in the offspring of children born to obese parents, are more important determinants in their increased risk for obesity.

6.2 Future Directions:

To understand how epigenetics may affect future generations by inducing obesity, there are multiple experimental options. First, keeping the offspring model for a longer time period may show if the risk of obesity is a conditional risk during the period of adulthood. For example, many studies had kept the animals model for months to observe the risk of obesity (Laker et al. 2014). Second, measuring DNA methylation is a valid experiment to be conducted to see how certain key regulator genes may affect the energy metabolism regulation in offspring born to obese parents. The techniques for measuring DNA methylation may be divided into two main categories: 1. Measuring whole genome methylation profiling, which can be used when the region of DNA methylation is unknown; and 2. Identifying the methylation in a known gene, which can be used when the region of methylation is known. For instance, whole genome methylation can be measured by using a special ELISA assay kit technique referred to as Methylflash Global DNA methylation, or by hydroxymethylation ELISA assay kit (Kurdyukov and Bullock 2016). Results from genome profiling will reveal many methylation alterations across the genome. Of those, there will be DNA methylation of metabolic genes that will affect the activity of some metabolic enzymes. Therefore, measuring the activity of enzymes by radiometric activity assay or by Western blotting will be critical for energy expenditure assessment.

Another valid future direction is performing other PCR experiments to measure mRNA expression in the muscle tissue for *Ucp*$_2$ in the HFD group to see

if similar results to the LFD group can be reached. If a trend in the *Ucps* expression is confirmed, then measuring mitochondrial functions will be another valid experiment to conduct. For instance, measuring the lipid content and assessing the ATP production by Magnetic Resonance Spectroscopy are two methods of testing the mitochondrial function. Finally, biochemical assessment for other collected tissues (skeletal muscles, white and brown adipose tissue, heart, liver, brain, and kidney) from both studies may take place to evaluate some of the results, especially if this could provide different observations of genders. These observations could possibly suggest different ways in treating obese individuals based on gender. Moreover, similar to previously conducted works, the *Ucp* KO mice model could be generated (von Essen et al. 2017). Then, the established model could be supplemented with HFD and compared to WT. KO mice are not expected to develop obesity. This approach supports previous conducted work on the role of *Ucp* in obesity and could be useful in the development of anti-obesity drugs.

The experiments described above will help to form an understanding of how molecular factors are passed on from obese parents to their offspring and thereby potentially improve health outcomes for obese children. Moreover, understanding how epigenetic modifications can cause early onset obesity in children may lead to the discovery of novel therapeutic agents to prevent the development of childhood obesity, thereby decreasing the prevalence of obesity in the future.

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Appendix

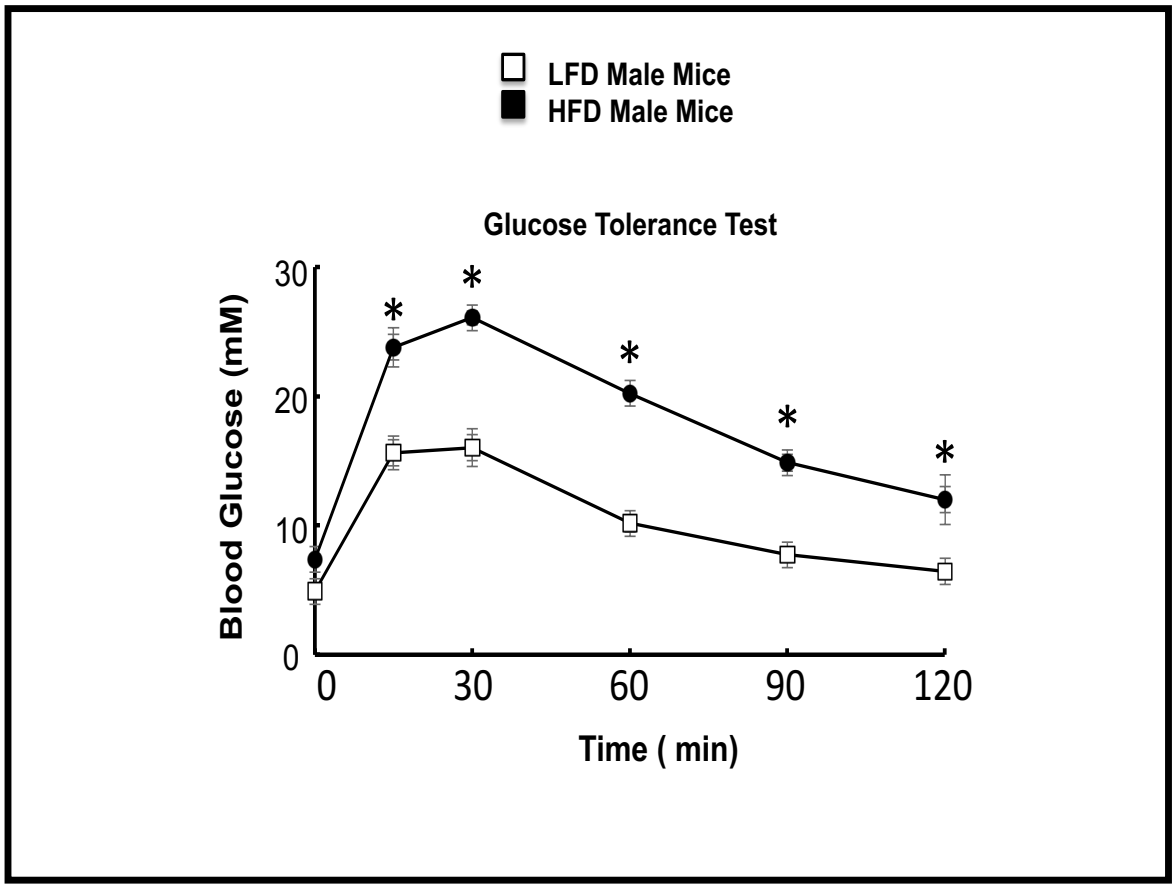

Figure A- 1 Glucose Tolerance Test for male mice of the paternal study. *P < 0.05.

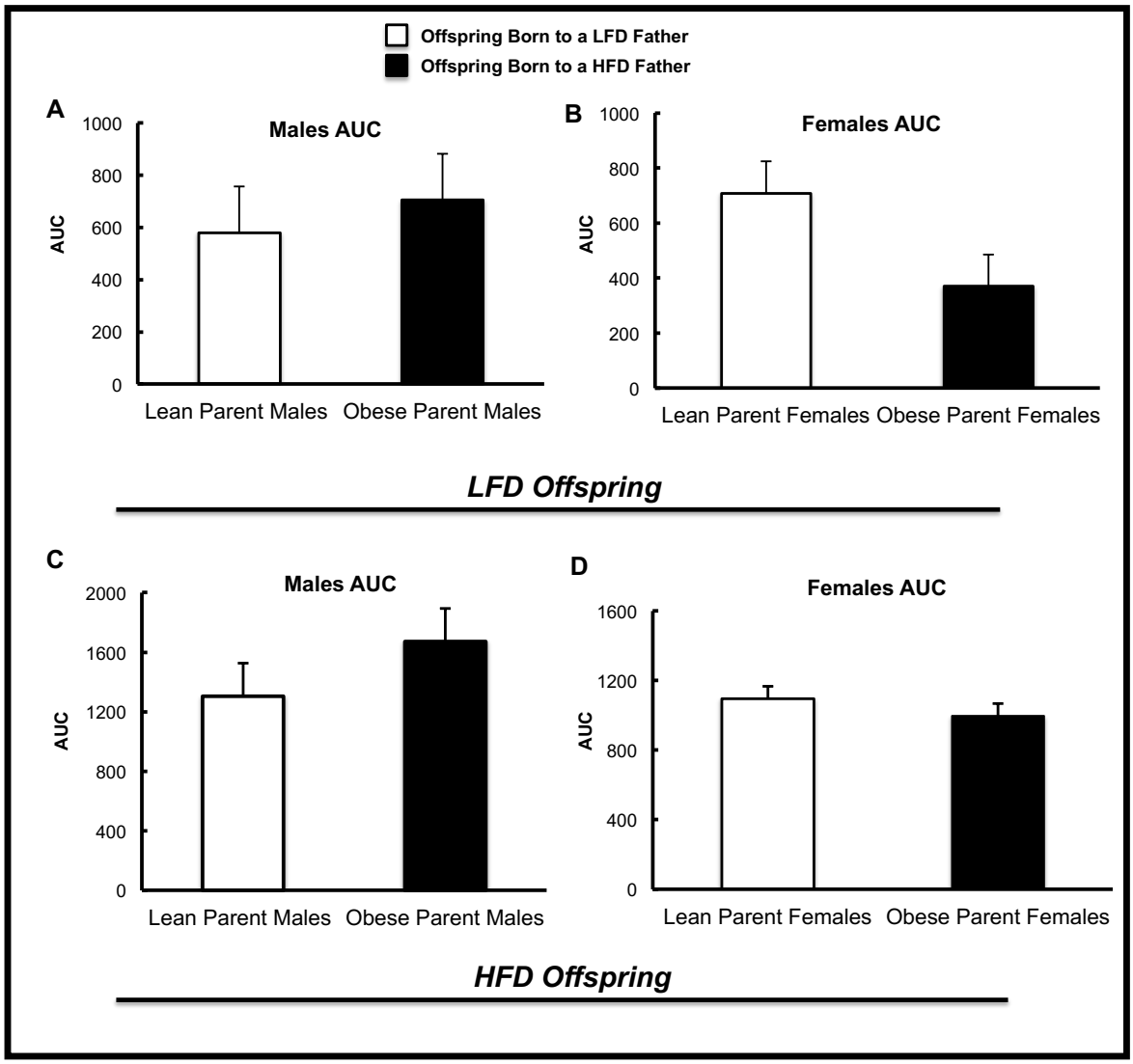

Figure A- 2 Area Under the Curves graphs of IP GTT for Obese male Study for (A and B) the LFD offspring group and (C and D) HFD offspring group. *P < 0.05.

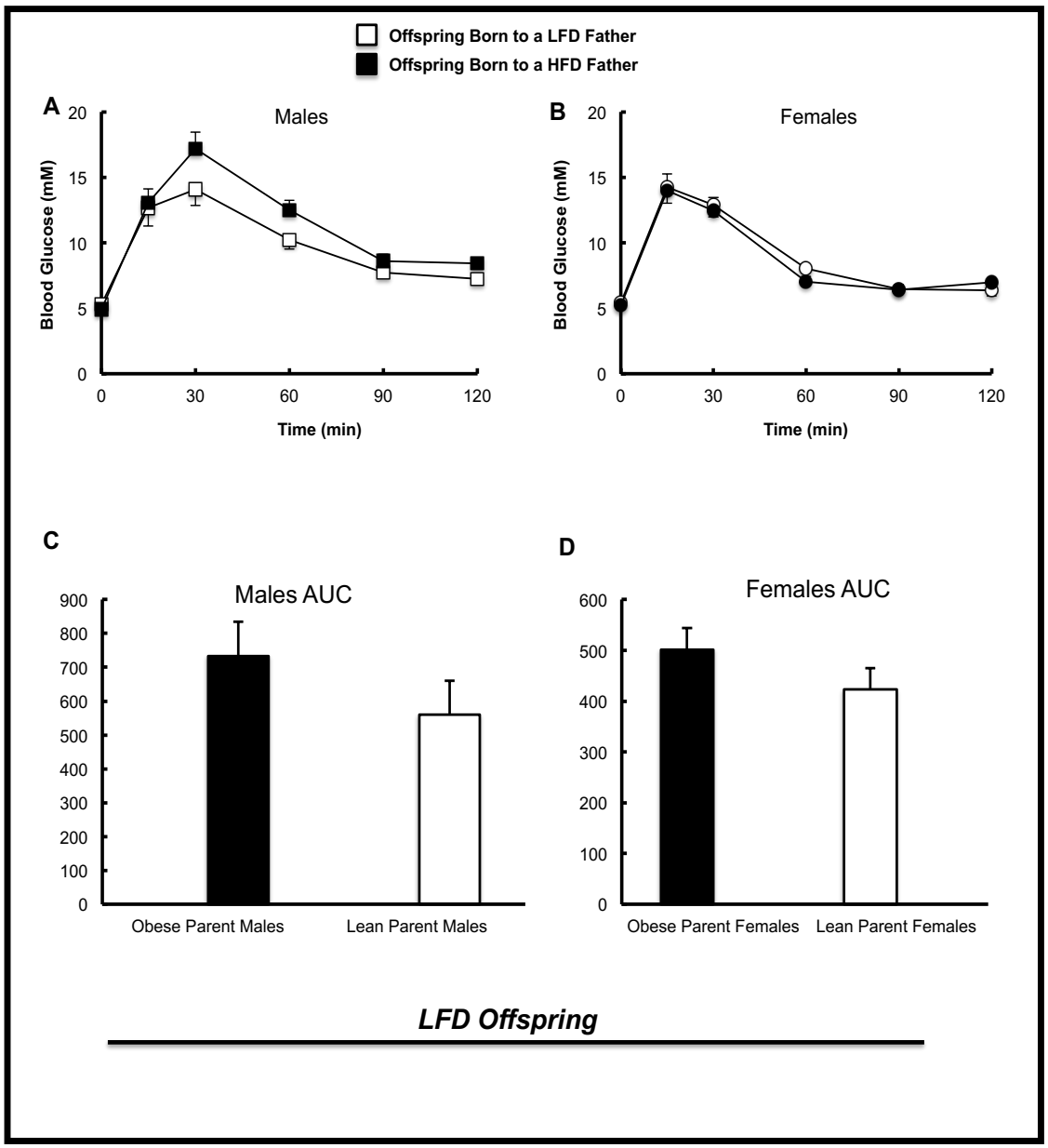

Figure A- 3 Oral GTT graphs for obese male mice Study (A and B). AUC for both graphs (C and D). *P < 0.05.

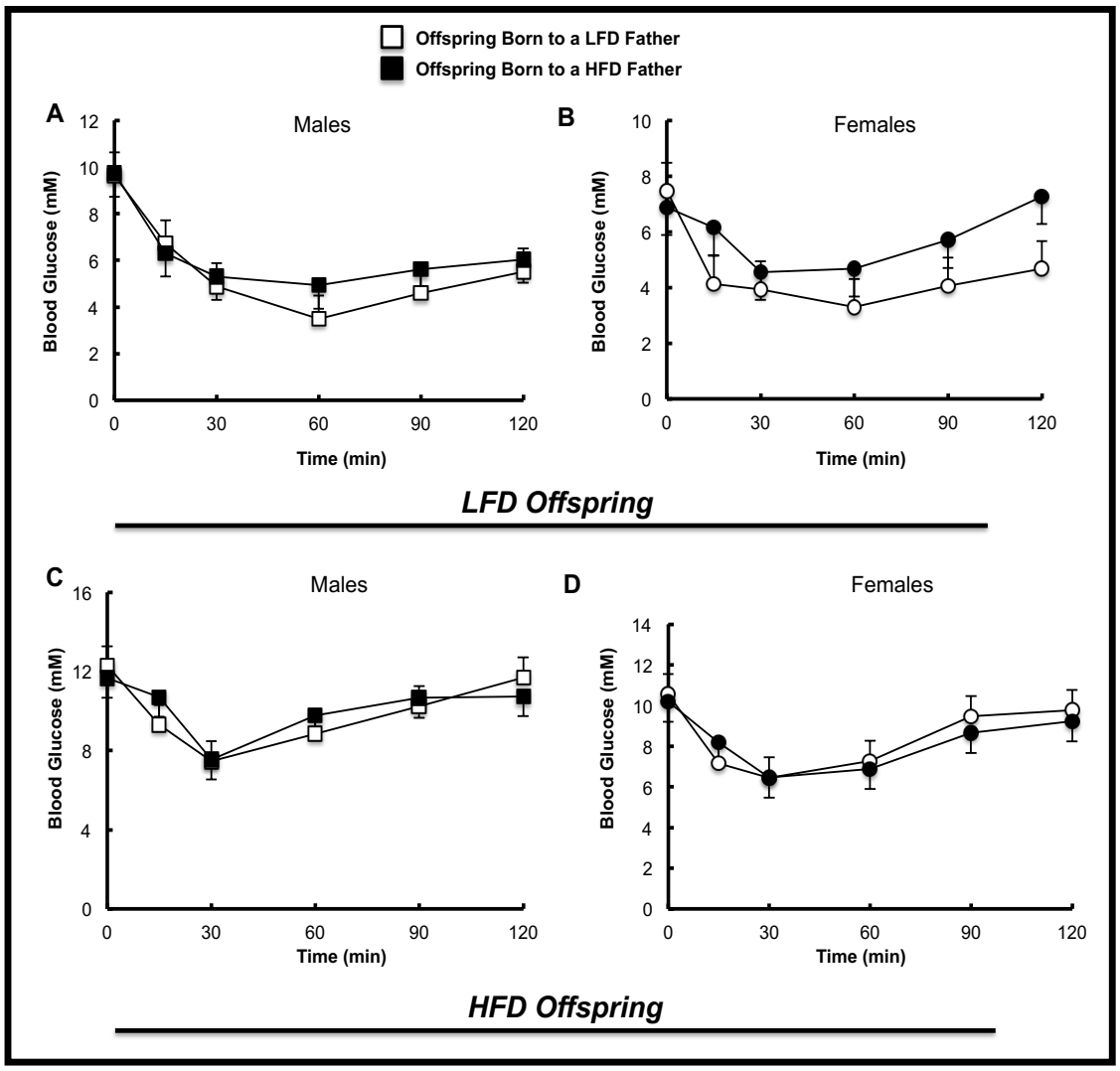

Figure A- 4 Insulin Tolerance Test graphs for Obese male mice Study for (A and B) the LFD offspring group and (C and D) HFD offspring group. *P < 0.05.

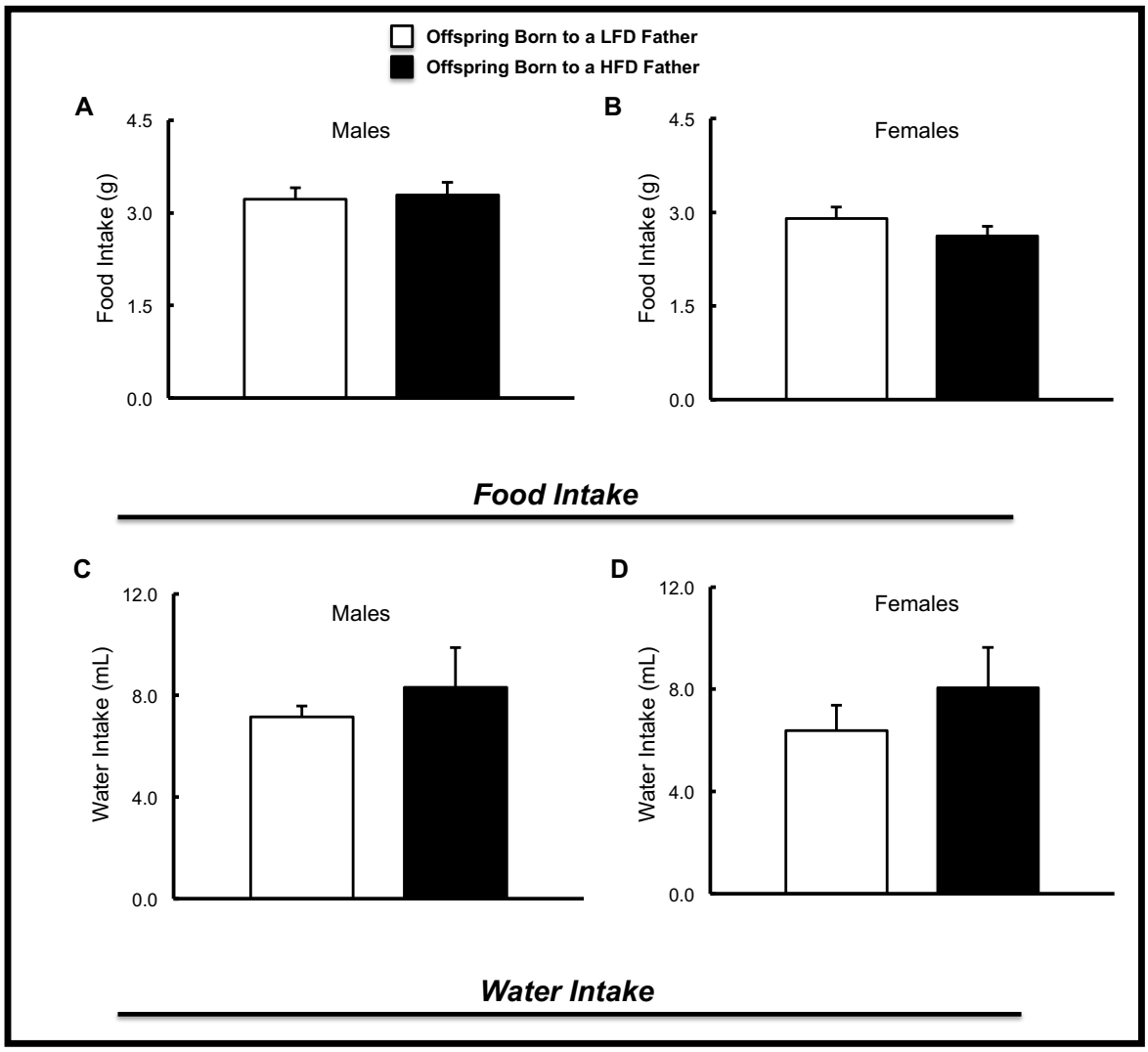

Figure A- 5 Food and Water Intake graphs from Obese male mice Study for the LFD offspring group (A and B) Males and Females food intake (C and D) Males and Females water intake. *P < 0.05.

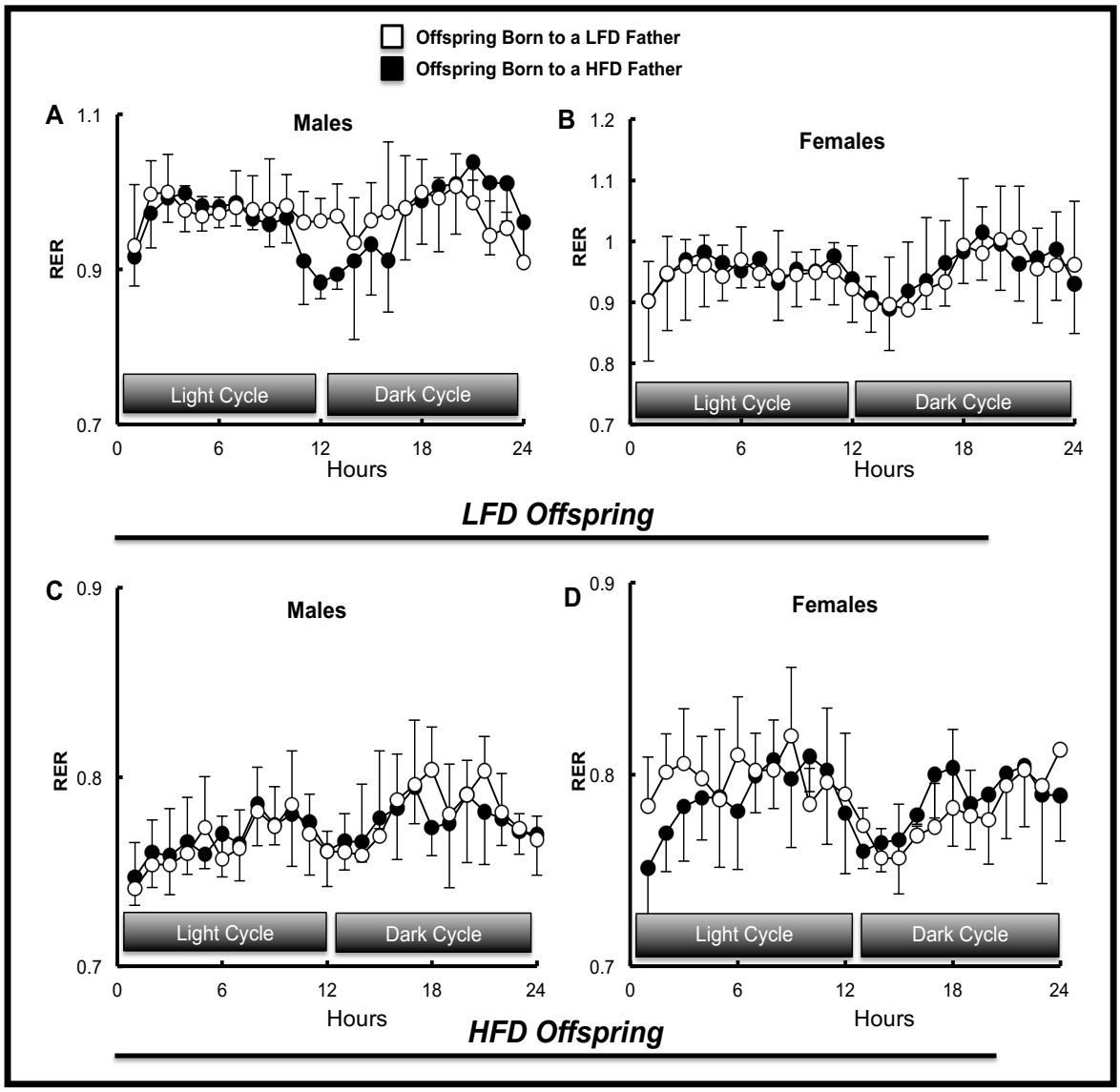

Figure A- 6 Respiratory Exchange Ratio graphs from Obese male mice Study for both offspring groups (A and B) LFD offspring group (C and D) HFD offspring group. *P < 0.05.

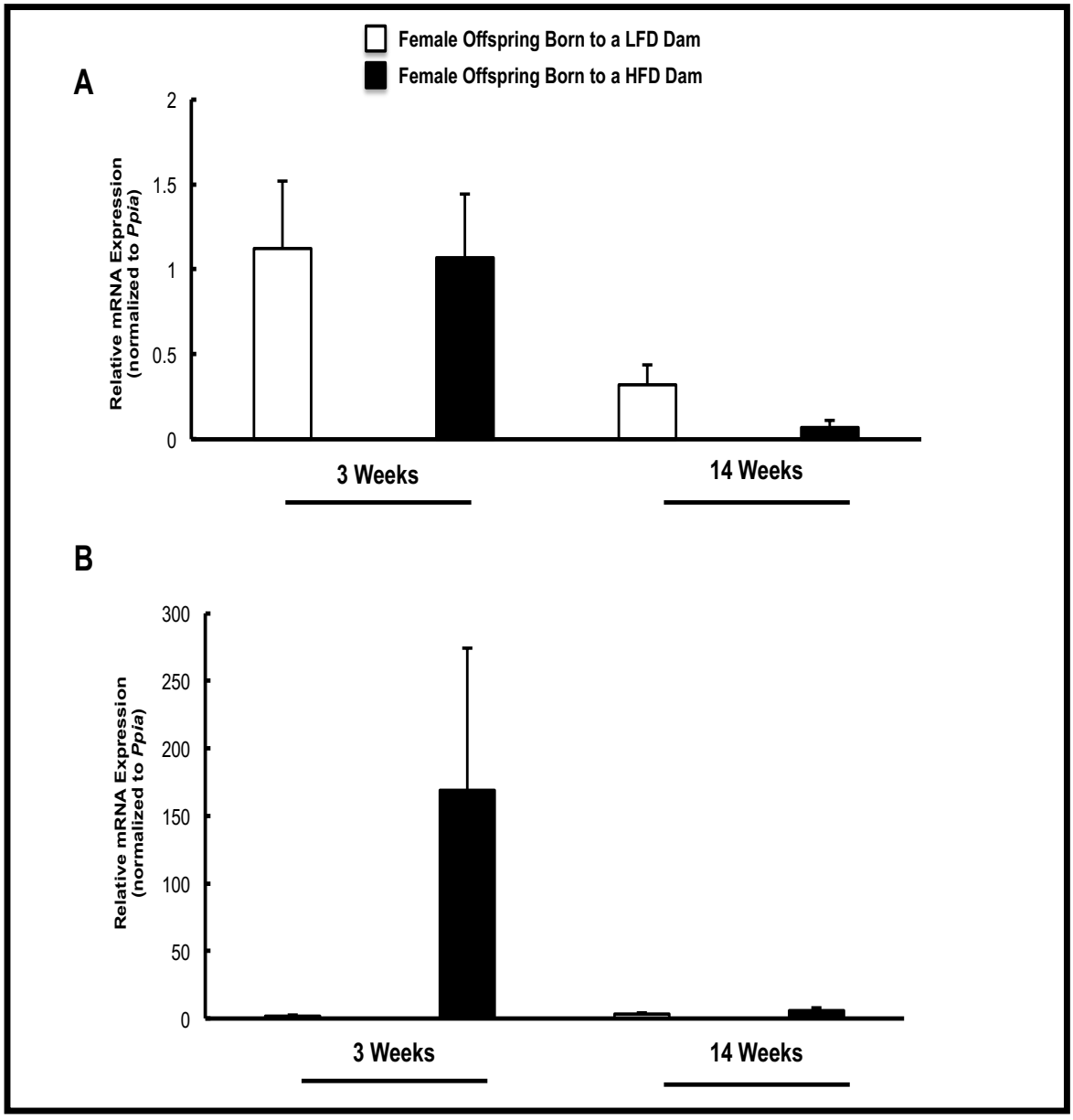

Figure A- 7 mRNA expressions of *Pdk4* in Gastrocnemius tissue and liver tissue of LFD offspring that were born to an obese dam. (A) Gastrocnemius tissue (3 weeks n=3, 4 weeks n=5). (B) Liver tissue (3 weeks n=3, weeks n=5). *P < 0.05.

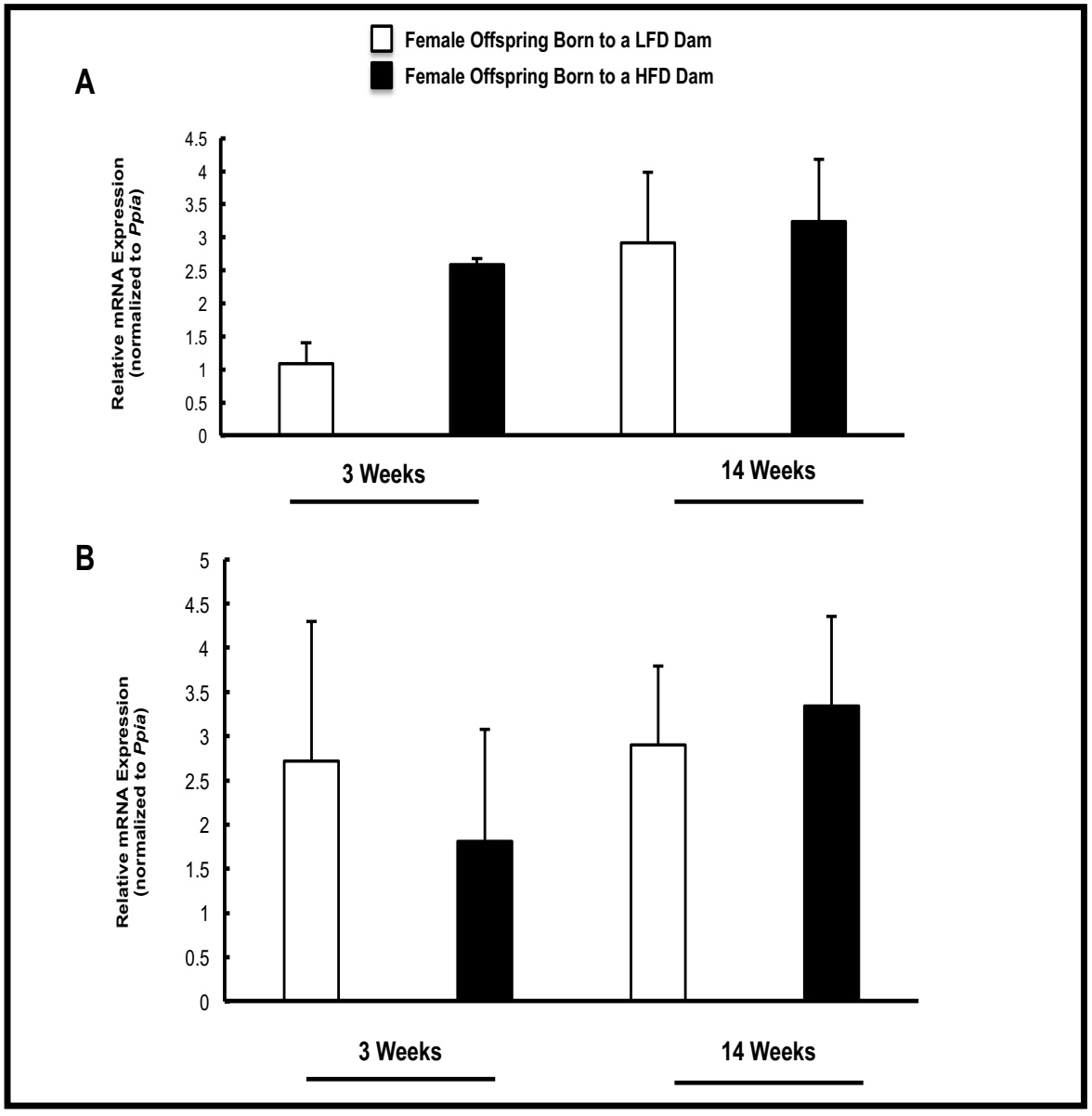

Figure A- 8 mRNA expressions of *Ppara* in Gastrocnemius tissue and liver tissue of LFD offspring that were born to an obese dam. (A) Gastrocnemius tissue (3 weeks n=3, 4 weeks n=5). (B) Liver tissue (3 weeks n=3, weeks n=5). *P < 0.05.

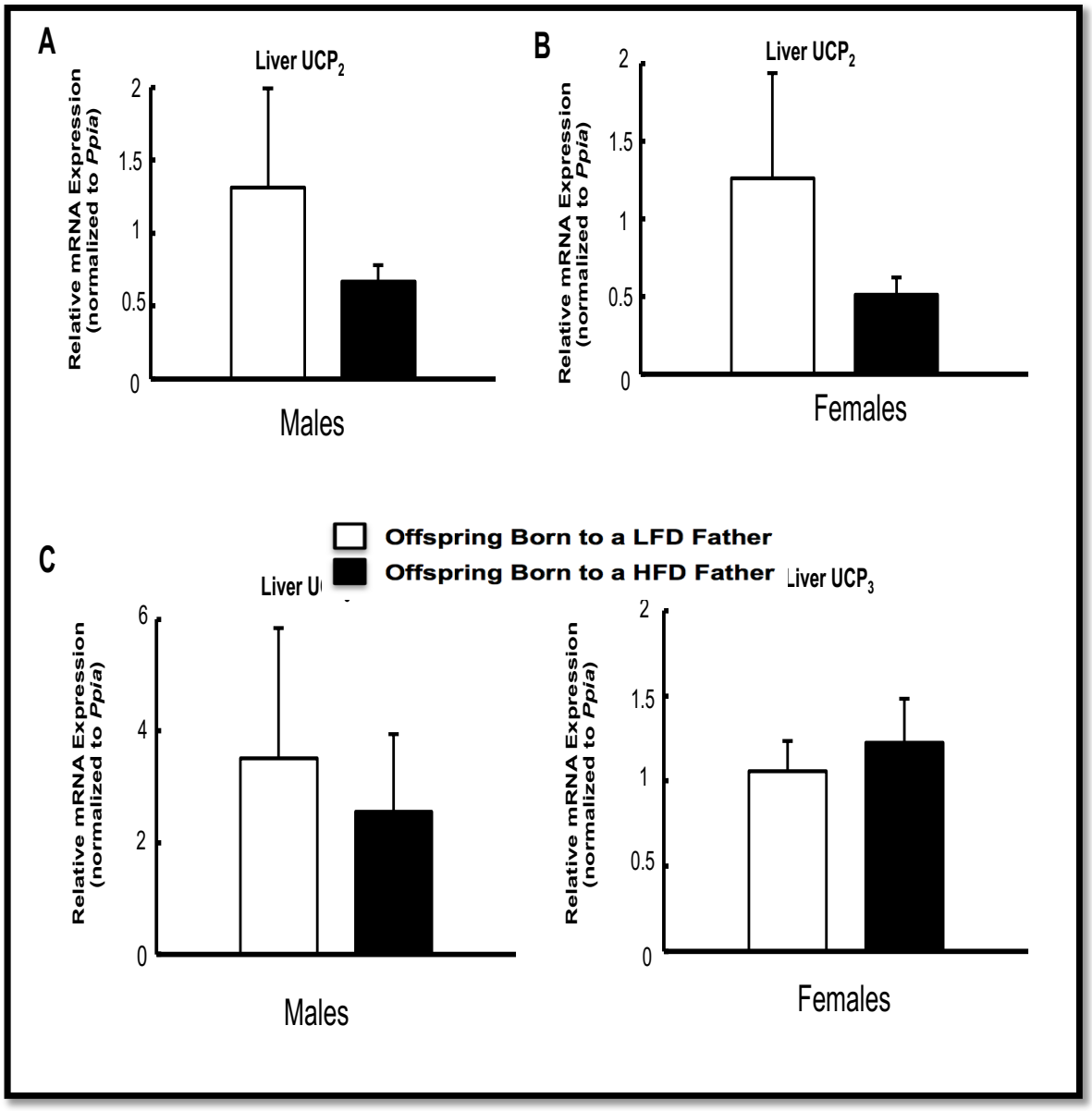

Figure A- 9 mRNA expressions of *Ucp*$_2$ and *Ucp*$_3$ in liver tissue of LFD offspring group. (A and B) showing results of the *Ucp*$_2$ (Males n=3,4, Females n=4,3). (C and D) *Ucp*$_3$ results (n=4). *P < 0.05.