MODELS OF GESTATIONAL DIABETES AND OFFSPRING OUTCOMES IN MICE

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled:

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DEDICATION

I dedicate my dissertation to those that have inspired, supported and advanced my academic pursuits. Firstly, to God from whom are all things, as “it is the glory of God to conceal a matter, but to search out a matter is the honor of kings.” To my husband C.J, for your strong support and encouragement. I am grateful to have had you with me on this journey. To our daughter, Noelle who has brought us an incredible amount of joy. To my parents, Drs. Alfred and Pamela Esangbedo, and siblings Ame and Enato; thank you for your love, provision, and for pushing me to excel. To my parents-in-law, Clay and Tracy Talton, thank you for providing a place of rest and for always cheering me on. To Dr. Peter Goldman, my first Biology professor, in whose home I had my first American Thanksgiving. To Convergence Church for being a family away from natural family. I am unable to write down the names of all the teachers, staff, friends, and countless other individuals that have left incredible impacts on my life, but I am grateful to all of you.

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MODELS OF GESTATIONAL DIABETES AND OFFSPRING OUTCOMES IN MICE

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ABSTRACT

Gestational diabetes mellitus (GDM) is the most common pregnancy disorder. GDM pregnancies result in offspring that are more likely to develop metabolic syndrome in adolescence than the background population. As offspring experience these adverse effects during their reproductive years, GDM has the potential to propagate disease for many generations.

Hyperleptinemia, a key characteristic of both GDM and maternal obesity has not been studied in isolation to determine its role in programming offspring outcomes. Hyperglycemia in the absence of obesity has also not been widely modeled without surgical or chemical means. My research goal was to study the offspring outcomes of these two facets of GDM in C57B6 mice.

We observed that maternal hyperleptinemia improved offspring insulin sensitivity, and protected the offspring from developing glucose intolerance. These outcomes were partly mediated by reduced fatty acid accumulation in the liver. Our findings suggest that maternal hyperleptinemia is protective of offspring glucose control.

Maternal hyperglycemia in lean dams increased offspring adiposity while glucose tolerance was unchanged. This effect was mediated by a preference for glucose over

lipids for substrate utilization, and multiple gene expression changes in the male adipose tissue and liver. Our results indicate that lean maternal hyperglycemia results in metabolically healthy obesity in offspring.

This work demonstrates that GDM in lean women may not negatively affect glucose tolerance, and that maternal hyperleptinemia may mediate this, through improving insulin sensitivity. It supports other data that suggest that the liver and adipose tissue are key regulators of whole body metabolism.

CHAPTER I

REVIEW OF THE LITERATURE

Gestational Diabetes – Definition, Prevalence and Risk Factors

Gestational diabetes mellitus (GDM), described as glucose intolerance during pregnancy [1], is the most common complication of gestation and affects one in seven pregnancies worldwide according to the International Diabetes Federation [2].

In 2010 the prevalence of GDM was up to 14% in the United States [3], with a relative increase of 122% between the late 1980s and the mid-2000s [4]. Recent studies place the global prevalence as high as 25% depending on the diagnostic criteria used [5], making GDM a global epidemic. The American Congress of Obstetricians and Gynecologists (ACOG) guidelines commonly used in the USA and International Association of the Diabetes and Pregnancy Study Groups (IADPSG) criteria preferred outside the United States provide the most common criteria for diagnosing GDM (Table 1).

Typically diagnosed in women during the middle of pregnancy, GDM has a number of risk factors. A meta-analysis of twenty GDM studies spanning 2000 to 2005 revealed that the odds ratio for developing GDM was 3.56 for obese women compared to lean women [6]. Obesity is accompanied by insulin resistance, and in obese women, this has been suggested to bethe basis for a higher risk of developing GDM [7]. In a study of 455 women, Hedderson et al showed that women from Hispanic and Asian backgrounds are more likely to develop GDM than Caucasians and African-Americans [8]. Aside from maternal BMI and ethnic background, a previous history of GDM [9] and parental

diabetes [10] are also risk factors. While the cause of GDM remains unknown, it involves prior insulin resistance [11], and impairments in the maternal adaptation to glucose control during pregnancy.

Normal versus GDM Pregnancy

Normal pregnancy is characterized by modulations in insulin sensitivity to mediate glucose control. During early pregnancy, insulin sensitivity is similar to that of the non-pregnant state. [12] This is partly to allow insulin-mediated lipogenesis to occur, building maternal fat stores for the latter part of pregnancy and lactation [13]. As gestation advances, insulin sensitivity declines and by the third trimester, insulin sensitivity is 50% lower in pregnant compared to non-pregnant women [14, 15]. This is partially facilitated through an increase in maternal estrogen, progesterone, and human placental lactogen, which inhibit insulin signaling in peripheral tissues [16]. These pregnancy hormones cause insulin resistance by decreasing levels and subsequent phosphorylation of the insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) in skeletal muscle [17]. PI-3 kinase activity downstream of IR is also inhibited by placental hormones further inhibiting insulin signaling [18]. Accompanying this increased insulin resistance is reduced blood glucose, which drops as pregnancy progresses. The cause for the decrease is the redirection of maternal glucose to the fetus, to accommodate its growing energy requirements [19].

In the fasted state, this maternal insulin resistance results in the preferential trafficking of glucose to the maternal-fetal interface, where glucose is diffused into fetal blood. Insulin resistance promotes this by reducing the uptake of glucose by maternal tissues and by

uninhibited hepatic glucose production [12]. Maternal feeding results in a rise in blood glucose which, under pregnancy-induced insulin resistance, would result in hyperglycemia. To compensate for the reduced insulin action in maternal tissues, and prevent post-prandial hyperglycemia during pregnancy, the pancreas expands to increase insulin secretion [20, 21]. This maintains maternal blood glucose levels and results in hyperinsulinemia as part of the normal milieu of pregnancy [19]. Thus, despite insulin resistance similar to what is observed in type 2 diabetics, pregnant women are not glucose intolerant.

In women with gestational diabetes, there is higher-than-normal post-prandial blood glucose in maternal circulation due to increased insulin resistance and inadequate insulin response to glucose [22-25]. Additionally, hepatic glucose production is higher in GDM pregnancies, due to increased insulin resistance [12]. As GDM pregnancies progress, insulin sensitivity decreases by the same proportion as it does in normal pregnancy. The apparent further reductions in insulin sensitivity are due to lower insulin sensitivity in the pre-gravid state, which has been observed in both lean and obese GDM women [26].

There remains much to be elucidated about the pathophysiology of GDM; however studies have revealed some of the mechanisms underlying the development of gestational hyperglycemia. Insulin resistance in GDM pregnancy involves further reductions of IR protein levels and reduced phosphorylation of IR as well [17]. The insulin response is further weakened by an inability of the pancreas to expand in response to the stress of pregnancy [27, 28]. Thus despite being high, insulin levels are not sufficient to overcome the degree of insulin resistance encountered by GDM women, and post-prandial hyperglycemia results.

Gestational diabetes is a health concern because of the complications that follow in affected pregnancies. Premature labor, preeclampsia and fetal macrosomia often resulting in shoulder dystocia are some of the common issues associated with GDM pregnancies [29-32]. The effects of GDM go beyond the pregnancy, affecting the future health of the mother. Women with GDM are seven times more likely to develop type 2 diabetes than women with normal glycemia during gestation [33], and more likely to experience recurring GDM in subsequent pregnancies [4].

Offspring Outcomes of GDM

The consequences of GDM are compounded by numerous adverse outcomes in the offspring. Left untreated, GDM fetuses have a four-fold higher risk of stillbirth. Less severe perinatal complications of GDM at the time of birth include shoulder dystocia, fetuses who are large for gestational age and fetal hypoglycemia [34, 35].

Of additional concern are the long-term negative effects in offspring, particularly when occurring in offspring of reproductive age. Children born to mothers with GDM are predisposed to diabetes and obesity in adulthood. The Diabetes in Pregnancy study, founded in Northwestern University, contains data on a large cohort of women enrolled between 1977 and 1983 and is used to study the offspring of women with GDM. Using these data, Silverman et al showed that maternal diabetes conferred an increase in glucose intolerance and hyperinsulinemia in adolescent offspring [36]. The Arizonian Pima Indian population has a high prevalence of diabetes, and glucose tolerance data has also been collected on that group to study diabetic pregnancies. Exposure to maternal diabetes is the major risk factor for the high prevalence of diabetes in the children of Pima Indians

[37]. In a Pima Indian study of siblings born to mothers before or after a diagnosis of diabetes, the siblings born after the diagnosis had an increased risk for developing diabetes when compared to siblings born prior, with an odds ratio of 3.7 [38]. Pettitt et al showed that the prevalence of non-insulin-dependent diabetes in Pima Indian offspring of GDM mothers is 45% compared to 1.4% in offspring not exposed to diabetes *in utero* [39]. Studies have also been conducted in populations at a low risk for developing GDM with similar results [40, 41], including a Danish study which determined that the prevalence of impaired glucose tolerance was 21% for 18-27 year old offspring born to GDM mothers compared to 4% for offspring of women from the background population [42].

A study of the Pima Indian population showed that offspring born to diabetic mothers had a higher incidence of obesity than those born to non-diabetic mothers [43]. In the same population, siblings had different birth weights depending on whether they were born before or after their mother was diagnosed with diabetes, with children born after the diagnosis weighing more [38]. A European study including over 280,000 men determined that exposure to maternal diabetes increased offspring BMI independently of maternal BMI [44]. Other human studies show the same higher risk for obesity in offspring of GDM mothers [45-48] including a multi-ethnic study showed that as maternal glycemia values from a glucose challenge test increased, so did offspring obesity in children aged 5-7 years [49]. Further evidence of the association between maternal GDM and offspring obesity comes from intervention studies, including a follow-up study in offspring born to GDM women who were treated with insulin [50]. Compared to offspring of untreated

mothers, the offspring that were exposed to insulin-treated GDM *in utero* had decreased adiposity.

Aside from obesity and diabetes, offspring born to mothers with gestational diabetes are also at an increased risk for developing GDM in their own pregnancies, with one study showing that women exposed to *in utero* hyperglycemia are more likely to develop GDM than the background population [51].

The rising rates of childhood obesity, diabetes, and metabolic syndrome are a cause for concern both for the present and future. While therapies exist to control diabetes and obesity, including drugs and lifestyle interventions, they are still leading causes of death, and confer a heavy burden on the economy. Since GDM pregnancies are implicated in promoting these offspring diseases [52], there is a compelling basis for research into how GDM affects offspring. Studies that can elucidate these mechanisms and present therapeutic strategies are essential in curbing the negative effects of the disease on individual lives as well as society at large.

Mechanisms Underlying GDM Offspring Outcomes

Genetic

Diabetes and obesity, often termed "diabesity" are the two main offspring outcomes associated with GDM. Combinations of genetic and environmental factors mediate the development of diabetes and obesity in offspring of GDM mothers. There are several gene variants associated with GDM and type 2 diabetes [53, 54], and studies have shown that mutations in maturity onset diabetes in the young (MODY) genes are also associated

with increased risk of GDM [55]. The clustering of GDM among families, and among certain ethnic groups also point toward genetic inheritance; in fact, one study shows that nearly 80% of type 2 diabetics have either one or both parents with diabetes [56]. Thus women with diabetes during pregnancy might carry GDM-associated polymorphisms and transmit them genetically to their offspring.

The aforementioned study demonstrated that the risk of developing diabetes was much higher if the mother had diabetes than the father [56], suggesting that maternal diabetes is a stronger determinant of the offspring phenotype. Numerous other studies have confirmed this stronger association of maternal diabetes with both diabetes and obesity in offspring [57-60], including a compelling study which showed that siblings born to mothers prior to a diagnosis of diabetes weighed less than their siblings born afterwards [38]. These data suggest that independent of genetic inheritance, the intrauterine environment also confers offspring outcomes.

Direct effects of Intrauterine Environment

The intrauterine environment of a GDM pregnancy is characterized by increased fetal availability of glucose, leading to fetuses that are large for gestational age [61, 62]. Maternal blood glucose is diffused across a concentration gradient to the fetus, as maternal blood in the intervillous space bathes the placental villi containing fetal blood vessels [63]. Higher post-prandial blood glucose levels in GDM mothers, due to insufficient insulin response, promote glucose uptake to the fetus, resulting in a macrosomic, or large-for-gestational age (LGA) fetus. Pedersen's hypothesis states that maternal hyperglycemia promotes fetal hyperglycemia which then promotes a

hyperactive fetal pancreas than secretes high levels of insulin in response to glucose which has angiogenic effects on the fetus [64]. Ample studies show that birth weight and cord insulin/C-peptide levels correlate, supporting this hypothesis [65-68].

The placenta responds to changes in maternal hormones, and alters fetal growth accordingly [69]. Insulin receptors are expressed on the human placenta, primarily on the maternal side in early pregnancy, with a progressive shift toward the fetal side with advancing gestation [70, 71]. Maternal and fetal insulin, elevated in GDM pregnancies, may act on placental receptors to affect fetal growth. Additionally, Jansson et al, along with other groups, have shown that elevated levels of insulin can increase the activity of amino acid transport systems in the placenta, potentially promoting fetal growth and metabolism [72, 73]. The larger placentas in GDM pregnancies may also increase nutrient transfer and fetal growth [74].

As such, the *in utero* environment of GDM pregnancies directly affects fetal outcomes, promoting fetal macrosomia, which confers an increased risk of developing obesity in adolescence [75, 76]. Studies have also found that high BMI increases the risk for type 2 diabetes [77, 78], thus there is evidence that offspring predisposition to diabetes and obesity is directly due to *in utero* insults. Examining the effect of offspring macrosomia on future risk for metabolic diseases such as obesity, high blood pressure, and diabetes in offspring, Boney et al showed that the prevalence of metabolic syndrome was higher in 6-11 year old children who were large for gestational age (LGA) and born to a mother with GDM than LGA offspring from a mother with normal glucose tolerance [79]. This implies that aside from macrosomia, exposure to maternal glucose intolerance *in utero* promotes long-term impaired health of the offspring.

Developmental Programming and Epigenetics

The study of how the maternal environment during early development can program offspring susceptibility for diseases later in life is referred to as the developmental origins of health and disease [80-82], and originates with the historical Dutch Hunger Winter studies. Women who were pregnant during Holland's 1944 famine were severely undernourished. They gave birth to offspring who went on to develop metabolic diseases such as diabetes, obesity, and hypertension in adulthood [83-85]. Santos et al. recently studied 7-year-old children of diabetic mothers and observed a positive association between maternal diabetes and offspring adiposity independent of maternal BMI and offspring birthweight [86]. This provides evidence that the effects of maternal diabetes on offspring adiposity are largely due to programming events in the uterus, and other studies indicate that the maternal milieu comprising GDM can alter fetal tissues conferring disease risk beyond genetic inheritance and beyond birth weight [87].

Epigenetics, which literally means "above genetics," refers to mitotic or meiotic inheritance without changes in DNA or gene sequence. Epigenetic modifications, including DNA methylation, histone acetylation and imprinting, affect which genes are silenced and expressed and can change the pattern of inheritance [88]. The external environment influences mitotic inheritance through changes in the epigenome [89, 90], and specifically, high insulin and glucose have been shown to affect the epigenetic regulation of gene expression in a hepatic cell line [91]. In pancreatic islets from patients with type 2 diabetes, differential DNA methylation was observed compared to non-diabetic control subjects [92]. In another study, a pancreatic beta cell line exposed to high glucose had increased DNA methylation of the insulin promoter compared to control

[93]. During early development, the epigenome is most susceptible to change; thus, changes in the maternal environment during fetal development have an increased potential to alter the methylation status [94]. In comparing genome-wide methylation patterns between fetuses from mothers with and without GDM, Haertle et al found that GDM offspring have a different epigenetic status, with methylation differences spanning multiple genes in fetal cord blood [95]. Other studies have shown that GDM alters placental and cord blood gene methylation [96, 97] in pathways related to metabolic disease [98]. Thus epigenetic changes *in utero* due to GDM might program a greater susceptibility to diseases like obesity and diabetes by changing the expression of genes involved in the development of these diseases.

Rodent models for GDM pregnancy

These studies in humans confirm that GDM programs offspring risk for metabolic disease, but they are limited in the scope of the work that can be done. Ethical boundaries, the lack of non-invasive imaging techniques, and sample size limitations do not allow detailed study into how GDM affects various tissues in offspring, and how these *in utero* effects result in long-term development of disease. Additionally, the difficulty in following up with children of diabetic mothers into adulthood, and improper completion of questionnaires and surveys typically used to collect data also present challenges. The aforementioned reasons detail the need for animal models to study the phenotypes observed in GDM offspring and their underlying mechanisms. Various animal models have been used to model GDM pregnancy, including sheep [99, 100], monkeys [101], and pigs [102, 103]. Conducting studies in these larger animals is cost-

inhibiting, which is part of the reason why rodents are the preferred models of GDM pregnancy [104].

Aside from their small size and the economics of their maintenance, there are many factors that make rodents an ideal model of GDM pregnancy. In studying the link between maternal insults and offspring outcomes, the placenta, as the site of nutrient and oxygen transfer, is of importance. While it is clear that no animal model can recapitulate human placentation, [105] rodents have an advantage over other animals in that they have structurally similar placentas to humans [106, 107]. Both possess a hemochorial placenta, in which the maternal blood is in direct contact with the fetal trophoblast cells.

Additionally, studies have shown molecular similarity with conserved genes between human and mouse sites of placental transfer [108], namely the villous tree and labyrinth in humans and mice respectively. The physiology of rodent pregnancy also confers multiple benefits. Pregnancy in mice and rats lasts approximately 3 weeks, which allows studies to be performed more quickly than in animals with longer gestation periods. Rodent pregnancy is also multiparous, which reduces the number of animals required to obtain an adequate offspring sample size.

Finally, mouse models of GDM are able to recapitulate many of the features observed in GDM such as reduced insulin response [109-111], spontaneous glucose intolerance [109, 112], impairments in pancreatic adaptation to pregnancy [109, 113, 114], as well as many of the offspring outcomes clinically observed such as macrosomia/fetal growth restriction [115-118], and a predisposition to type 2 diabetes and obesity in adulthood [118-121].

Current Rodent Models of GDM

Surgical

The beta cells of the pancreas are the site of insulin production. During early pregnancy, hyperinsulinemia develops to promote the passive diffusion of glucose to the fetus, and this diversion of glucose is maintained in late pregnancy by elevated levels of placental hormones, which promote insulin resistance [20, 122]. Insulin production must increase to protect against maternal hyperglycemia in late pregnancy, and this is facilitated by beta cell expansion through hypertrophy [20, 114, 123]. In human pregnancy, a postmortem study has suggested that deficient beta cell expansion drives the pathophysiology of GDM [28]. Consequently pancreas excision has been used a model of GDM.

Surgical removal of part of the pancreas and the beta cells therein recapitulates inadequate beta cell proliferation and produces a mild diabetic phenotype during pregnancy; however, not many studies have been performed using this technique in rodents [124, 125], and only one group has examined fetal outcomes to my knowledge. The fetal mortality rate among offspring of pancreatectomized rats was higher than in controls, and the offspring had higher birth weights and aberrant glucose tolerance compared to controls [126]. The invasiveness of surgery, high mortality and high abortion rates have contributed to the disuse of pancreatectomized rodents in GDM studies [104]. In addition, removal of whole portions of the pancreas attenuate both endocrine and exocrine pancreas functions, which has implications beyond maternal hyperglycemia for food processing and digestion [127].

Chemically induced

An alternative to pancreatectomy is the selective ablation of the insulin producing beta cells which proliferate during gestation [114]. This has the benefit of leaving the remainder of the pancreas intact for exocrine functions, and can be accomplished through the administration of drugs that selectively destroy beta cells. Alloxan and streptozotocin (STZ) are two drugs that kill beta cells upon administration, and while alloxan was developed first, the latter has less dangerous side effects, and is consequently the preferred drug for use in animal models [127]. STZ is similar in structure to glucose, such that it can enter the GLUT2 channel receptors located on pancreatic beta cells. Upon entry, STZ educes an inflammatory response resulting in beta cell death [128].

GDM models using STZ vary in the dosage and timing of STZ administration. High doses result in more severe maternal hyperglycemia than that observed in typical GDM, while lower doses give moderate hyperglycemia [129]. Deeds et al have reviewed the STZ doses typically used in mice [128].

The various streptozotocin treatments result in different fetal outcomes. Moderate maternal hyperglycemia from single or low doses of STZ can result in macrosomic offspring or offspring with no differences in birth weight [129, 130]. Correspondingly, when dams are given one or more high doses of streptozotocin resulting in severe maternal hyperglycemia, their offspring present with low birth weights (37,[129]. Both fetal outcomes are observed in GDM pregnancies at a higher rate than in the background population [131, 132]. In adulthood, offspring of STZ diabetic dams display aberrant glucose tolerance regardless of the dosage [121, 129, 130].

STZ-treated dams make good models of hyperglycemia that is due to insulin-deficiency in pregnancy [133], and are particularly useful in studying populations with pre-existing diabetes prior to pregnancy since the model more closely resembles type 1 diabetes than GDM [133]. Additionally, STZ models of GDM recapitulate the incidence of neural tube defects observed in children of GDM mothers [134]. As a result this model has been widely used to study the development of these congenital malformations in offspring from diabetic pregnancies [134-136].

STZ, particularly in one or more high doses has proven toxic to other tissues, and can cause nephropathy, among other diseases [137-139]. To avoid these side effects of the STZ drug on pregnancy and embryogenesis, the drug must be given prior to pregnancy, resulting in diabetes that carries over to pregnancy, rather than diabetes that develops during pregnancy [140]. Additionally, treatment during pregnancy risks direct effects of STZ on the fetus; one study has shown that STZ crosses the placenta in rhesus monkeys [141], although in this study, no further effects were observed. Prager and Padmanabhan have separately shown that STZ induces morphological changes in the rat placenta [142, 143] which has implications for fetal growth and may be responsible for the growth restriction observed in fetuses of STZ-induced diabetic dams.

An additional caveat of STZ and surgical models of diabetes is that typically, GDM women develop hyperglycemia spontaneously, rather than as a direct consequence of an intervention. Additionally, women with GDM do not display the same severity of hyperglycemia observed in most STZ models [129]. As a result, the offspring phenotype produced may be due to any number of the maternal insults caused by STZ administration.

Diet-induced

Feeding rodents a long-term high fat diet results in hyperglycemia and abnormal glucose tolerance, along with obesity. When fed a 45% kcal/fat diet 6 weeks prior to mating and throughout gestation and lactation, Sprague-Dawley rats more than doubled in weight and developed glucose intolerance prior to pregnancy, but did not exhibit fasting hyperinsulinemia and hyperglycemia until mid-gestation [115]. Offspring were macrosomic at birth and by 7 weeks of age, they exhibited increased body weight. Other phenotypes in the offspring of the diet-induced GDM dams included increased adiposity, hyperinsulinemia, markedly reduced expression of IR-beta and liver steatosis compared to offspring of lean controls [115]. A limitation of this model is that glucose intolerance develops prior to pregnancy, thus is more suitable as a model of type 2 diabetes during pregnancy than of GDM.

Models of long-term (4-12 weeks) high-fat (45% - 60% kcal/fat)-induced GDM exist wherein glucose intolerance begins during gestation [111, 144-146], and offspring outcomes are similar with a propensity towards obesity, non-alcoholic fatty liver disease and diabetes beginning at 13-15 weeks of age [145, 146]. The high-fat fed rodent is commonly used to model GDM as it is a largely side-effect-free method of inducing GDM, as well as being effective and relatively simple. The spontaneous development of glucose intolerance only during pregnancy is an added benefit, as it is similar to the pathophysiology of clinical GDM in obese women.

However studies have shown that high maternal BMI causes adverse pregnancy outcomes independently of maternal hyperglycemia [147, 148], including a lot of the

same offspring outcomes that are observed in diet-induced GDM models, such as weight gain, inflammation, and elevated lipid accumulation in the liver [149]. Maternal high-fat feeding has been found to alter the developmental programming of offspring, with effects on the epigenome predisposing the offspring for metabolic syndrome [150-153]. Additionally, obesity has implications for germ cell development. Studies by the Moley lab have shown that diet-induced obesity in mice results in oocyte abnormalities [154-156]. In one study, Jungheim et al showed that feeding C57BL6 mice a 35.8% g/fat diet for 16 weeks resulted in increased follicular death, and decreased size and growth of oocytes [157]. Thus the effects of maternal high fat feeding on the oocytes might also affect offspring development.

In spite of these concerns, the high-fat-induced GDM model remains ideal for recapitulating the effects of obese GDM on offspring outcomes, which is present in about 42% percent of the GDM human population [158]. More women of child-bearing age are obese now than ever in history [159], and obesity is a major risk factor for the development of GDM [160]. It also serves to confirm and mimic studies characterizing the effects of maternal hyperglycemia on offspring in humans that are also often performed in obese individuals [65, 161].

It is important to separate maternal hyperglycemia from maternal obesity in studying their respective outcomes on offspring. Indeed, many studies on the effects of maternal obesity on offspring health make no mention of maternal glycemia, whereas the aforementioned studies show that the two comorbidities often present together. Epidemiological research has shown that around 30% of women with GDM are lean [162], outlining the need for GDM models that are representative of this population.

Catalano et al have shown that there are differences in the pathophysiology of lean and obese GDM. While both lean and obese women who will go on to develop GDM begin their pregnancies with slight insulin resistance, the two groups diverge as pregnancy progresses. In obese women, first and second phase insulin responses are increased and hyperinsulinemia develops whereas in lean women, the first phase insulin response is reduced and overall insulin secretion is lower [26]. One recent study showed that the effects of obese GDM on pregnancy outcomes are worse than those of lean GDM in women carrying twins [163], further illustrating the need for a more accurate model for lean GDM. Furthermore, isolating specific features of GDM and studying their respective offspring outcomes would prove useful in the development of therapeutic strategies.

In response to this need, we previously developed a high-fat, high-sucrose fed model of GDM in which dams exhibit glucose intolerance and reduced insulin response to glucose challenge only during pregnancy, without accompanying obesity [109]. When female C57B6 mice were fed a high-fat, high-sucrose (HFHS) diet (45% kcal/fat, 17% kcal/sucrose) one week prior to mating and throughout gestation for a total of 4 weeks, they exhibited normal glucose tolerance and no weight difference at day 0 of pregnancy. By mid-pregnancy, glucose tolerance was impaired in the HFHS fed dams, and they maintained similar weights to controls. A blunted insulin response to glucose was observed, which was attributed to insufficient beta cell expansion. This model aptly recapitulates spontaneous glucose intolerance that begins during pregnancy and is resolved postpartum. In a similar model, dams fed a diet high in sugar and fat (HFHS diet: fat, 30g%; protein, 17g%; CHO, 53g% (simple sugar, 36%); 18.3 MJ/kg) from D1 of gestation to term exhibited hyperinsulinemia and hyperglycemia on D16, although

they weighed less than control dams [164]. The isolation of GDM from the confounding variable of maternal obesity provides a promising model for studying the etiology of lean GDM, and its offspring outcomes.

Genetic

Prolactin

Knockouts in a number of genes have resulted in the development of spontaneous hyperglycemia during pregnancy, including knockouts of the prolactin and leptin receptors.

During mouse and human pregnancy, prolactin levels are elevated, as estrogen and progesterone promote pituitary secretion of prolactin that is required for mammary gland development [165]. Prolactin, along with placental lactogen, acts through the prolactin receptor to stimulate pancreatic beta cell proliferation to counteract heightened insulin resistance [20, 21]. As such, mice lacking the prolactin receptor are deficient in the beta cell remodeling required for glucose control during pregnancy. Banerjee et al demonstrated that conditional inactivation of the prolactin receptor on beta cell islets in mice results in decreased beta cell mass, reduced insulin secretion and glucose intolerance in mice during pregnancy [166].

Knockout mice are more widely used as GDM models. Homozygous $Prlr^{-/-}$ mice are glucose intolerant prior to [167] and during pregnancy [168] however, they also exhibit severely decreased fertility and, when pregnancy is successful, do not lactate [169], whereas heterozygotes ($Prlr^{+/-}$) are fertile and display hyperglycemia, decreased islet

mass, and impaired beta cell proliferation during but not before pregnancy [113]. Female offspring of *Prlr*$^{+/-}$ mice have a higher likelihood of developing GDM in their own pregnancies, due to decreased beta cell proliferation during pregnancy [170, 171] consistent with human data showing that there is a higher risk of GDM in pregnancies of women born to mothers with GDM [51, 172].

Leptin

Pregnancy is a state of hyperleptinemia and leptin resistance, and the action of leptin has been widely studied during pregnancy. Leptin, a 16 kDa protein encoded by the obesity gene, was discovered in 1994 by Zhang et al [173] and is best known for regulating food intake, which led to it being aptly named for the Greek word "leptos" meaning thin. Leptin is a cytokine produced by adipocytes that reflects adipose tissue mass. Secretion of leptin by adipocytes signals satiety, decreasing food intake, and increasing energy expenditure [174-176]. In the years since its discovery, the known functions of leptin have quickly expanded from a homeostatic regulator, to, among many other things, a key reproductive hormone [177]. Leptin is required for pregnancy, as proven by studies which show that leptin null animals are infertile, and that leptin administration to null mice reverses this [178, 179]. In the non-pregnant state, leptin acts through its receptors to maintain energy balance and influence reproduction. The leptin receptor (LEPR) is a receptor tyrosine kinase and member of the class I cytokine family of receptors [180]. Leptin receptors exist as 6 different isoforms, as a result of alternative splicing of the same gene; LEPRa-f. LEPb is the primary signaling form of the receptor, and highly expressed in the hypothalamus in humans and mice. The remaining isoforms have limited ability to transduce leptin signals, with LEPRe lacking the transmembrane and

intracellular domains [181]. The leptin receptor intracellular domain contains docking sites for Janus kinase (JAK) and other kinases, which activate signaling cascades [181].

During pregnancy, adipose tissue mass expands to increase maternal nutrient stores [182] and consequently, leptin secretion is elevated [183]. However hyperleptinemia is present prior to increased adiposity [184] suggesting that it is promoted by other factors as well. In fact, there is evidence that the human placenta produces and secretes leptin, which contributes to the elevated leptin levels in maternal circulation during pregnancy [185, 186]. Leptin levels are elevated to ~ 2 fold in humans [187] and ~10 fold in rodents [188] In rodents, the placenta does not produce leptin, but the increased production of the soluble form of the leptin receptor, LEPRe, from the rat and mouse placentae is largely responsible for increased serum levels of leptin by increasing the half-life of circulating leptin [189, 190].

The purpose for elevated leptin levels is not fully known. The high levels of leptin in maternal circulation during pregnancy are unaccompanied by decreased food intake and a reduction in adipose mass, thus pregnancy is a state of leptin resistance. Ladyman et al have performed elegant studies detailing the attenuation of the JAK/STAT signaling pathways of leptin during pregnancy. Upon leptin administration, hypothalamic STAT3 phosphorylation was reduced in pregnant rats [191] and mice [192] compared to controls, and food intake was not suppressed. Leptin does not cross the placenta [193, 194] but both human and mouse placentas contain leptin receptors [195, 196]. Moreover, leptin has been shown to regulate placental nutrient transport [72], indicating that it plays a role in the growth and development of the fetus. In GDM, hyperleptinemia beyond that observed in normal pregnancy is observed [197, 198].

Homozygous leptin receptor mutant mice (Lepr-/-) have been used as a model for diabetes, however, the null mice are sterile, precluding their use in programming studies [199]. Mice that are heterozygous for the leptin receptor mutation are severely hyperleptinemic and display spontaneous glucose intolerance during pregnancy, leading to their use as a model of GDM [200]. Yamashita et al observed that offspring of Lepr+/- dams are macrosomic, and as adults the female offspring were hyperinsulinemic [112].

Unfortunately, other groups have been unable to reproduce the GDM phenotype. Plows et al. performed controlled experiments to identify possible contributors to the presence of GDM in some studies, and absence in others [201]. They did not observe GDM in any of their experimental models, and there were no clear associations with any potential contributors, leading to the conclusion that the Lepr+/- mouse is not an appropriate model of GDM. Nonetheless, the model consistently displays hyperleptinemia above normal pregnancy levels [201], as observed in GDM [202], and may be useful for determining what role this characteristic of GDM pregnancy plays in conferring offspring outcomes.

Research Goals

Deficient in the literature are isolated studies, where specific facets of GDM are assessed to determine their role in mediating the outcome measures observed in GDM, specifically diabetes and obesity. This is essential in understanding the pathophysiology of GDM, and its method of altering offspring phenotypes. It is also necessary in identifying factors that can be modulated by lifestyle and drug interventions to curb the negative effects of the disease.

The aim of the first study was to determine the role that hyperleptinemia, which is exacerbated in GDM pregnancies, played in programming offspring metabolic outcomes. Leptin has been implicated in the developmental programming of offspring health [203-206], and is present in many rodent models of GDM and maternal obesity. However, in these animal models it is accompanied by maternal glucose intolerance, and obesity, and its independent effects on offspring metabolism had not been shown. Our study compared offspring from two maternal hyperleptinemic models to their respective controls. We developed the first model of hyperleptinemia using the Lepr+/- mouse. Lepr-/-) mice have a mutation in the gene that encodes the leptin receptor, resulting in a truncated long form of the leptin receptor. Homozygous mice are hyperleptinemic, obese, diabetic, and infertile. As previously mentioned, GDM is spontaneously developed during the pregnancies of some heterozygous mice, but it did not develop in ours. Our heterozygous Lepr+/- mice had a normal glucose tolerance phenotype, but exhibited hyperleptinemia. For the second hyperleptinemic model, wildtype females were implanted with mini-osmotic pumps which release leptin (350ng/hr), or saline prior to mating, and throughout gestation. The LEP dams have mild hyperleptinemia compared to the DB dams, allowing for dose-dependent studies. Weights and activity had previously been analyzed in the offspring of these mice, with offspring of hyperleptinemic dams having lower weights and increased activity [204]. Assessments of offspring metabolism including glucose and lipid control suggested that maternal hyperleptinemia alone does not cause metabolic disease in offspring, and may be protective of insulin and leptin sensitivity.

The aim of the second study was to study the isolated effects of maternal glucose intolerance during gestation on offspring outcomes. As models of GDM are often

confounded by maternal obesity, or the side effects of drug treatment and genetic manipulations, we wanted to remove those factors. Additionally, we wanted a model that was representative of lean GDM women. Using the lean GDM model developed by Pennington et al [109] and previously described, we assessed offspring metabolism. Our findings indicate that maternal glucose intolerance in lean dams has moderate effects on offspring, programming increased adiposity without predisposing the offspring to develop diabetes.

	IADSPG	**ACOG**
24-28 week test	Fasted 2hr 75g OGTT	Non-fasting 1hr 50g GCT
		Fasted 3hr 100g OGTT
Basis of GDM Diagnosis	1 abnormal value on OGTT	1 positive screen, 2 abnormal values on OGTT
Glucose Target Level for Diagnosis		Screen: >130 mg/dL
	Fasting glucose: 92 mg/dL	Fasting glucose: 95 mg/dL
	1hr glucose: 180 mg/dL	1hr glucose: 180 mg/dL
	2hr glucose: 153 mg/dL	2hr glucose: 155 mg/dL
		3hr glucose: 140 mg/dL

Table 1. IADSPG and ACOG criteria for diagnosing gestational diabetes mellitus [207].

CHAPTER II

MATERNAL HYPERLEPTINEMIA IMPROVES OFFSPRING INSULIN SENSITIVITY IN MICE

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Abbreviations: GDM (gestational diabetes mellitus), NAFLD (non-alcoholic fatty liver disease), GTT (glucose tolerance test), CD (chow diet), HFD (high fat, high sucrose diet)

Abstract

Maternal obesity and gestational diabetes are prevalent worldwide. Offspring of mothers with these conditions weigh more and are predisposed to metabolic syndrome. A hallmark of both conditions is maternal hyperleptinemia, but the role of elevated leptin levels during pregnancy on developmental programming is largely unknown. We previously found that offspring of hyperleptinemic mothers weighed less, and had increased activity. The goal of this study was to determine whether maternal leptin affects offspring insulin sensitivity by investigating offspring glucose metabolism and lipid accumulation. Offspring from two maternal hyperleptinemic models were compared. The first model of hyperleptinemia is the $Lepr^{db/+}$ mouse, which has a mutation in one copy of the gene that encodes the leptin receptor, resulting in a truncated long form of the receptor, and hyperleptinemia. Wildtype females served as the control for the $Lepr^{db/+}$ females. For the second hyperleptinemic model, wildtype females were implanted with mini-osmotic pumps which released leptin (350ng/hr), or saline (as the control) just prior to mating, and throughout gestation. In the offspring of these dams, we measured glucose tolerance, serum leptin, insulin, and triglyceride levels, liver triglycerides, pancreatic alpha and beta cell numbers, body composition, incidence of non-alcoholic fatty acid disease (NAFLD), and the expression of key metabolic genes in liver and adipose tissue. We found that the offspring of hyperleptinemic dams exhibited improved glucose tolerance, reduced insulin and leptin concentrations, reduced liver triglycerides, and a lower incidence of NAFLD. Overall, maternal hyperleptinemia was beneficial for offspring glucose and lipid metabolism.

Introduction

Pregnancies complicated by gestational diabetes mellitus (GDM) are on the rise globally. Recent data place the prevalence of GDM in the United States at 9.2%, or up to 18%, by some diagnostic criteria [3]. GDM has emerged as a risk factor not only for adverse maternal health outcomes [208, 209], but for a number of childhood conditions related to metabolic syndrome [210], wherein researchers have found that children born to women with GDM have an increased risk for obesity [211, 212] and type 2 diabetes [213, 214].

There is compelling evidence linking GDM to the development of offspring type 2 diabetes. Damm et al. showed that children born to women with GDM were over seven times more likely to develop type 2 diabetes than the background population [215], and Hamman et al. found that the association between GDM and offspring diabetes prevailed regardless of age, sex or ethnicity [216]. In one study, children born to the same mother prior to, or after a maternal diagnosis of diabetes were compared. The children exposed to diabetes *in utero* had a risk of developing diabetes exceeding threefold that of siblings born prior to the diagnosis [38].

Leptin, an adipose-derived satiety hormone that is elevated during pregnancy [217], is significantly higher in pregnant women with GDM [197, 202, 218], suggestive of leptin resistance. In the non-pregnant state, leptin promotes energy expenditure and decreases food intake [219-221]. Leptin has also been shown to increase insulin sensitivity [222], and in leptin-null mice, leptin treatment normalizes serum insulin and improves glucose tolerance, correcting diabetes [223]. During obesity, leptin levels are high (hyperleptinemia) but do not serve to reduce food intake or BMI, indicating leptin

resistance [224]. Additionally, elevating plasma leptin to a level similar to that observed in overweight and insulin resistant people decreased glucose-mediated insulin secretion [225]. This suggests that under high leptin conditions, leptin resistance leads to a lack of leptin action that promotes insulin resistance and obesity.

The *in utero* environment affects offspring metabolism, a phenomenon referred to as developmental programming [226-228]. While maternal leptin does not cross the placenta to directly act on the fetus [229, 230] it has been implicated in developmental programming. We have previously hypothesized that elevations in maternal leptin during maternal obesity and GDM contribute to the mal-programming of the fetus [204]. Alternatively, maternal leptin signaling may improve offspring metabolism, such that leptin resistance impairs it. To test this, we studied the offspring of hyperleptinemic dams using a pharmacological hyperleptinemia model (dams infused with leptin via osmotic pumps compared to saline treated controls), and a genetic hyperleptinemia model ($Lepr^{db/+}$ dams compared to wildtype controls). Rather than promoting offspring macrosomia and obesity, exposure to high maternal leptin resulted in lower offspring weights and increased offspring activity [204]. Others have also found that maternal hyperleptinemia resulting from acute leptin treatment during late gestation [205], or genetic disposition [231] reduced diet-induced obesity in offspring.

These studies indicate that leptin resistance, rather than high leptin itself, may be problematic in GDM and maternal obesity. That is, a lack of leptin action promotes the programming of offspring obesity, which normal leptin action protects against. In the present study, the aim was to determine whether maternal leptin also protects insulin sensitivity in offspring, by examining glucose tolerance and insulin concentrations in the

same offspring in which weights and behavior were previously assessed [204]. Additionally, factors that may influence insulin sensitivity, including offspring body fat percentage, liver triglyceride accumulation, and serum leptin levels were examined.

Hepatic steatosis, and non-alcoholic fatty liver disease (NAFLD) are implicated in the pathogenesis of type 2 diabetes. Furthermore, NAFLD can be developmentally programmed by an adverse maternal environment, such as that of high fat fed dams [232]. We previously found evidence that the combination of hyperleptinemia and caloric restriction in dams alters the risk of NAFLD [233]. Thus, in the present study, liver histopathology and triglyceride content were examined, as well as the expression of two genes, *Cd36* and *Srebp1c*, which transport fatty acids into the liver. These have been implicated in NAFLD, and the developmental programming of NAFLD [232, 234-240]. Additionally, we assessed levels of transcripts encoding *Hsd11b*, which metabolizes cortisol, and which is altered in the liver of sheep that have increased lipid accumulation following developmental exposure to maternal undernutrition [241].

Materials and Methods

Animals and Tissue Collection

Animal procedures were approved by the University of Missouri Institutional Animal Care and Use Committee and performed according to the NIH Guide for the Care and Use of Laboratory Animals. The $Lepr^{db}$ colony was established as previously described [204] by mating $Lepr^{db/+}$ males from Jackson Laboratories (Bar Harbor, Maine) with wildtype females from the University of Missouri. Data reported here were collected from mice involved in behavioral experiments that have been published previously [204].

We studied the effects of maternal hyperleptinemia using two different models; a genetic model (WT vs. DB/+) and a pharmacological model (SAL vs. LEP).

In the genotypic model, wildtype females (treatment group WT) served as the control group for $Lepr^{db/+}$ females (DB/+). DB/+ females have a point mutation in one copy of *Leprb*; the long form of the leptin receptor, excising the portion of the cytoplasmic region of the receptor that is responsible for leptin signaling [242]. The DB/+ females were mated with wildtype males, and reciprocal crosses (wildtype females mated with $Lepr^{db/+}$ males) were performed to ensure that each litter contained an equal proportion of offspring genotypes [204].

In the pharmacological model, wildtype females infused with saline (SAL) throughout gestation via insertion of a subcutaneous Alzet Mini-Osmotic Pump (Model 2004, Durect Corporation, Cupertino, CA) served as the controls for wildtype females with subcutaneously inserted leptin-filled pumps (LEP). Surgeries were performed a week before mating with wildtype males [204].

Maternal characteristics were reported previously [204]. Briefly, DB/+ females exhibited severe hyperleptinemia (127.0 ng/ml vs 53.1 ng/ml WT), while LEP females exhibited milder hyperleptinemia (47.7 ng/ml vs 31.4 ng/ml SAL), allowing for dose dependent studies. DB/+ dams weighed significantly more than WT controls, while LEP dam weights were only numerically higher than SAL controls. Maternal fasting glucose, serum insulin, and serum leptin were not different among treatment groups [204].

Forty-nine females were mated, producing 20 litters (7 WT, 3 DB/+, 4 SAL, 6 LEP). Two months later, an additional 63 females were mated, producing 32 litters (6 WT, 8

DB/+, 10 SAL, 8 LEP). Pups were genotyped at weaning, as previously described [204] and up to two male and two female wildtype offspring from each litter were retained for study. Offspring from the first mating were kept on a standard chow diet (LabDiet 5008 Purina, St. Louis, MO) along with two male offspring from dams mated in the second period, to provide a sufficient sample size for the chow diet cohort (male offspring: n= 11 WT, 8 DB/+, 8 SAL, 10 LEP; female offspring: n= 11 WT, 6 DB/+, 8 SAL, 10 LEP). Offspring from the second mating period were placed on a high fat, high sucrose diet (45% kcal/fat DIO HFD D12451, Research Diets, New Brunswick, NJ) at 23 weeks of age (male offspring: n= 9 WT, 12 DB/+, 20 SAL, 14 LEP; female offspring: 8 WT, 9 DB/+, 18 SAL, 16 LEP). Weights and behavior data for these offspring were reported previously [204].

All offspring were sacrificed by CO_2 inhalation at 31 weeks of age, following *ad libitum* food access. At sacrifice, blood was collected via cardiac puncture, and then centrifuged to obtain serum which was stored at -20^{o}C. Liver, subcutaneous fat and visceral fat were excised and snap-frozen in liquid nitrogen prior to storage at -80^{o}C. Pancreatic samples were fixed in 4% paraformaldehyde (PFA), paraffin-embedded and stored at room temperature. Liver samples were also fixed in 4%PFA, and embedded in Optimum Cutting Temperature Compound (Fisher Scientific, Pittsburgh, PA) prior to storage at −80^{o}C.

Glucose Tolerance Tests

At 30 weeks of age, glucose tolerance was assessed in the offspring following the Animal Models of Diabetic Complications Consortium protocol. The OneTouch Ultra glucose

meter was used to measure blood glucose levels as instructed by the manufacturer (LifeScan Inc.). Following a 6 hour fast, blood glucose was measured from a sample collected from a tail vein incision. Offspring were then given an intra-peritoneal injection of 1mg/g body weight glucose, and blood was collected at 15, 30, 60 and 120 minutes post-injection for glucose determination. GraphPad Prism (GraphPad Software, San Diego California USA) was used to obtain a value for the area under the curve for each animal as a measure of glucose tolerance.

Serum Insulin

Serum insulin was measured in offspring in terminal blood collections from mice with free access to food. Insulin concentrations were measured using the Rat/Mouse Insulin ELISA kit (Millipore, St. Charles, MO intra-assay variation: 14%, inter-assay variation: 3%) according to the manufacturer's instructions, save that the primary antibody was incubated overnight.

RNA Isolation and qRT-PCR

RNA was isolated from offspring liver, subcutaneous adipose tissue, and visceral adipose tissue samples collected upon sacrifice at 31 weeks. Liver samples of approximately 30mg were homogenized in 500ml TRI Reagent (Sigma-Aldrich, St. Louis MO) on a General Laboratory Homogenizer (OMNI International, Kennesaw, GA). Phase-lock gel tubes (5 Prime Inc., Gaithersburg, MD) were used to separate the aqueous phase and further RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA) by using the manufacturer's protocol. Adipose tissue samples of approximately 60 mg were homogenized in 1 mL TRI Reagent. Samples were centrifuged to separate and

remove the lipid layer and RNA isolation was performed using the Nucleospin RNA Clean-up kit (Clonetech, Mountain View, CA; Mancherey-Nagel, Bethlehem, PA) following the manufacturer's protocol. Genomic DNA was eliminated from all RNA samples using the Turbo DNA-Free Kit (Ambion by Life Technologies, Grand Island, NY).

One µg of liver RNA, or 500 ng adipose RNA were reverse transcribed using the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol with random hexamer primers. Real-time PCR with SYBR Green Master Mix (Superarray, Qiagen, Valencia, CA) was performed to quantify the relative mRNA concentrations of genes involved in (1) glucose metabolism: *Gck, Ghr, Igf1r, Igfbp1 and Insr;* and (2) lipid accumulation: *Cd36, Hsd11b1 and Srebp1c* in liver and of *Lep, Leprb, Insr* and *Srebp1c* in adipose tissue. *Gapdh* was used as the internal reference gene for liver, and *Actb* and *Hprt* were used as the references for adipose tissue. PCR cycling was as follows: 50.0 °C for 2 min (1 rep), 95°C for 10 min (1 rep) and 95°C for 15 sec, followed by 60°C for 1 min (40 reps). *Cd36* and *Srebp1c* primers have previously been published [243] and *Gck, Hprt, Hsd11b1, Igf1r, Gapdh, Igfbp1*and *Insr* primers were designed with Primer Express (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA). *Ghr* and *Lep* primers were designed and synthesized by Integrated DNA Technologies. All primers were tested for efficiency relative to the internal control, by using serial dilutions of template. Internal control genes were determined not to differ in expression across treatment groups. PCR results are expressed as fold change relative to CD WT, by the ΔΔCt method [244]. Primer sequences are detailed in Table 1.

Pancreatic Morphology

Pancreatic tissue was assessed by immunohistochemistry as previously described [233]. Pancreas sections were incubated overnight with primary antibodies mouse anti-glucagon (Abcam ab10988) to mark alpha cells and rabbit anti-insulin (Abcam ab63820) to mark beta cells at 1:200 and 1:250 dilutions, correspondingly. Alexa Fluor 568 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit were used as secondary antibodies at 1:500 (Invitrogen Life Technologies, Carlsbad, CA). Six 5μm sections separated by 50μm intervals were examined in each pancreas. At least 3 islets per section, up to 16 islets total, were photographed from each pancreas, and then 12 of these images were selected for analysis by using a random number generator. Alpha cells, beta cells and nuclei were pseudo-colored and alpha and beta cell numbers were counted by an operator blinded to treatment group using ImageJ software, and the average number of cells per islet per section was calculated for each animal.

Magnetic Resonance Imaging

Body composition was measured in the offspring at 29 weeks of age. A Micro-MRI high performance 7T MR Imaging and Spectroscopy system (Bruker Corp., Billerica, MA) equipped with a 86 mm inner diameter volume coil was used to determine body composition as described elsewhere [245]. Data were analyzed with Mnova7 Software (Mestrelab Research, Santiago de Compostela, Spain).

Serum Leptin

At 31 weeks of age, upon sacrifice of mice with free access to food, offspring blood samples were collected by cardiac puncture for serum analysis. Serum leptin

concentrations were measured using the Mouse Leptin ELISA kit (Millipore, St. Charles, MO, intra-assay variation: 8.9% inter-assay variation: 10%) according to the manufacturer's instructions, save that the primary antibody was incubated overnight.

Serum and Liver Triglycerides

Triglyceride and free glycerol concentrations were measured in the terminal serum samples via the Serum Triglyceride Determination Kit (Sigma-Aldrich, St. Louis, MO). We modified the manufacturer's protocol, by using 96-well plates instead of cuvets. Triglyceride concentrations were measured in liver samples using a modified Folch protocol [246]. Briefly, liver samples of ~100mg were homogenized in chloroform/methanol (2:1) and washed with 0.9% saline solution, then centrifuged at 4000 RPM for 10 min, after which the organic phase was collected. The organic phase was dried and resuspended in glycerol reagent, and triglycerides were measured following the same protocol.

NAFLD Assessment

Liver histopathology was performed on offspring to evaluate non-alcoholic fatty liver disease (NAFLD) as previously described [245]. In brief, slides with liver sections were stained with Oil Red O (Sigma) as instructed in the Biological Stain Commission guidelines [247], then counter-stained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO). Liver sections were graded on a scale of 0-3 (0 = less than 33% of the sample contains the defect, 1 = 33-50%, 2 = 50-66%, and 3 = 66-100%) for common characteristics of NAFLD, namely hepatocellular ballooning, steatosis and portal

inflammation. The score for each sample is the sum of the scores for each of the three features. Three 8μm sections separated by 50μm intervals were examined per animal.

Statistical Analysis

All data were analyzed using SAS(R) (SAS Institute, Cary, NC). In each test, the wildtype offspring of hyperleptinemic models were compared to their respective controls (WT vs. DB/+ and SAL vs. LEP). Glucose concentrations across the time points of the GTT were analyzed by nested repeated measures three-way ANOVA, with offspring sex, diet, and maternal treatment group as fixed effects, and with offspring nested in mother. All other parameters (including GTT area-under-the-curve) were analyzed by nested three-way ANOVAs with offspring sex, diet, and maternal treatment group as fixed effects and with offspring nested in mother. Real-time RT-PCR data were analyzed by comparing ΔCt values. In order to correct for unequal variance, insulin concentrations were log transformed prior to analysis.

Results

Regulation of Glucose Tolerance in Offspring

Maternal hyperleptinemia significantly improved offspring insulin sensitivity and glucose tolerance. At age 30 weeks, fasting blood glucose was not different among offspring from any of the maternal treatment groups (Fig. 1A-D). In a glucose tolerance test, as measured by the area under the curve, glucose tolerance was significantly improved in offspring of DB/+ dams, independent of offspring sex or diet (Fig. 1 A,C, $p<0.05$). There was no difference in glucose tolerance between the offspring of SAL and LEP dams on

either diet (Fig. 1, B,D). Glucose tolerance deteriorated in each offspring group following high fat, high sucrose feeding.

Insulin was measured at sacrifice, in mice with *ad libitum* access to food. Offspring of DB/+ dams had significantly lower insulin than offspring of WT dams (p= 0.007) (Fig. 1E), and offspring of LEP dams had significantly lower insulin than offspring of SAL dams (p=0.002) regardless of sex or diet (Fig. 1F)

Alpha and beta cells were identified by immunostaining (Fig. 2 A-D) in a random sample of islets to determine whether differences in insulin production were driven by differences in beta cell number. There was no difference in the number of alpha or beta cells per pancreatic islet between the male offspring of control and hyperleptinemic dams whether the offspring were fed chow or HFD (Fig. 2 E,F). Consuming HFD increased beta cell numbers and reduced alpha:beta cell ratios in male offspring (Fig. 2 E-H), whereas it increased alpha cell numbers in SAL and LEP females (Fig. 2J). Female offspring of DB/+ dams had a significantly higher alpha to beta cell ratio (p=0.013) than controls when consuming the HFD (Fig. 2K). Female offspring of LEP dams on the chow diet also had a significantly higher alpha to beta cell ratio (p=0.05) than controls (Fig. 2L).

Real-time RT-PCR was used to examine expression of key insulin-related genes in the liver that were previously shown to be altered by concomitant maternal hyperleptinemia and food restriction [233]. HFD-fed offspring of DB/+ dams had significantly lower *Igfbp1* mRNA (p=0.004) than HFD-fed offspring of WT controls in pairwise comparisons (Fig. 3A,B). Male offspring of LEP dams had significantly reduced liver

Insr mRNA (p=0.02) compared to controls in pairwise comparisons, independent of diet (Fig. 3B). Corresponding to an improved insulin response, transcript levels of *Gck*, a hexokinase responsible for the initial step of glucose phosphorylation that precedes glycogen synthesis or glycolysis, significantly higher in female offspring of DB/+ dams independently of diet, and *Insr* transcript levels were higher on CD. (Fig. 3C)

Adipose Tissue and Lipid Metabolism in Offspring

We next investigated whether differences in insulin sensitivity among offspring were associated with differences in adiposity and lipid accumulation that can reduce insulin sensitivity. Despite previously observed differences in body weight [204], at 29 weeks of age, neither the offspring of DB/+ or LEP dams differed in body fat percentage from the offspring of WT and SAL dams respectively (Fig. 4A,B). Placement on HFD increased the body fat percentage in all groups, and it was not further affected by maternal treatment.

At 31 weeks of age, leptin concentrations in the offspring of DB/+ dams were significantly lower than in offspring of WT controls on the chow diet (p=0.01). High fat, high sucrose feeding significantly elevated serum leptin and abrogated the effect of maternal leptin (Fig. 4C). Serum leptin concentrations exhibited the same trend within the offspring of SAL and LEP, but were not significantly different (Fig 4D).

Total serum triglycerides were significantly higher in the offspring of DB/+ dams compared to WT controls, but neither free glycerol nor true serum triglycerides were different (Fig. 4E). In the offspring of LEP and SAL dams, HFD decreased total serum triglycerides, free glycerol and true serum triglyceride content (Fig. 4F).

Real-time RT-PCR was used to investigate the source of differences in serum leptin. High fat, high sucrose feeding increased *Lep* mRNA in both visceral and subcutaneous fat, concomitant with the increase in serum leptin (Fig 5A-F). In contrast, *Lep* mRNA did not differ between offspring of DB/+ and WT dams in visceral fat (Fig 5A) and female offspring of DB/+ dams actually had significantly higher expression of *Lep* ($p=0.02$) than offspring of WT controls in subcutaneous fat (Fig. 5E).

However, differences in expression in visceral fat of *Leprb*, the main signaling isoform of the leptin receptor, and a determinant of leptin sensitivity, were inversely related to serum leptin concentrations. In visceral fat, offspring of DB/+ dams expressed higher *Leprb* mRNA than offspring of WT controls ($p=0.03$) on the chow diet (Fig. 5A). In all groups, HFD feeding decreased the expression of *Leprb* by visceral fat (Fig. 5a, b). There were no differences in Leprb expression in subcutaneous fat (Fig. 5C-F).

In parallel with increases in serum insulin (Fig. 1), adipose tissue *Insr* mRNA decreased with HFD in visceral fat from all offspring groups, and in subcutaneous fat from offspring of SAL and LEP dams (Fig. 5A-F). In subcutaneous fat, male offspring of LEP dams had significantly higher *Insr* expression ($p=0.047$) compared to SAL controls (Fig. 5D), consistent with greater insulin sensitivity and lower serum insulin.

Expression of the genes encoding fatty acid transporters Srebp1c and CD-36 were examined in adipose tissue and liver (Fig. 5-6). In offspring of LEP and SAL dams, HFD decreased *Srebp1c* mRNA expression in both visceral and subcutaneous fat, though it was not affected by diet in the offspring of WT and DB/+ dams. There was less mRNA

for fatty acid transporter *Cd-36* (p=0.0002) in the liver of male offspring of LEP dams than of SAL dams, independent of offspring diet (Fig. 6B).

To determine whether this reduction in Cd-36 expression resulted in less hepatic lipid accumulation in the offspring of hyperleptinemic dams, hepatic triglycerides were measured. Offspring of DB/+ dams had significantly reduced hepatic triglycerides when compared to WT controls, and offspring of LEP dams also had reduced hepatic triglycerides compared to SAL controls, though only on the HFD (Fig. 7A,B). Accumulation of fat in the liver was also analyzed by histopathological scoring, as non-alcoholic fatty liver disease (NAFLD) is associated with insulin resistance (Fig. 7C-F). As expected, on the HFD, the mean score for NAFLD was significantly higher than it was on the chow diet (p=0.001), where there was no indication of NAFLD, with scores of zero for all but one animal. Males on the HFD displayed an increased severity of NAFLD compared to females (p=.002). Compared to the male offspring of SAL dams, male offspring of LEP dams had significantly less severe NAFLD (p=0.05) (Fig. 7D).

Discussion

We have previously shown that high maternal leptin reduces offspring weights from age 23 weeks through sacrifice at age 31 weeks, and increases spontaneous activity, pointing to an anti-obesity effect of maternal hyperleptinemia on adult offspring [204]. Here we sought to examine the role of maternal hyperleptinemia in programming of offspring insulin sensitivity. Overall, we found that offspring born to hyperleptinemic dams had improved insulin sensitivity at 30 weeks of age. Exposure to maternal hyperleptinemia was also associated with lower serum leptin and reduced hepatic lipid accumulation in

adult offspring. There was no difference in overall body fat percentage, which, combined with the reduction in body weights, indicates a proportional reduction in total fat and lean mass. Thus, maternal leptin may have protected offspring by changing the distribution of lipids, and reducing overall growth, but not by altering body composition. These benefits may have been programmed directly, or may be secondary to the reduced body weight and increased activity levels that we documented previously [204]. Together, these data show that high maternal leptin promotes long-term metabolic health in offspring.

Improved Insulin Sensitivity

The mechanisms by which GDM predisposes the fetus to diabetes and obesity have not been clearly outlined, but a number of studies have shown that exposure to GDM decreases offspring insulin sensitivity [248, 249] and impairs glucose tolerance, increasing risk of type 2 diabetes [213]. Here we found that prenatal exposure to maternal hyperleptinemia improves offspring insulin sensitivity regardless of offspring diet. Increased insulin sensitivity in male offspring of dams treated with leptin during late gestation was seen in a previous study [205], consistent with this finding. Offspring of both models of maternal hyperleptinemia had reduced insulin concentrations, with no increase in blood glucose, suggesting an improved response to insulin [250]. There were no changes in beta cell number associated with maternal hyperleptinemia, supporting the conclusion that differences in insulin sensitivity, rather than insulin production, underlie the serum differences. The reduction in insulin concentrations in offspring of hyperleptinemic dams was maintained on the HFD, although the diet increased insulin concentrations in all groups. Similarly, although HFD raised blood glucose concentrations in the GTT, on HFD offspring of DB/+ dams still had significantly better

glucose tolerance than offspring of controls. These results parallel our initial observations of offspring weights, [204] in that maternal hyperleptinemia does not abolish the impact of high fat, high sucrose diet, but does significantly protect offspring from its full consequences.

Differences in *Insr* and *Gck* gene expression in liver and subcutaneous fat among offspring with differing developmental leptin exposures were closely related to changes in insulin sensitivity, with the exception of *Insr* expression in the liver of male offspring of LEP. Unfortunately, skeletal muscle, a major determinant of insulin sensitivity, was not collected, and insulin signaling in this tissue should be examined in future studies in order to develop a clear mechanism. There was also a pattern of improved lipid metabolism that may underlie the improved insulin sensitivity in offspring of hyperleptinemic dams.

Improved Lipid Metabolism

Infants born to mothers with GDM have elevated leptin levels [251] along with increased weights [252] indicating greater adiposity, and potentially leptin resistance, which has been implicated in the pathogenesis of type 2 diabetes [253, 254]. While body fat percentage of adult offspring was not affected by maternal leptin, offspring of DB/+ dams had lower serum leptin concentrations on the chow diet. In addition, maternal hyperleptinemia resulted in increased visceral fat expression of *Leprb* in these offspring, indicating improved leptin sensitivity. While female offspring of DB/+ dams expressed more *Lep* mRNA than controls, this was not reflected by protein levels, suggesting increased leptin clearance, or decreased translation or release. These differences were lost

on HFD; *Lep* transcript levels were significantly increased regardless of maternal treatment group, and there were no differences in serum leptin concentrations among treatment groups, showing that some of the effects of maternal hyperleptinemia are dependent on offspring diet. Unexpectedly, serum leptin did not increase with the increase in transcript levels.

Greater metabolic activity in fat was suggested by excess free glycerol in the offspring DB/+ dams, which, although not statistically significant, contributed to the significant increase in total serum triglycerides in these offspring of DB/+ dams. Higher serum glycerol suggests increased lipolysis, a process stimulated by leptin to metabolize fat tissue [255, 256]. In addition to lipolysis, leptin also promotes fatty acid oxidation preventing tissue lipotoxicity [257]; thus improved leptin sensitivity in offspring of DB/+ dams may protect against lipid accumulation, reducing the risk of inflammation and resultant type 2 diabetes [258, 259].

There was consistent evidence of reduced hepatic lipid accumulation in offspring of hyperleptinemic dams. Hepatic triglyceride levels were reduced in the liver from offspring of DB/+ under both diet conditions. Offspring of LEP exhibited decreased hepatic triglyceride levels, reduced NAFLD in male offspring on HFD and reduced Cd-36 expression under both diet conditions. *Cd-36* is a free fatty acid transporter that contributes to the pathogenesis of NAFLD [260] and the development of type 2 diabetes, and thus, its reduced expression may protect offspring of hyperleptinemic dams [235]. Collectively, these data indicate that the programming of the insulin sensitive phenotype in these offspring by maternal leptin likely involves decreasing fatty acid accumulation in the liver.

Differences in hyperleptinemic models.

We used two models of hyperleptinemia (DB/+ dams and LEP dams) to determine if the degree of maternal hyperleptinemia would determine the metabolic consequence in the offspring, and to control for potential confounding factors specific to each model. LEP dams had lower serum leptin than DB/+ dams, and we observed some dose-dependent outcomes. For instance, the offspring of DB/+ dams had significantly lower serum leptin compared to controls on the chow diet, while the offspring of LEP dams had numerically, but not significantly, reduced leptin compared to controls. This was also true of glucose tolerance curves on HFD, and a similar dose-dependency was seen in the offspring weights [204].

However, not all of the observed changes can be credited to the concentration of leptin exposure in utero. Particularly, NAFLD assessment, and some of the gene expression differences we observed were only present in the offspring of LEP or offspring of DB/+ dams compared to their respective controls. HFD feeding resulted in decreased mRNA expression of *Srebp-1c* in both fat depots in offspring of SAL and LEP dams, but not in offspring of WT or DB/+ dams, independent of maternal leptin level. Some of these differences may be due to differences in sample size, as the SAL/LEP arm was larger [204]. There may also be differences in the two models. Unlike the wildtype LEP dams, the DB/+ dams lack one copy of the gene for the leptin receptor, and the impact on leptin sensitivity has not been characterized. These mice had extremely high serum leptin, yet only moderate overweight, and normal or improved glucose tolerance, suggesting mild or mixed resistance. Conversely, SAL and LEP dams underwent pump surgeries and maintained the pumps throughout pregnancy, while WT and DB/+ dams were not

similarly stressed, which may contribute to differences between the hyperleptinemic models.

While it increases the complexity of our results, using two models concurrently is instructive in clarifying whether the outcomes observed are due to maternal hyperleptinemia, or experimental manipulations. That both models resulted in improved insulin sensitivity suggests that this is a specific effect of maternal hyperleptinemia, and not an artifact of experimental design.

Comparisons with other models of maternal hyperleptinemia

We previously examined the effects of maternal hyperleptinemia during early pregnancy only, with concomitant maternal food restriction [233], and found effects on offspring essentially opposite to what is reported here, namely increased offspring weights, and reduced insulin sensitivity. There, hepatic expression of *Insr*, *Igfbp1*, *Gck*, and *Igf1r* were altered in HFD-fed offspring of restricted, leptin-treated dams relative to HFD-fed offspring of either control or restricted dams [233, 243]. Here, only *Igfbp1*expression in HFD-fed, male offspring of DB/+ dams was affected. While different from the study of high leptin with food restriction, the results of the present study are consistent with studies from other laboratories of maternal hyperleptinemia in well-fed mothers [231, 261]. Thus, the long term effects of maternal leptin on offspring are dependent on the nutritional milieu of pregnancy. This may also explain why hyperleptinemic $Lepr^{db/+}$ dams have an obesogenic effect on offspring metabolism when the dams are also diabetic [112]. These differences in effects of maternal leptin, combined with the inability of leptin to cross the placenta [229, 230], suggest that maternal leptin affects offspring by

acting in concert with the nutritional environment to change maternal and/or placental metabolism. Finally, the effects of maternal hyperleptinemia are different than those of direct fetal and neonatal leptin treatment. Offspring of rat dams treated with from the latter half of pregnancy through lactation were protected from weight gain and insulin resistance, similarly to the offspring in this study [261]. In contrast, acute leptin treatment of neonates has been shown to both protect and predispose offspring to obesity, depending on both timing and prior exposures of the offspring [206, 262-264], and injection of leptin directly into the fetal brain inhibits adult glucose tolerance [265].

Conclusion

The focus of this study was to elucidate the effect of elevated leptin levels during pregnancy on the programming of insulin response. Previous studies have shown that leptin alone does not predispose offspring to obesity [231]. This study demonstrates that maternal leptin improves insulin sensitivity and reduces hepatic fat accumulation in offspring. These findings are potentially relevant to human pregnancies complicated by hyperleptinemia and leptin resistance. Outside of pregnancy, obesity leads to hyperleptinemia, and eventually to diminished transport of leptin to the brain and inhibition of leptin signaling [266, 267]. It is difficult to assess directly whether leptin resistance complicates some pregnancies, as some resistance to the appetite-suppressing and weight-reducing effects of leptin is present even in normal pregnancies [192, 268]. Nonetheless, the combination of high serum leptin with lack of leptin-induced weight loss is strongly suggestive of leptin resistance. Recent meta-analyses found that leptin concentrations are elevated in early pregnancy in women who will go on to develop GDM, even when controlling for BMI [269]. The leptin response to OGTT is also

dysregulated in women with prior GDM pregnancy [270] Likewise, pregnancies complicated by obesity are associated with higher maternal leptin concentrations, and do not show the normal association between weight gain and leptin concentrations[271, 272]. It has been proposed that this elevated maternal leptin may mediate negative impacts of GDM or obesity on offspring metabolism [227]. The present study suggests that it is instead a lack of maternal leptin action, i.e. leptin resistance that is responsible. Whether the protective actions of leptin identified here are maintained in the presence of other features of GDM or maternal obesity remains to be tested.

Gene of Interest	Primer	Sequence (5'-3')
Leprb	Sense	GAGCCCTGAACCCATTTCAGAAG
	Antisense	ACCATAGCTGCTGGGACCAT
Actb	Sense	GATGACCCAGATCATGTTTGAGACC
	Antisense	AGATGGGCACAGTGTGGGTGA
Cd36	Sense	GGTCCTTACACTACAGAGTTCGTTA
	Antisense	CATTGGGCTGTACAAAAGACACA
Srebp1c	Sense	TGGTGGGCACTGAAGCAAA
	Antisense	GCAAGAAGCGGATGTAGTCGAT
Gck	Sense	ATGTGAGCTCGGCATGATTGT
	Antisense	CCTTCCACCAGCTCCACATT
Ghr	Sense	CTTCGCTGAACTCGCTGTA
	Antisense	AGAAGTAAATGAATCAAAATGGAAAGTG
Hsd11b1	Sense	GGCGGACTGGACATGCTT
	Antisense	GAGTGGATGTCGTCATGGAAGAG
Igf1r	Sense	GCATTTAGAGAAACGAACATTCC
	Antisense	CAAGTCCAAATATGGTCCATGCT
Igfbp1	Sense	GGAGATTTCCTCATCGTCTCACA
	Antisense	TATGGGACGCAGCTTTCCA
Insr	Sense	CCACCAAGAACTCGTGAAAGG
	Antisense	TGCACGCAGGAAAGAACCT
Gapdh	Sense	TGCACCACCAACTGCTTAGC
	Antisense	GGCATGGACTGTGGTCATGAG
Hprt	Sense	TGACACTGGCAAAACAATGCA
	Antisense	GGTCCTTTTCACCAGCAAGCT
Lep	Sense	GTGAAATGTCATTGATCCTGGTG
	Antisense	GTGGCTTTGGTCCTATCTGTC

Table 1. Primer Table

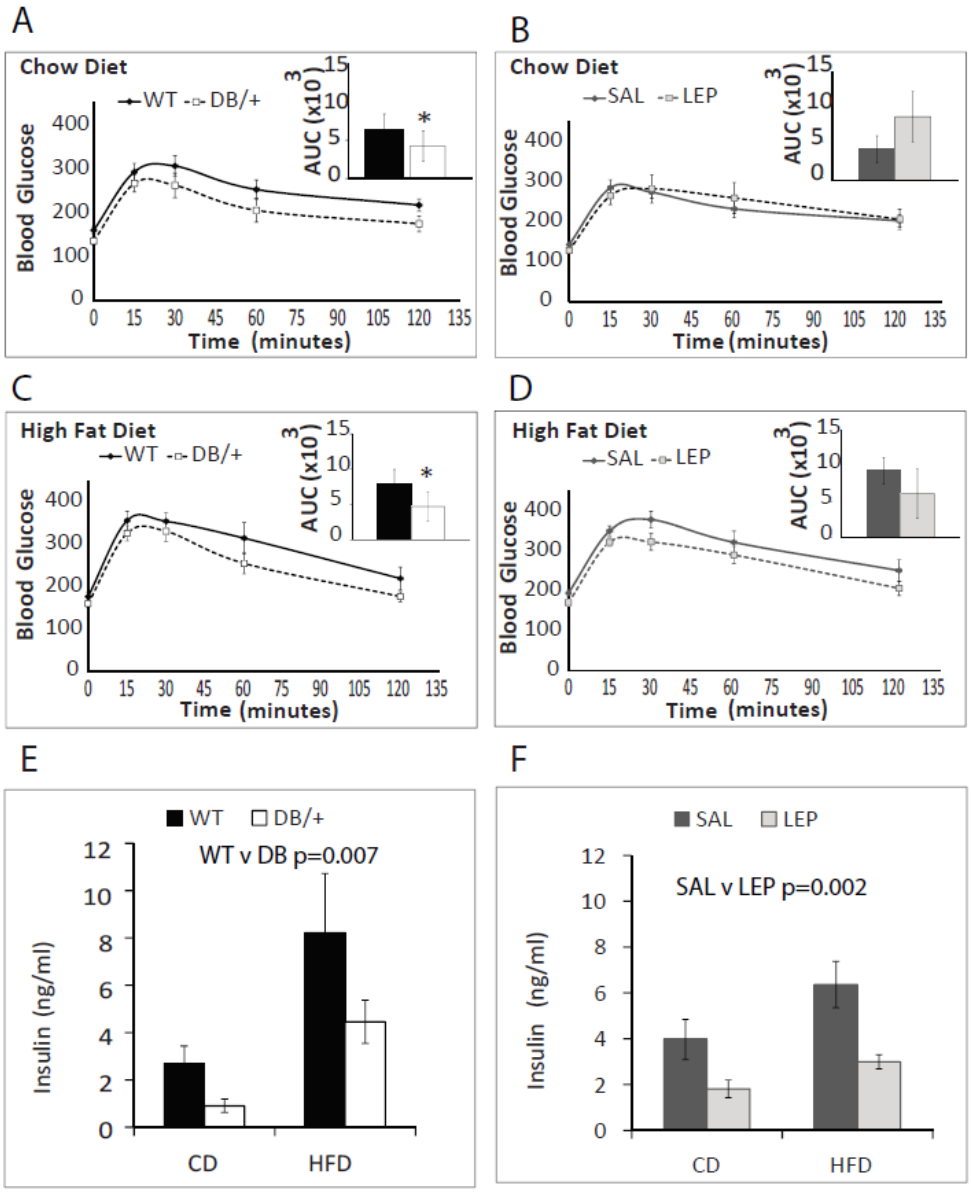

Figure 1: Insulin response in wildtype offspring. Glucose tolerance tests were performed at 30 weeks of age in offspring of (**A,C**) WT and DB/+ dams and in offspring of (**B,D**) SAL and LEP dams fed either (**A, B**) chow diet (CD) throughout or (**C,D**)switched to high fat, high sucrose diet (HFD) at age 23 weeks. Area under the curve is shown inset. Sample size ($offspring_{dam}$): (**A**) Males: WT 7_7, DB/+ 5_5 Females: WT 6_6, DB/+ 3_3 (**B**) Males: SAL 4_4, LEP 5_5 Females: SAL 4_4, LEP 5_5 (**C**) Males: WT 6_6, DB/+ 5_5 Females: WT 4_4, DB/+ 6_6 (**D**) Males: SAL 9_9, LEP 8_8 Females: SAL 10_{10}, LEP 7_7. Serum insulin concentrations at age 31 weeks in *ad libitum* fed offspring of (**E**) WT and DB/+ dams and (**F**) SAL and LEP dams. Male Sample size: (CD, HFD) WT 8_7,3_3 DB/+ 3_3,7_7 SAL 4_4,9_9 LEP 6_6,8_8. Female Sample size: (CD, HFD) WT 6_6,5_5 DB/+ 3_3,6_6 SAL 4_4,8_8 LEP 5_5,8_8. * indicates significantly different than control ($p < 0.05$). Data are represented as mean ± SEM.

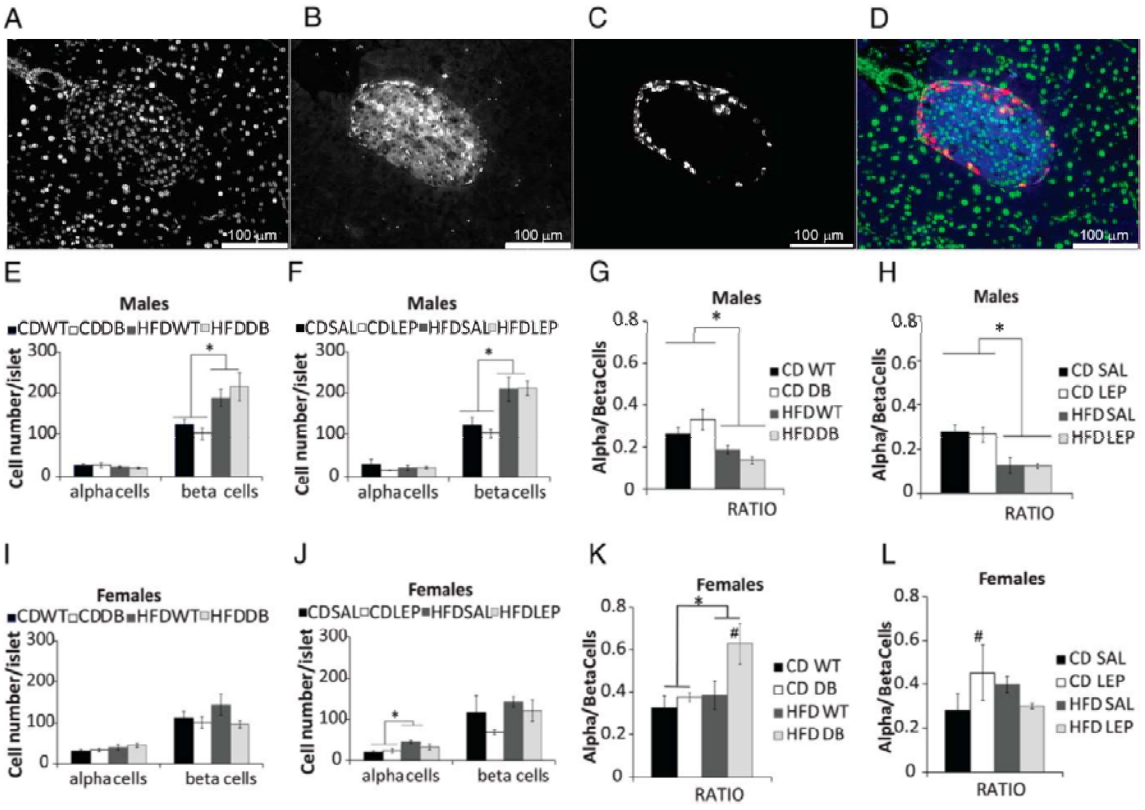

Figure 2: Pancreatic endocrine cell counts. Representative images of an islet immunostained for nuclei (**A**), insulin (**B**), glucagon (**C**) and a merge of insulin-positive β-cells (blue) and glucagon-positive α-cells (red) counterstained with DAPI (pseudocolored green) (**D**). The number of alpha and beta cells per islet, per level in (**E,F**) male and(**I,J**) female wildtype offspring of (**E, I**) WT and DB/+ or (**F,J)** SAL and LEP dams. Alpha to beta cell ratio in male (**G,H**) and female (**K, L**) offspring. CD=chow diet; HFD=high fat, high sucrose diet. Male sample size ($offspring_{dam}$): (CD,HFD) WT $6_5,6_5$ DB/+ $5_3,5_5$ SAL $4_3,6_5$ LEP $5_4,6_6$. Female sample size ($offspring_{dam}$): (CD,HFD) WT $6_5,6_5$ DB/+ $3_3,4_4$ SAL $3_3,9_9$ LEP $4_4,5_5$ *indicates significant difference between diets($p < 0.05$). **K)** # $p<0.05$ vs. WT HFD. **L)**# $p<0.05$ vs SAL CD. Data are represented as mean ± SEM.

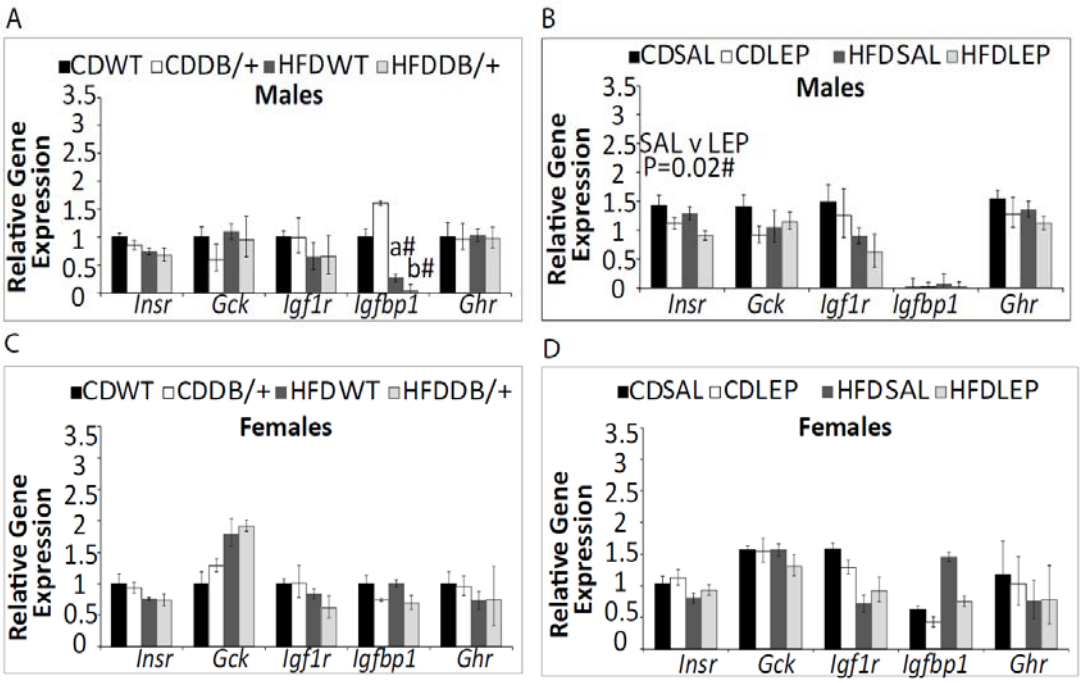

Figure 3: Real-time PCR analysis of glucose metabolic gene expression in liver from male and female offspring of (**A,C**) WT and DB/+ dams and (**B,D**) SAL and LEP dams at 31 weeks of age. Data are represented as mean fold change relative to control group mean on chow diet. Error bars represent range of fold changes based on SEM of ΔΔCt.. # $p<0.05$ in pairwise comparisons between maternal groups within diet and sex. Male sample size ($offspring_{dam}$): (CD,HFD) = WT $7_7,6_6$ DB/+ $3_3,7_7$ SAL $4_4,8_8$ LEP $5_5,8_8$. Female sample size ($offspring_{dam}$): (CD,HFD) WT $5_5,4_4$ DB/+ $4_2,5_5$ SAL $4_4,10_{10}$ LEP $5_5,7_7$. For *Igf1r* female offspring of DB/+ dams on CD n =2_2.

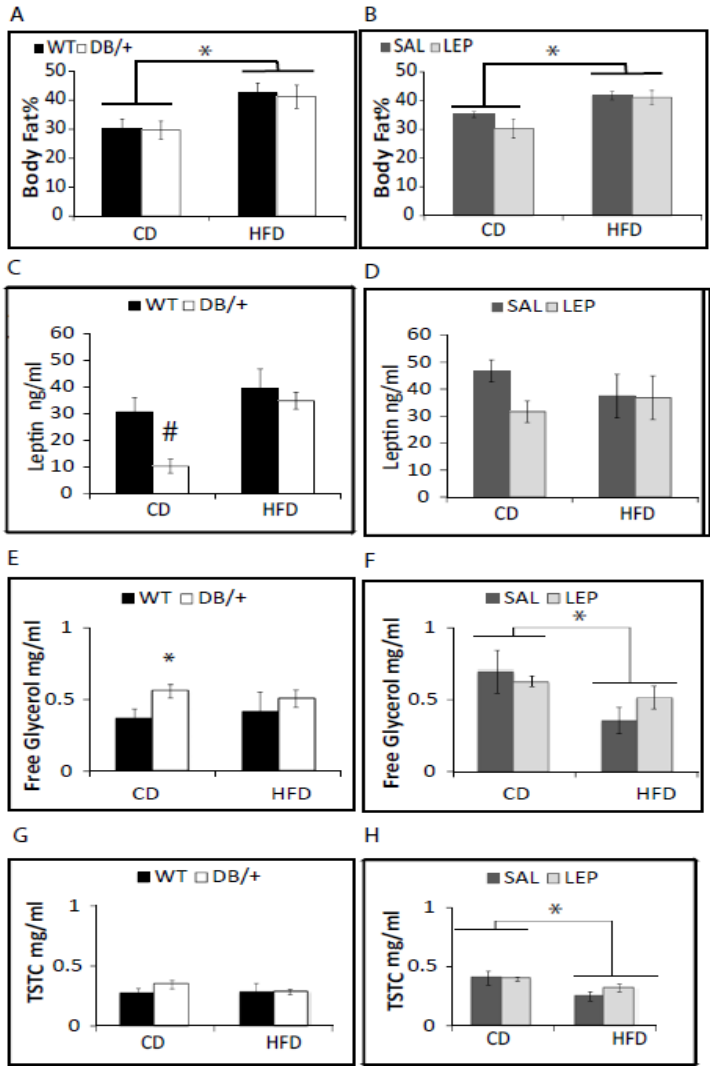

Figure 4: Magnetic Resonance Imaging and serum measurements in adult offspring. Body fat percentage in offspring of (**A**) WT and DB/+ dams and (**B**) SAL and LEP dams at 29 weeks of age. * indicates significant difference between diet groups ($p<0.05$). Male Sample size ($offspring_{dam}$): (CD,HFD) WT $7_7,3_3$ DB/+ $3_3,5_5$ SAL $4_4,9_9$ LEP $5_5,6_6$. Female Sample size ($offspring_{dam}$): (CD,HFD) WT $6_6,3_3$ DB/+ $3_3,3_3$ SAL $4_4,7_7$ LEP $4_4,8_8$. Serum leptin concentrations at 31 weeks in *ad libitum* fed offspring of (**C**) WT and DB/+ dams and (**D**) SAL and LEP dams. # Significantly different from WT CD in pairwise comparison ($p < 0.05$). Data are represented as mean ± SEM. Male Sample size ($offspring_{dam}$): (CD,HFD) WT $7_7,4_4$ DB/+ $3_3,6_6$ SAL $4_4,2_2$ LEP $5_5,4_4$. Female Sample size ($offspring_{dam}$): (CD,HFD) WT $6_6,2_2$ DB/+ $3_3,3_3$ SAL $4_4,8_8$ LEP $5_5,7_7$. Total serum triglyceride, free glycerol and true serum triglyceride concentrations in offspring of (**E**) WT and DB/+ dams and (**F**) SAL and LEP dams at 31 weeks. *significant difference ($p < 0.05$). Data are represented as mean ± SEM. Male Sample size ($offspring_{dam}$): (CD,HFD) WT $11_6,3_3$ DB/+ $6_3,7_7$ SAL $8_4,19_{10}$ LEP $11_6,14_8$. Female Sample size ($offspring_{dam}$): (CD,HFD) WT $11_6,8_5$ DB/+ $5_3,9_6$ SAL $8_4,18_{10}$ LEP$10_5,16_8$.

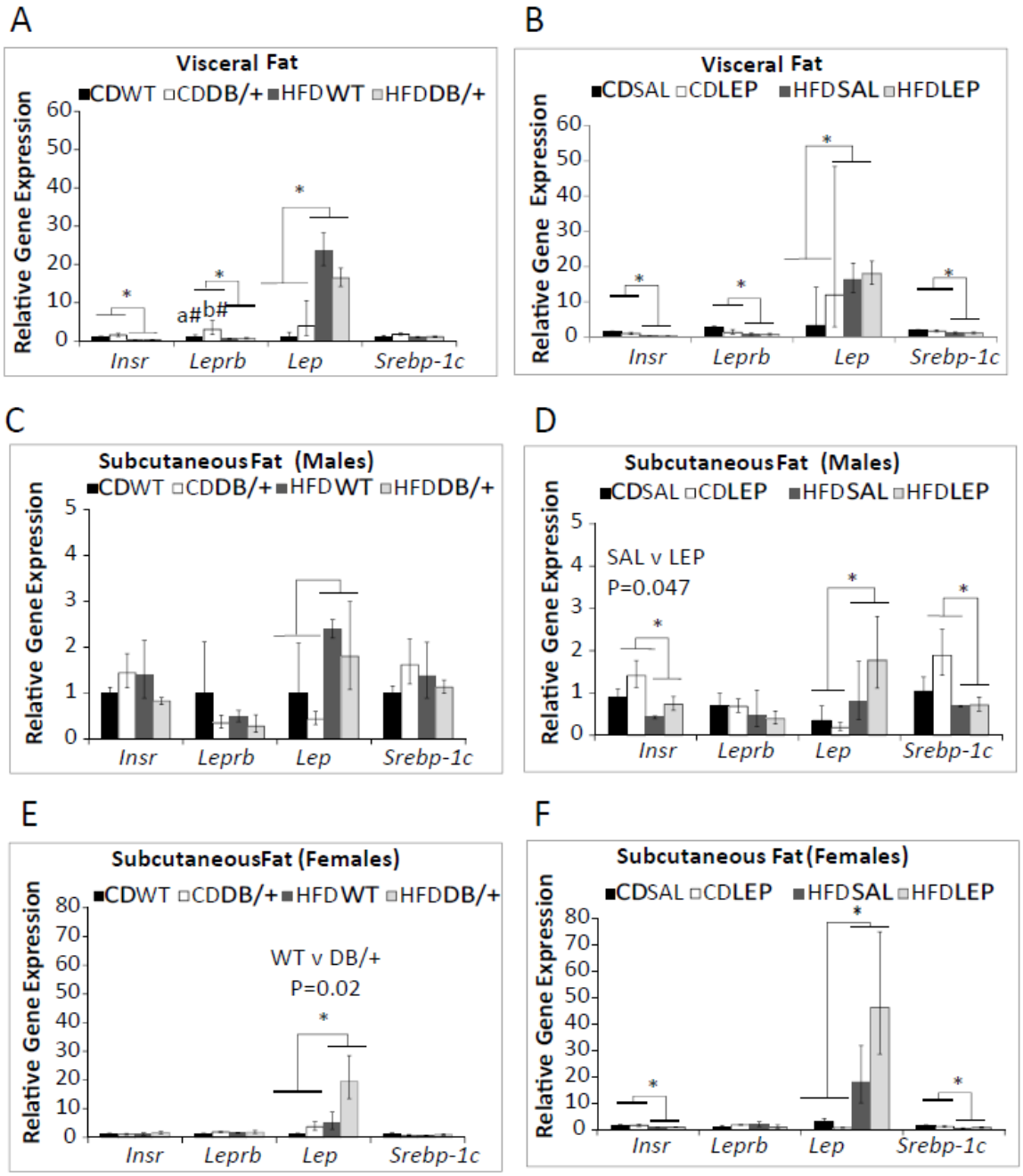

Figure 5: Real-time PCR analysis of lipid metabolic gene expression in adipose tissue at 31 weeks. Relative gene expression levels in (**A,B**) visceral fat and (**C-F**) subcutaneous fat. Data from males and females are combined for visceral fat, as there were no differences by sex. Data are represented as mean fold change relative to CD WT mean. Error bars represent range of fold changes based on SEM of ΔΔCt. * significant difference between diets. # signficantly different from WT CD in pairwise comparison. Sample size (offspring$_{dam}$): (CD,HFD) (**A,B**) Males: WT $4_4,7_5$ DB/+ $3_3,4_4$ SAL $4_4,3_3$ LEP$7_5,3_3$ Females: WT $3_3,4_3$ DB/+ $3_2,3_3$ SAL $5_4,3_3$ LEP$4_4,3_3$; (**C,D**))= WT $3_3,3_2$ DB/+ $5_4,5_5$ SAL $5_4,3_3$ LEP$5_4,6_6$; (**E,F**) WT$3_3,4_3$ DB/+$5_3,5_4$ SAL$5_3,3_3$ LEP$3_3,3_3$.

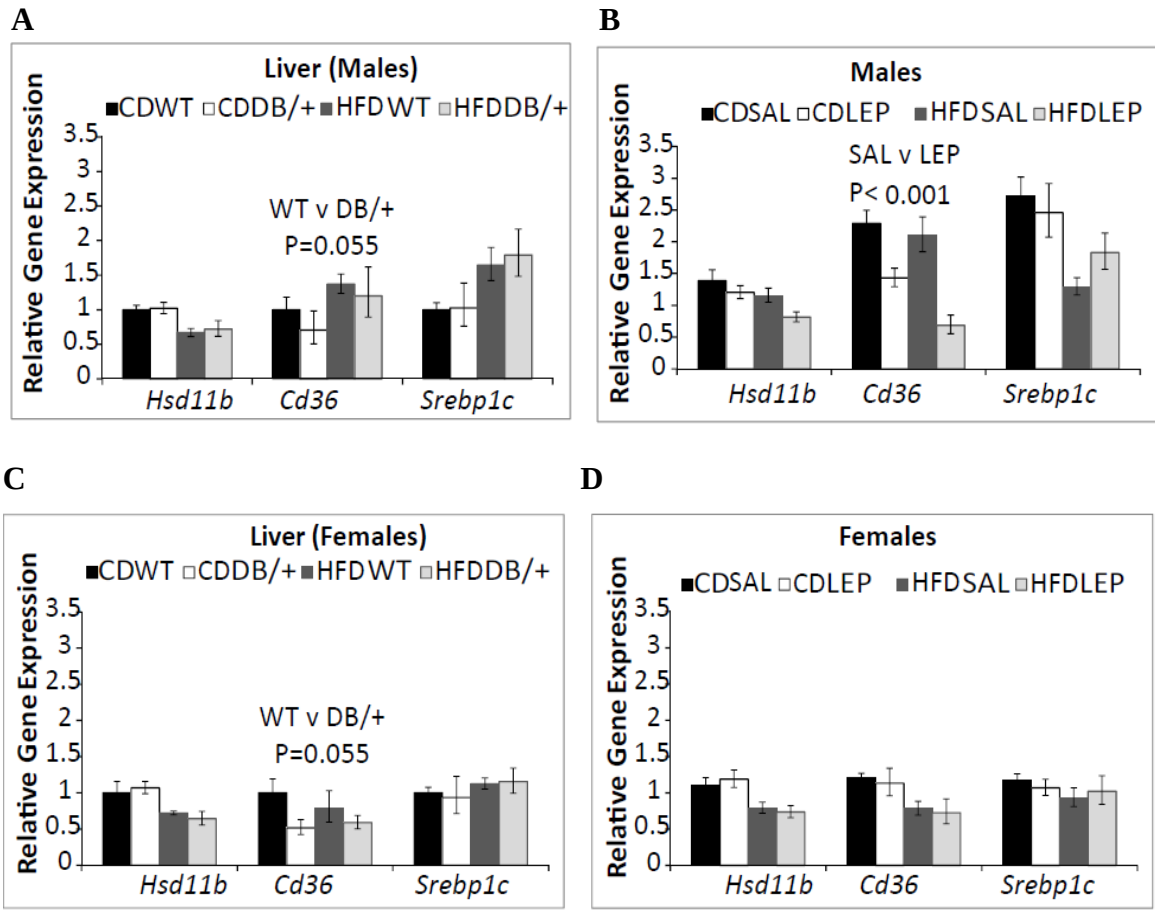

Figure 6: Real-time PCR analysis of lipid metabolic gene expression in liver tissue at 31 weeks. Relative gene expression levels liver (**A-D**). Data are represented as mean fold change relative to CD WT mean. Error bars represent range of fold changes based on SEM of ΔΔCt. * significant difference between diets. # signficantly different from WT CD in pairwise comparison. Sample size ($offspring_{dam}$): (CD,HFD) (**A,B**) WT $7_7,6_6$ DB/+ $3_3,7_7$ SAL $4_4,8_8$ LEP $5_5,8_8$; and (**C,D**) WT $5_5,4_4$ DB/+ $4_2,5_5$ SAL $4_4,10_{10}$ LEP $5_5,7_7$.

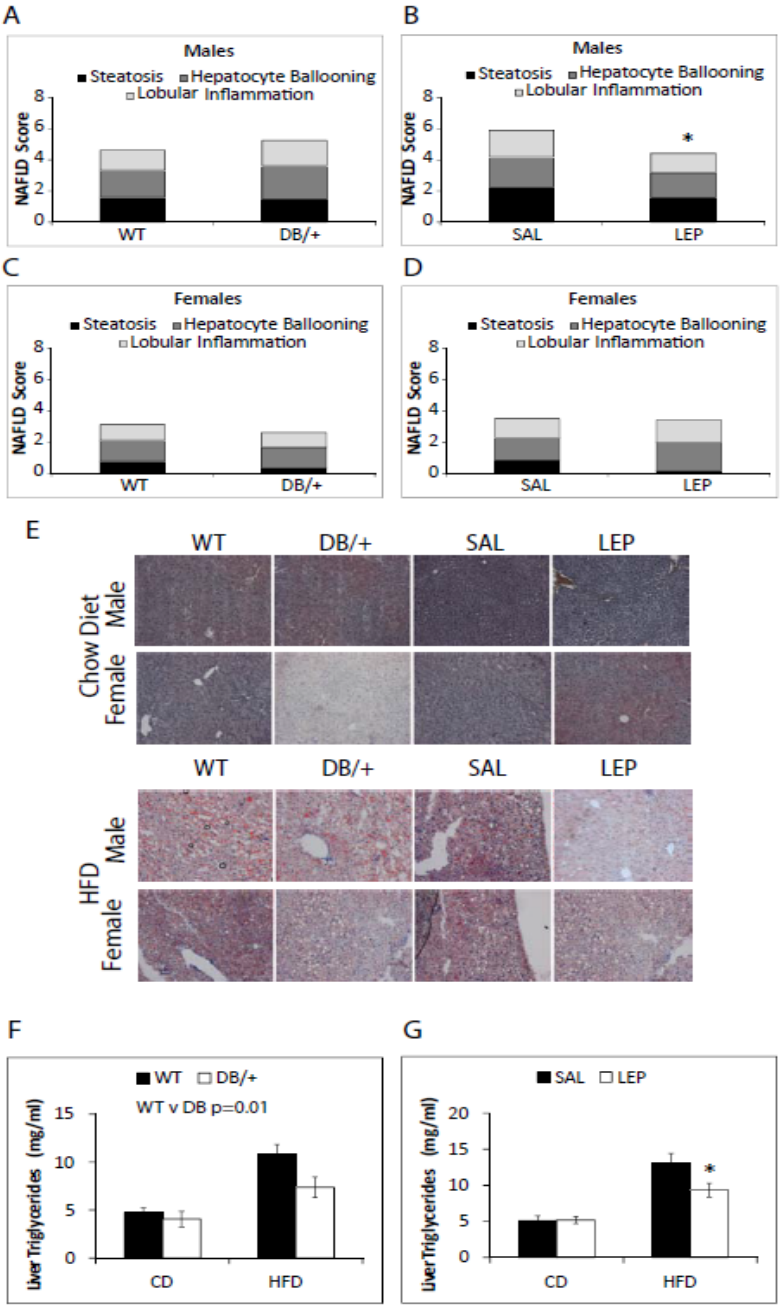

Figure 7: Lipid accumulation in livers of offspring at 31 weeks. (**A,B**) Triglyceride concentrations in livers from offspring of (**A**) WT and DB/+ dams and (**B**) SAL and LEP dams. # $p<0.05$ vs. SAL HFD. Male sample size ($offspring_{dam}$): (CD,HFD) = WT 5_5,5_4, DB/+ 3_3,5_5, SAL 5_4,13_9, LEP 5_5,11_7. Female Sample size ($offspring_{dam}$): (CD,HFD) = WT 6_6,5_5, DB/+ 3_3,6_6, SAL 4_4,7_6, LEP 5_5,6_6. Mean NAFLD score ± SEM in (**C,D**) male and (**E,F**) female offspring. *indicates significant difference ($p < 0.05$). Male sample size ($offspring_{dam}$): WT 6_6, DB/+ 7_7, SAL 10_9, LEP 8_8; Female sample size ($offspring_{dam}$) : WT 6_5, DB/+6_5, SAL 10_{10}, LEP8_8. (**G**)Representative images of NAFLD in offspring from each maternal condition and offspring diet group. Black arrows indicate steatosis, white arrows indicate hepatocyte ballooning, black circles indicate lobular inflammation. Lipid droplets are stained with Oil red O.

CHAPTER III

LEAN MATERNAL GLUCOSE INTOLERANCE INCREASES OFFSPRING ADIPOSE MASS AND IMPROVES INSULIN SENSITIVITY IN MICE

This chapter is in preparation for submission to the journal *Endocrinology*

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Abbreviations: GDM (gestational diabetes mellitus), GTT (glucose tolerance test), CD (chow diet), HFHS (high fat, high sucrose diet)

Abstract

Gestational diabetes mellitus (GDM) increases the risks of metabolic syndrome in offspring. We previously developed a lean model of GDM in which dams exhibit glucose intolerance and reduced insulin response to glucose challenge. Here, we aimed to determine effects on offspring metabolism.

One cohort of offspring was sacrificed at 19 weeks, and half of the offspring in the second cohort were placed on a high fat high sucrose (HFHS) diet at 23 weeks, prior to sacrifice at 31 weeks. We examined offspring weight, body composition, food consumption, activity, glucose tolerance, adipose and liver gene expression, liver and serum triglycerides, serum insulin and leptin.

Exposure to maternal glucose intolerance increased weights of HFHS-fed offspring, and adiposity of offspring fed both diets. Increased adiposity in offspring of GDM dams was accompanied by increased respiratory quotient (CO_2 produced /O_2 consumed) which is suggestive of preferential utilization of glucose for energy over lipids. Increased mRNA levels of *Pparg, Adipoq Insr* and *Lpl* in adult male offspring subcutaneous fat suggest greater capacity for fatty acid uptake. Glucose control was unaffected by exposure to maternal glucose intolerance, potentially due to increased mRNA levels of *Insr* in both liver and subcutaneous fat.

Our findings show that GDM comprising glucose intolerance only during pregnancy in lean dams programs increased adiposity in offspring, and increases the insulin sensitivity of subcutaneous adipose tissue and liver.

Introduction

Gestational diabetes mellitus (GDM), defined as maternal hyperglycemia first diagnosed during pregnancy, is an emerging global epidemic [273]. It affects up to 25% of pregnancies [5], contingent on the diagnostic criteria and population studied, and is on the rise worldwide. Diabetic pregnancies can increase the risk of serious complications for the mother such as hypertensive disorders like preeclampsia [274], premature delivery [275], stillbirth and cesarean delivery [35]. GDM also has consequences for the offspring, both perinatally and long-term.

The incidence of fetal macrosomia is higher in offspring born to mothers with gestational diabetes [61], and these offspring also have increased risks for diabetes [276-279] and obesity [277, 280-282] in late childhood and early adulthood. Baptiste-Roberts et al showed that offspring born to GDM mothers are 61% more likely to be overweight at the age of 7 than their counterparts [283], while Clausen et al showed that offspring exposed to GDM in utero are at an 8-fold higher risk of developing type 2 diabetes [215]. In 2011, the International Diabetes Federation stated that type 2 diabetes is responsible for one death every seven seconds, amounting to 4.6 million deaths per year[284]. The incidence of metabolic syndrome in young people born to GDM mothers indicates that the global rise of diabetes, obesity, and other metabolic diseases is partly due to the rise of GDM [285], however the mechanisms by which maternal hyperglycemia programs offspring metabolism remain unclear.

Among the challenges in understanding how GDM affects offspring is the variety of clinical characteristics found among women with GDM. For example, obesity is a major

risk factor for GDM, and roughly half of women with GDM are also obese [162]. Maternal obesity is itself a risk factor for obesity and diabetes in offspring [286] and may interact in complex ways with maternal diabetes to influence fetal growth [287]. One study found that overweight in offspring was a consequence of GDM in obese, but not lean mothers [288]. Secondly, preexisting type I and type II diabetes make up approximately 26% of diabetes cases during pregnancy [289] and these can affect oocyte and early embryonic development, which occur before onset of symptoms in other GDM cases [290-292]. Researchers have modeled various aspects of GDM impacts on offspring using rodent models. One such model is long-term feeding of a 40-60% fat, high sugar diet for at least 4 weeks prior to mating [111, 144], which leads to obesity, insulin resistance and glucose intolerance during gestation. Offspring weights vary, with some studies reporting macrosomia [115, 116], others reporting no weight difference, or growth restriction at birth [117, 120, 293]. Later in life, offspring of obese, insulin-resistant rodents weigh more, and are at a higher risk of type 2 diabetes [119, 120, 294].

Streptozotocin (STZ)-treated rodents are models of non-obese, insulin-deficient diabetes, with onset depending on the timing of administration [295, 296]. STZ permanently destroys pancreatic beta cells, and can have toxic effects on other tissues as well [297]. Thus, the maternal phenotype most closely resembles type 1 diabetes [133].These animals display mild or severe hyperglycemia, depending on the dosage of STZ [298] with varying offspring outcomes. Mild maternal hyperglycemia results in macrosomic or normal weight offspring [129, 130] while severely hyperglycemic dams have offspring with low birth weights [129, 299], which also occurs at higher-than-normal frequency in

GDM pregnancies [131, 132]. Adult offspring are insulin-resistant regardless of the severity of maternal hyperglycemia [121, 130].

Previously, we developed a mouse model of GDM [109] that resembles the 28% of women with GDM who are not overweight or obese [162]. Among lean women with GDM, there is subclinical insulin resistance pre gravidas, and impaired glucose-stimulated insulin response during mid-late pregnancy compared to pregnant women with normal glucose tolerance [300]. In our model, mice are fed a high fat, high sucrose diet a week prior to, and through the three weeks of gestation, for a total of 4 weeks. These mice have mild insulin resistance and normal glucose tolerance at the time of conception, spontaneously develop glucose intolerance and reduced insulin response to glucose in the latter half of pregnancy, and return to normal glycemia postpartum [109]. These dams do not differ in weight to controls, allowing us to hone in on the effects of pregnancy-specific hyperglycemia in lean dams. The goal of the present study is to use this model to determine how moderate gestational glucose intolerance, in the absence of maternal obesity affects offspring risk of metabolic dysfunction, particularly obesity and insulin resistance.

Methods

Animals and tissue collection

Animal procedures were approved by the University of Missouri Institutional Animal Care and Use Committee and animals were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Gestational diabetes was induced as previously described [109]. Briefly, 14 female wildtype C57Bl6/J mice (JAX) were fed a high fat, high sucrose (HFHS) diet (D12451, Research Diets Inc.) one week prior to mating to WT sires, and for the duration of gestation, for a total of four weeks. Twenty control females were fed a chow breeder diet (LabDiet 5008, Purina) throughout. The GDM dams produced 7 litters, for a total of 34 offspring (18 males, 16 females) and the CON dams produced 16 litters for a total of 82 offspring (52 males, 30 females).

Two male and two female offspring from each litter were maintained on the chow diet; one of each was sacrificed at 19 weeks of age, and one at 31 weeks of age. An additional male and female offspring from each litter was kept on the chow diet until 23 weeks of age, and then fed HFHS diet until sacrifice at 31 weeks of age.

Beginning at 4 weeks of age, offspring were weighed weekly. A subset of the offspring was sacrificed by CO_2 inhalation followed by cervical dislocation at 19 or 31 weeks of age after *ad libitum* food access. Another subset was anesthetized with isoflurane and underwent blood pressure assessment for a cardiovascular study prior to cervical dislocation at 19 (n=16) and 31 (n=47) weeks of age after *ad libitum* food access. Blood was collected at the time of sacrifice by cardiac puncture, and centrifuged to acquire serum, which was stored at −20°C. Samples of liver and subcutaneous fat were removed and snap-frozen in liquid nitrogen before storage at −80°C.

Magnetic resonance imaging

At 4, 12, 20 and 28 weeks of age, body composition was measured in CD fed male and female offspring via nuclear magnetic resonance imaging (Echo MRI mouse, Echo Medical Systems). Body composition was also measured in HFHS-fed male and female offspring from each treatment group at 30 weeks. Each conscious mouse was inserted into the MRI chamber in a plastic cylinder, and lean mass, fat mass and water were quantified.

Metabolic Cage Assessments

Male and female CD-fed offspring of CON and GDM dams at 4, 12, 20 and 28 weeks of age, and HFHS-fed offspring of CON and GDM dams at 30 weeks of age were placed in metabolic monitoring systems (PromethION; Sable Systems International). Animals were singly housed with *ad libitum* access to food and water and metabolic parameters were assessed for 3 days, consisting of three light and dark cycles of 12 hours each. Animals were placed in the cages in the middle of the light cycle and data from the first light and dark cycles were excluded from the analysis to account for acclimation; data from the following 48 hours were assessed. The metabolic cages assess food intake, energy expenditure, distance travelled and respiratory quotient using algorithms designed by the manufacturer (Sable Systems International). Food mass is weighed by sensors continuously, to determine food intake. Energy expenditure is calculated according to the Weir equation, where kcal per hour = 60*(0.003941*Vol O2+0.001106*Vol CO2) and where VO2 and VCO2 are in ml/min. Distance travelled is the sum of all distances traveled within the beam break system in meters, including fine movement (such as

grooming and scratching) and direct locomotion. Respiratory quotient is calculated as Vol CO2/Vol O2.

Glucose tolerance and Insulin Sensitivity

At 19 and 30 weeks of age, glucose tolerance tests (GTT) were performed on the offspring according to the Animal Models of Diabetic Complications Consortium protocol (https://www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=11). Blood glucose was measured using dual ReliOn PRIME glucometers (Walmart) according to the manufacturer's instructions. Offspring were fasted for 6 hours, following which blood was collected from the tail vein for assessment of fasting glucose and insulin. Offspring were injected intraperitoneally with 1 mg of glucose per kg body weight, and blood glucose was measured at 15, 30, 60, and 120 minutes post- injection. GraphPad Prism (GraphPad Software) was used to calculate the area under the curve for each animal.

The concentration of insulin was measured using the Rat/Mouse Insulin ELISA kit (Millipore) (intraassay variation: 6.7%, interassay variation: 6.0%) according to the manufacturer's protocol, with the exception that the primary antibody incubation was performed overnight.

RNA isolation and quantitative RT-PCR

RNA was isolated from offspring liver and subcutaneous adipose tissue samples collected at both 19 and 31 weeks of age by homogenization in TRI Reagent (Sigma-Aldrich), using a General Laboratory homogenizer (OMNI International). Adipose tissue samples

were centrifuged to separate and remove the lipid layer. Following phase separation according to TRI reagent instructions, RNA from both liver and adipose samples was further purified using the Nucleospin RNA clean-up kit (Clontech; Mancherey-Nagel) according to the manufacturer's protocol. Genomic DNA was eliminated from RNA samples using the Turbo DNA-free kit (ThermoFisher Scientific).

Reverse transcription was performed using the SuperScript First-Strand Synthesis System (ThermoFisher Scientific) according to the manufacturer's protocol. Briefly, 1 µg of liver RNA or 500 ng adipose RNA was reverse transcribed using random hexamer primers. Relative mRNA levels were quantified by real-time PCR with SYBR Green Master Mix (Qiagen) for the following genes in liver: *Insr, Acaca, Cpt1a and Ppara*; and in adipose tissue: *Insr, Srebp1c, Pparg, Pgc1a, Pck1, Il6, Adipoq, Lep, Lpl, Tnfa, and Ucp1*. *Gapdh* and *Hprt* were used as the internal reference genes for liver, and *Actb* and *Hprt* were used as the internal reference genes for adipose tissue. PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories) with the following cycling conditions: 95°C for 10 minutes (1 rep), and 95°C for 15 seconds, followed by 60°C for 1 minute (40 reps).

Insr, Lep, Gapdh, Hprt and *Srebp1c* primer sequences have previously been published [301],and *Acaca, Pparg, Il6, Adipoq, Lpl, Tnfa, Ucp1, Cpt1a, Pgc1a, Pck1 and Ppara* primers were designed by Integrated DNA Technologies, which synthesized all primers (Table 1). Primer efficiencies were validated by using serial dilutions of the respective template. Reference genes did not differ in expression across the treatment groups. PCR results are displayed as fold change relative to CON offspring, by the ΔΔcycle threshold method [244].

Serum leptin

The concentration of leptin was measured in terminal serum samples by using the Mouse Leptin ELISA kit (Millipore; intraassay variation: 8.9%; interassay variation: 10%) according to the manufacturer's protocol, with the exception that the primary antibody incubation was performed overnight.

Serum and liver triglycerides

Serum triglyceride concentrations were measured in the terminal serum samples by using the Serum Triglyceride Determination kit (Sigma-Aldrich) according to the manufacturer's protocol with the exception that 96-well plates, with a total reaction volume of 250 uL were used instead of cuvettes. An adapted Folch protocol was used to extract liver triglycerides as previously described [246, 301], and then measured by using the serum triglyceride determination kit.

Statistical analysis

Data analysis was performed using SAS (SAS Institute).

Each of the metabolic cage response variables (food intake, energy expenditure and respiratory quotient) were regressed on the maternal effect (GDM or control), age effect (4^{th}, 12^{th} or 20^{th} week since birth), cycle effect (day or night), offspring sex effect (male or female), and interactions among these factors. The models were constructed by both biological and statistical considerations. To model the correlations among the repeated measurements that were obtained at different time points for each offspring, the

compound symmetry covariance structure was used. The mothers are considered a random effect in the models to account for the correlations among offspring of the same dam. Optimal models were selected by AIC, BIC, and AICC criteria. We used the studentized residual plot and normal quantile plot for checking model fitting. For pairwise comparisons, we used Tukey-Kramer method for multiple test adjustment.

The metabolic cage data corresponding to the 28^{th} and 30^{th} weeks were analyzed separately. For average respiratory quotient, total food uptake and total distance of locomotion a model was built including maternal effect (GDM or control), offspring diet effect (high fat diet or control), cycle effect (day or night), offspring sex effect (male or female), and interactions among these factors. The mothers are considered a random effect in the models to account for the correlations among offspring of the same dam. Optimal models were selected by AIC, BIC, and AICC criteria. We used the studentized residual plot and normal quantile plot for checking model fitting. For the pairwise comparisons, we used Tukey-Kramer method for multiple test adjustment. To comply with the normality assumption, the total distance of locomotion was transformed to log scale, and the square root transformation was used for the total food uptake.

Data from the glucose tolerance tests at 19 weeks were analyzed by nested, two-way ANOVA, with maternal treatment (GDM or control) and offspring sex effect (male or female) as fixed effects, with time as a repeated measure, and with dam considered a random effect. Data from the glucose tolerance tests at 30 weeks were analyzed by nested, three-way ANOVA, with maternal treatment (GDM or control), offspring diet (high fat diet or control), and offspring sex effect (male or female) as fixed effects, with time as a repeated measure, and with dam considered a random effect.

Weights from age 3-22 weeks were analyzed by nested, three-way ANOVA, with maternal treatment, offspring sex and offspring age as fixed effects, with time as a repeated measure, and with dam considered a random effect. Weights from age 23-31 weeks were analyzed by nested, four-way ANOVA, with maternal treatment, offspring sex, age and diet as fixed effects, with time as a repeated measure, and with dam considered a random effect.

For all additional analyses of samples collected at 19 weeks, data were analyzed by nested, two-way ANOVA with maternal treatment and offspring sex as the fixed effects and with dam considered a random effect. For samples collected at 31 weeks of age, data were analyzed by nested, three-way ANOVA with maternal treatment, offspring diet, and offspring sex as the fixed effects and with dam considered a random effect. The ΔΔcycle threshold values were compared for the real-time RT-PCR data analysis. To comply with the normality assumption, the Δcycle threshold values for liver *Insr* were transformed to log scale, and the square root transformation was used for the Δcycle threshold values for liver *Ppara*.

Results

Energy Balance in Mice Exposed to Gestational Diabetes in Utero

Prenatal GDM exposure increased offspring obesity risk. Offspring weights (Fig. 1) were not different when they were maintained on the CD. However, when fed a HFHS diet, the offspring of GDM dams weighed significantly more than offspring of CON ($p=0.0002$) fed the HFHS diet, independent of offspring sex. Additionally, exposure to maternal glucose intolerance resulted in increased adiposity on the CD in both male and female

offspring of GDM dams at 4, 12, and 20 weeks of age (p=0.001) (Fig. 2). Thirty week old offspring fed the HFHS but not the CD also had higher body fat percentages than offspring of CON (p=0.026).

To determine whether this energy imbalance resulted from increased energy intake or reduced energy expenditure, food intake, locomotor activity, and total energy expenditure were measured in metabolic cages over a three-day period each time at 4 (juvenile), 12 (post-pubertal), 20 (young adult) and 28 (middle adult) weeks of age. Offspring food consumption was not different among treatment groups, although offspring of GDM, but not CON, dams reduced their food consumption at 30 weeks when fed the HFHS diet (p=0.0075) (Fig. 3a-d). Energy expenditure was not different among treatment groups (Fig. 3e-h.), and there were no major differences observed in distance travelled (Supplemental Figure 1).

Lipid Metabolism in Mice Exposed to Gestational Diabetes in Utero

Next, we explored the hypothesis that increased adiposity in offspring of GDM dams was due not to overall energy imbalance, but rather to differences in the utilization and storage of lipids. Metabolic cages were used to determine respiratory quotient (RQ), the ratio of CO2 produced to O2 consumed, which reflects substrate utilization. An RQ close to 1 reflects pure carbohydrate oxidation, while ratios close to 0.7 reflect pure fatty acid oxidation. RQ were not different between the offspring of GDM and CON dams at 4 and 12 weeks, but in offspring of CON dams, RQ decreased after 4 weeks (p<0.0001) while in offspring of GDM dams it did not (Fig. 4). In male offspring at 20 weeks RQ (p=0.01) maternal glucose intolerance resulted in higher RQs (Fig 4a,c). At 28 weeks, offspring of

GDM dams also had higher RQs ($p=0.004$) on both diets independent of sex, but in separate pairwise comparisons, the difference was only significant in males ($p<0.0001$).

Fatty acid oxidation in the liver involves the action of major transcriptional regulator SREBP1C (Fig. 5). Steady-state mRNA levels of SREBPC1c and other genes related to beta oxidation were not changed in the liver, suggesting that the increase in adiposity in these animals attributed to decreased fatty acid oxidation is not mediated by these gene transcripts in the liver.

The steady-state mRNA levels of genes that facilitate fatty acid uptake were elevated in subcutaneous adipose tissue of adult male offspring (Fig. 6). The mRNA levels of *Pparg* ($p=0.013$) *and Adipoq* ($P=0.002$) were elevated in 31 week old CD-fed male offspring of GDM dams, while 31 week old male offspring of GDM dams had higher mRNA levels of *Lpl* ($p=0.047$) on both diets. There was some indication of an increase in *Pck1* mRNA levels in male and female 31 week old CD-fed offspring of GDM dams compared to controls, though it was not significant ($p=0.058$).

At 19 weeks, there were no differences in serum leptin or triglyceride concentrations (Fig. 7a, c). At 31 weeks, leptin levels were elevated in female offspring of GDM dams fed the HFHS diet ($p=0.0005$) (Fig. 7b). Liver triglycerides were not different (Fig. 7e, f), and serum triglycerides were numerically lower in offspring of GDM dams at 31 weeks on either diet, but not significantly different ($p=0.052$) (Fig 7d).

Glucose Tolerance, Insulin and its Receptor in Mice Exposed to Gestational Diabetes in Utero

Thirty-one week old male offspring of GDM dams exhibited higher mRNA levels of *Insr* on both diets in the liver ($p=0.033$) (Fig. 5a) and in subcutaneous fat ($p=0.001$) (Fig 6a). There were no differences in offspring insulin concentrations as a result of maternal GDM, and glucose tolerance was not different among the treatment groups. Males had higher insulin than females at 19 weeks ($p=0.0245$) and at 30 weeks ($p<0.0001$) (Fig. 8a, b). Males also had higher blood glucose over time when compared to females at every time-point measured ($p<0.0001$) (Fig 8c-h). HFHS feeding also elevated blood glucose in the offspring, regardless of maternal treatment or sex ($p<0.0001$).

Discussion

Epidemiological studies clearly demonstrate increased risks of obesity and insulin resistance in the offspring of pregnancies complicated by maternal diabetes [65, 278, 279, 281]. In such studies, GDM is often accompanied by maternal obesity or preexisting diabetes, which also impact offspring health [117, 302, 303]. By using an animal model, we have been able to separately examine the impact of maternal glucose intolerance, limited to gestation, without maternal obesity. While the prevalence of GDM is higher among obese women [304], about a third of women diagnosed with GDM are lean. Due to the absence of typical risk factors for GDM, this population is not widely studied [305]. Studying this population is not only useful from a clinical perspective [306], but also from a mechanistic perspective, particularly when combined with data from other models. Taken together, our findings suggest that maternal glucose intolerance of mid-

late pregnancy alone increases offspring risk of obesity without impairing offspring insulin sensitivity.

Prenatal GDM Exposure Increases Adiposity

The most striking finding of this study is that *in utero* exposure to maternal glucose intolerance promotes fat accumulation in offspring. This fat accumulation does not seem to result from an imbalance between energy intake and energy output, but rather from alterations in lipid metabolism. First, changes in gene expression in subcutaneous fat are suggestive of greater sensitivity to insulin stimulation of lipid storage. There were increased levels of *Insr, Lpl, Pparg and Adipoq* mRNA in subcutaneous fat of adult male offspring of GDM dams. *Adipoq* enhances whole body insulin action, and *Lpl* and *Pparg* promote insulin action in the adipose tissue. PPARG increases the expression and secretion of adiponectin [307], and also promotes adipogenesis, protecting against lipid overload in other places [308, 309]. Adiponectin expression improves whole body insulin sensitivity, partly decreasing triglyceride content in muscle and liver in obese mice [310, 311]. Insulin acts through its receptor to upregulate adipose lipoprotein lipase [312], and adipose tissue takes up fatty acids via the action of LPL [313]. Thus, working in tandem, *Pparg, Adipoq*, *Insr*, and *Lpl*, promote insulin action in adipose tissue, increasing adipose tissue lipid accumulation and GLUT4 mediated glucose uptake, thereby preventing ectopic fat storage and the resulting insulin resistance in other peripheral tissues [314].

Second, increased RQ measurements at 20 and 28 weeks suggest that the increase in adiposity in the offspring of GDM dams is also due to a preference for carbohydrate utilization for energy over fatty acids. However, genes related to fatty acid oxidation

were not decreased in the liver. Protein quantification or isotope-labelled analysis of fatty acid oxidation might clarify the role of hepatic beta oxidation in mediating these differences in RQ. Alternatively, beta oxidation elsewhere in the body might be reduced.

"Healthy Fat"?

There is a well-established relationship between obesity and insulin resistance [315-317]. Increased adiposity leads to inflammatory responses that interfere with insulin signaling, promoting insulin resistance, and adipose tissue lipolysis, and lipid accumulation in other tissues [318]. For example, feeding mice a high fat diet for 10 weeks promotes tissue inflammation and insulin resistance [319]. Early animal studies have also shown that the presence of inflammatory cytokines in adipose tissue disrupt insulin signaling [320]. Inhibition of insulin action in adipose tissue leads to uncontrolled lipolysis, increasing the triglycerides in the bloodstream and in peripheral tissues, such as the liver [321, 322]. Ectopic deposition of lipids in the liver promotes the development of NAFLD and liver-specific insulin resistance, which prevents gluconeogenesis inhibition, leading to high blood glucose [153].

In the present study, we found no evidence of these further consequences of adiposity in offspring of GDM dams. There was no evidence of inflammation, as inflammatory markers *Tnfa* and *Il6* in adipose tissue were not elevated. Neither serum nor liver triglycerides were higher in offspring of GDM animals. Fasting insulin also wasn't higher, and the ability to clear glucose was unaffected in offspring of GDM dams, even after they became more obese than offspring of CON dams following HFHS feeding. It should be noted, however, that offspring were only followed through 31 weeks of age,

after just 8 weeks of HFHS challenge. It is possible that further aging or a more prolonged HFHS feeding may have led to more of the negative consequences of obesity.

Several changes in transcript levels observed in the offspring of GDM mice may contribute to protecting their insulin sensitivity, despite altered lipid metabolism. For example, we observed an increase in insulin receptor mRNA in the liver and adipose tissue of adult male offspring that persisted when the animals were placed on HFHS feeding. In addition to insulin receptor, levels of mRNA encoding adiponectin, which has been shown to prevent insulin resistance in the liver, [323] were elevated. Yamauchi et al. showed that adiponectin ameliorates insulin resistance and hyperglycemia in *ob/ob* mice, which display obesity, diabetes and other facets of metabolic syndrome [324]. Adipose overexpression of phosphoenolpyruvate carboxykinase (PEPCK) activity can lead to obesity without insulin resistance [325], as it facilitates fatty acid re-esterification and promotes fat storage [326]. We measured adipose levels of *Pck1* transcript in our animals to determine if this was a mechanism through which they maintained glucose tolerance in spite of increased adiposity. While not quite statistically significant ($p=0.0579$), there is some evidence that the levels of *Pck1* in subcutaneous fat are upregulated to protect the offspring against insulin resistance.

Collectively, these findings point to a "healthy fat" phenomenon [327, 328], with the offspring of GDM dams having no excess insulin resistance despite high fat mass. The gene expression data suggest that the adipose tissue exhibits hyperplastic rather than hypertrophic propensities. Hypertrophy, or increase in adipocyte size, is accompanied by increased expression inflammatory cytokines such as IL-6 in [329], which is not elevated in the offspring of our GDM dams. In humans, adipocyte size has been positively

correlated with insulin resistance and decreased adiponectin gene expression [330, 331]. Hyperplasia, an increase in adipocyte number, or adipogenesis, is a mechanism by which adipose tissue mass can increase without hypertrophy. Hyperplasia is facilitated by an increase in adipogenic transcription factors such as PPARG [332] and prevents inflammation and insulin resistance [318]. It has been shown that adipose tissue can adapt protective mechanisms to maintain insulin sensitivity in spite of high adiposity and obesity [333]. In humans, insulin sensitive obesity is associated with differential adipose gene expression, including increased expression of adiponectin and a higher ratio of subcutaneous to visceral fat [334, 335]. The storage of fats in the adipose tissue may itself prevent ectopic fat storage and resultant lipotoxicity [336]. Adipose tissue accretion has been previously shown to positively affect metabolism, with mouse models that lack the capacity to promote adipogenesis developing hepatic steatosis and glucose intolerance [337]. This suggests that offspring of lean vs obese GDM mothers may exhibit increased adiposity while remaining metabolically healthy, without an increased risk for diabetes.

Potential Role of Leptin

One factor protecting offspring may have been *in utero* exposure to elevated concentrations of maternal leptin. We and others have previously shown that in dams with normal glucose tolerance, hyperleptinemia protects offspring from developing insulin resistance [231, 301, 338]. The GDM model dams exhibit hyperleptinemia despite normal pregnancy weights [109]. It is not clear whether this hyperleptinemia is a direct result of the HFHS diet composition, or an indication of leptin resistance [339]. There is also not yet a full understanding of the leptin resistance associated with obesity or with normal pregnancy [267, 268]. Thus, additional study is needed to understand how

maternal leptin protects offspring insulin sensitivity, and whether this might differ in obese and lean GDM.

Broader Relevance

Overall our results suggest that some of the well-documented impacts of GDM on offspring may not occur in lean GDM. This underscores the importance of separately studying the facets of GDM to determine how offspring outcomes are conferred.

The pathophysiology of lean GDM may be somewhat different from obese GDM, with women displaying lower glucose-stimulated insulin secretion, similar to our mouse model [23]. Our data suggest that maternal glucose intolerance in lean mothers does not promote offspring insulin resistance and glucose intolerance, but does elevate adiposity. Offspring insulin resistance may instead be a result of factors like glucose intolerance prior to, or in early pregnancy, or of obesity in combination with GDM.

Mouse studies of maternal obesity with hyperglycemia support this. Nivoit et al showed that diet induced obesity led to offspring with insulin resistance and increased adiposity [340], and Jungheim et al fed dams a 35.8% fat g/g diet that resulted in offspring obesity as well as alterations in offspring glucose tolerance [157]. Unfortunately, maternal obesity studies do not always report maternal glucose tolerance [119] making it difficult to determine whether offspring outcomes can be attributed to maternal obesity alone, or to maternal hyperglycemia as well. One study showed that in humans, increased fat mass is only present in offspring of mothers with both maternal obesity and GDM versus maternal obesity alone [302], consistent with the present finding that increased offspring adiposity is caused by maternal glucose intolerance.

Here, the effects of maternal glucose intolerance alone appear to be limited to adiposity, and offspring may even be protected against the development of type 2 diabetes through increased adipose storage of fat. This is mediated by a preferential utilization of carbohydrates to lipids in the offspring of GDM dams. In adult male offspring the increased expression of genes related to fat storage in subcutaneous adipose tissue appears to contribute to the increased adiposity. The sex-specific gene expression differences suggest that the programming of increased adiposity in the offspring is partly modulated by offspring sex hormones.

Collectively, our data suggest that while maternal glucose intolerance alone does not alter offspring glucose tolerance, GDM even in normal weight mothers affects offspring adiposity, and promotes obesity on a western obesogenic diet.

Gene of Interest	Primer	Sequence (5'-3')
Actb	Sense	GATGACCCAGATCATGTTTGAGACC
	Antisense	AGATGGGCACAGTGTGGGTGA
Srebp1c	Sense	TGGTGGGCACTGAAGCAAA
	Antisense	GCAAGAAGCGGATGTAGTCGAT
Insr	Sense	CCACCAAGAACTCGTGAAAGG
	Antisense	TGCACGCAGGAAAGAACCT
Gapdh	Sense	TGCACCACCAACTGCTTAGC
	Antisense	GGCATGGACTGTGGTCATGAG
Hprt	Sense	TGACACTGGCAAAACAATGCA
	Antisense	GGTCCTTTTCACCAGCAAGCT
Lep	Sense	GTGAAATGTCATTGATCCTGGTG
	Antisense	GTGGCTTTGGTCCTATCTGTC
Pck1	Antisense	GCGAGTCTGTCAGTTCAATACC
	Sense	GGATGTCGGAAGAGGACTTTG
Cpt1a	Antisense	AGTGTCCATCCTCTGAGTAGC
	Sense	CAGCAAGATAGGCATAAACGC
Acaca	Antisense	GTCCAACAGAACATCGCTGA
	Sense	AACATCCCCACGCTAAACAG
Ppara	Antisense	TGCAACTTCTCAATGTAGCCT
	Sense	AATGCCTTAGAACTGGATGACA
Pgc1a	Antisense	TCGCTCAATAGTCTTGTTCTCAA
	Sense	AGAAGTCCCATACACAACCG
Il6	Antisense	TCCAGTTGCCTTCTTGGGAC
	Sense	AGTCTCCTCTCCGGACTTGT
Ucp1	Antisense	ACTGCCACACCTCCAGTCATT
	Sense	CTTTGCCTCACTCAGGATTGG
Adipoq	Antisense	GCAGGATTAAGAGGAACAGGAG
	Sense	TGTCTGTACGATTGTCAGTGG
Tnfa	Antisense	TCTTTGAGATCCATGCCGTTG
	Antisense	AGACCCTCACACTCAGATCA
Pparg	Sense	TGCAGGTTCTACTTTGATCGC
	Antisense	CTGCTCCACACTATGAAGACAT
Lpl	Sense	CTTCTGCATACTCAAAGTTAGGC
	Antisense	CTAGACAACGTCCACCTCTTAG

Table 1. Primer Table

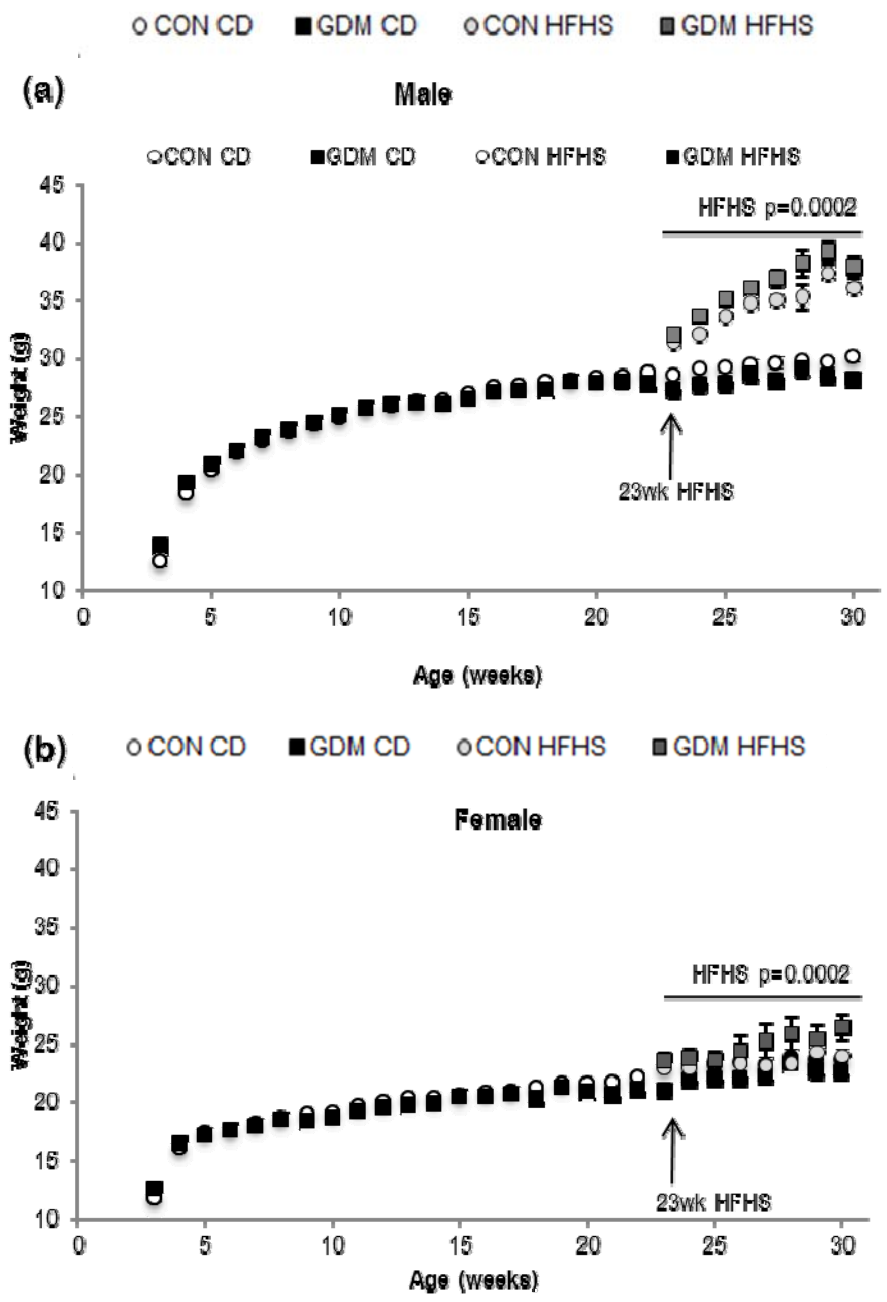

Figure 1: Offspring weights. Male (a) and Female (b) offspring weights measured weekly from 3 – 31 weeks of age. Offspring were maintained on a CD except for a subset of offspring placed on HFHS feeding at 23 weeks of age. Sample size ($offspring_{dam}$): Male CD ($CON11_{21}$, GDM 6_{13}) Male HFHS ($CON11_{11}$, GDM 5_{5}) Female CD ($CON12_{17}$, GDM 7_{10}) Female HFHS ($CON7_{7}$, GDM 6_{6}). Data are represented as mean ± SEM.

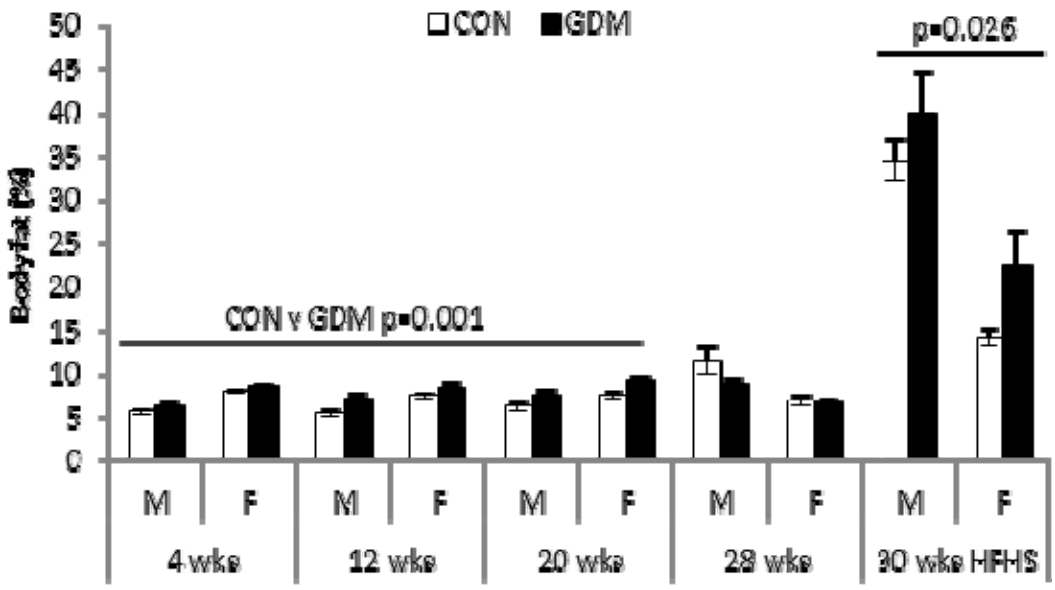

Figure 2: Body fat percentage measured by Magnetic Resonance Imaging in offspring at 4, 12, 20 and 28 weeks of age on CD and at 30 weeks on HFHS. Sample size ($offspring_{dam}$): Male CD (4wk $CON9_9$, GDM 5_5, 12wk $CON10_{10}$, GDM 6_6, 20wk $CON10_{10}$, GDM 6_6, 28wk $CON10_{10}$, GDM 6_6) Male HFHS ($CON10_{10}$, GDM 5_5) Female CD (4wk $CON9_9$, GDM 5_5, 12wk $CON9_9$, GDM 7_7, 20wk $CON9_9$, GDM 7_7, 28wk $CON9_9$, GDM 6_6) Female HFHS ($CON7_7$, GDM 6_6). Data are represented as mean ± SEM.

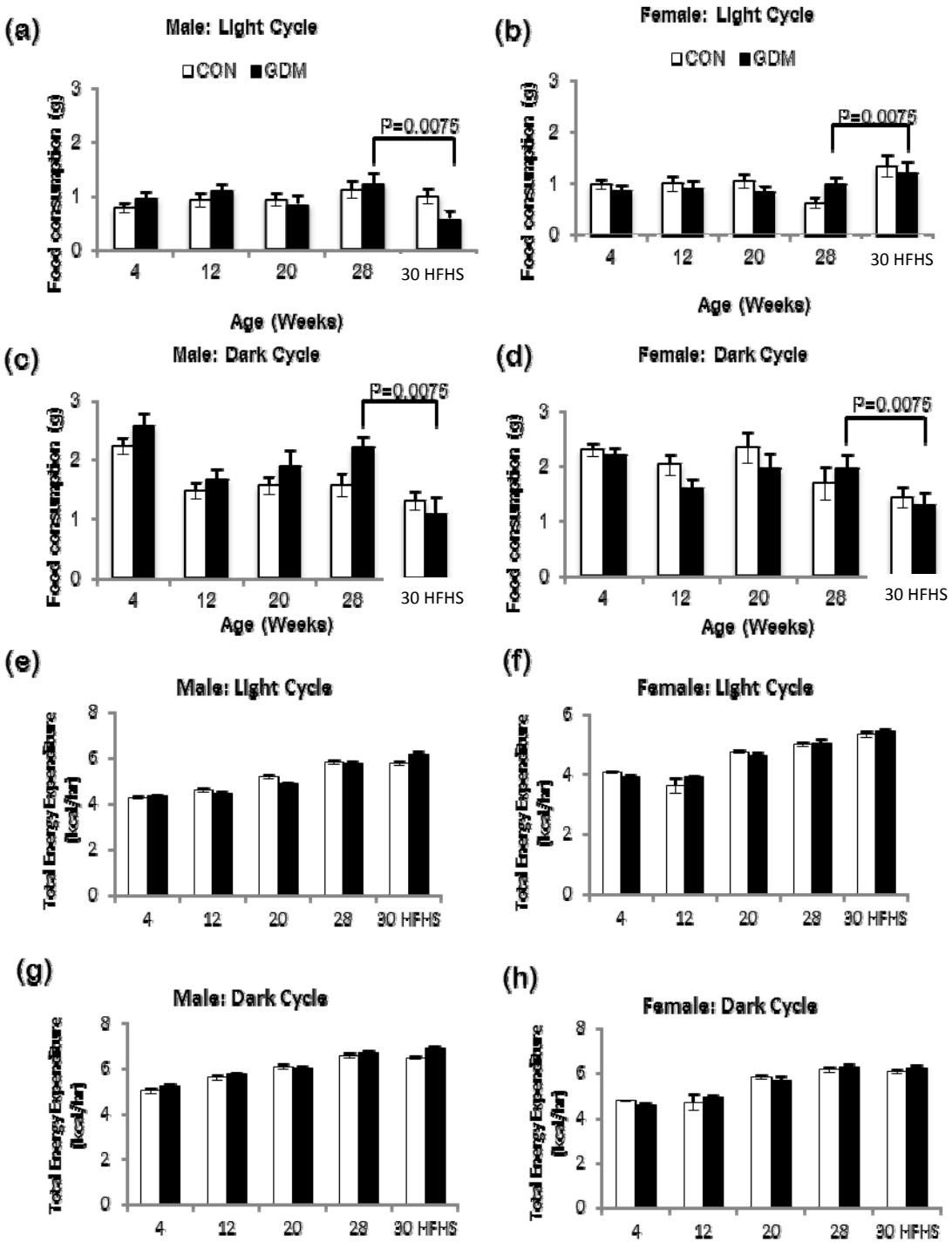

Figure 3: Food consumption (a-d) and energy expenditure (e-h) assessed in offspring at 4, 12, 20 and 28 weeks of age on CD and at 30 weeks on HFHS. Sample size ($offspring_{dam}$): Male CD (4wk $CON9_9$, GDM 5_5, 12wk $CON10_{10}$, GDM 6_6, 20wk $CON10_{10}$, GDM 6_6, 28wk $CON10_{10}$, GDM 6_6) Male HFHS ($CON10_{10}$, GDM 5_5) Female CD (4wk $CON9_9$, GDM 5_5, 12wk $CON9_9$, GDM 7_7, 20wk $CON9_9$, GDM 7_7, 28wk $CON9_9$, GDM 6_6) Female HFHS ($CON7_7$, GDM 6_6). Data are represented as mean ± SEM.

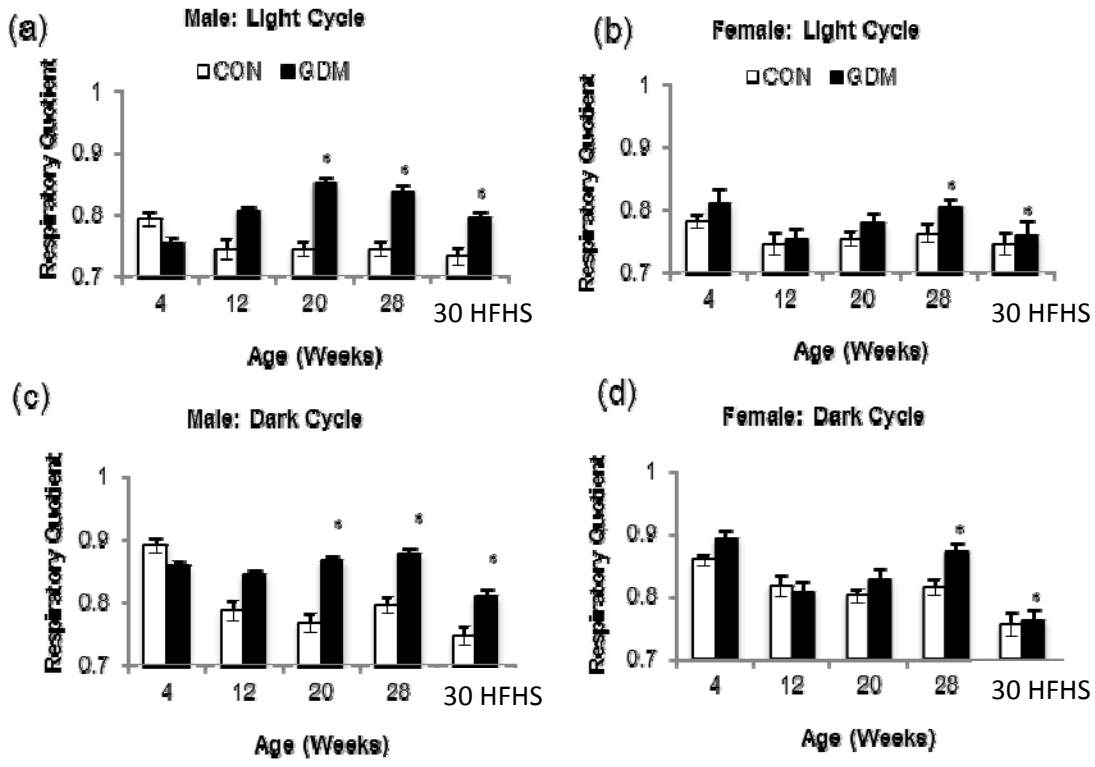

Figure 4: Respiratory quotient assessed in male (a, c) and female (b, d) offspring at 4, 12, 20 and 28 weeks of age on CD and at 30 weeks on HFHS. Sample size ($offspring_{dam}$): Male CD (4wk $CON9_9$, GDM 5_5, 12wk $CON10_{10}$, GDM 6_6, 20wk $CON10_{10}$, GDM 6_6, 28wk $CON10_{10}$, GDM 6_6) Male HFHS ($CON10_{10}$, GDM 5_5) Female CD (4wk $CON9_9$, GDM 5_5, 12wk $CON9_9$, GDM 7_7, 20wk $CON9_9$, GDM 7_7, 28wk $CON9_9$, GDM 6_6) Female HFHS ($CON8_8$, GDM 6_6).
* indicates significantly different than control ($p < 0.05$). Data are represented as mean ± SEM.

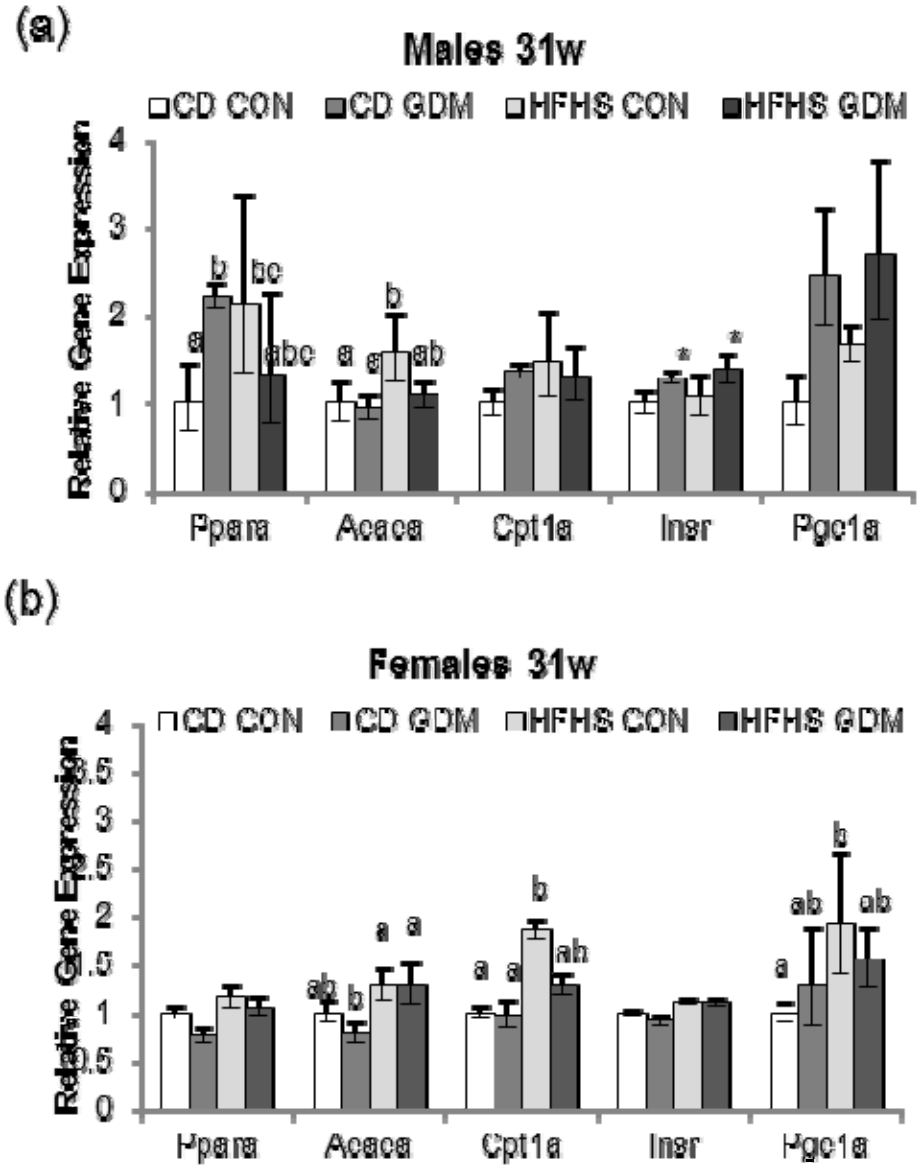

Figure 5: Steady state mRNA levels of genes related to beta oxidation in male (a) and female (b) offspring liver assessed at 31 weeks of age. Sample size ($offspring_{dam}$): Male CD (19wk $CON7_7$, GDM 4_6, 31wk $CON10_{10}$, GDM 6_6) Male HFHS ($CON5_5$, GDM 5_5) Female CD (19wk $CON5_5$, GDM 3_3, 31wk $CON12_{12}$, GDM 6_6) Female HFHS ($CON6_6$, GDM 6_6). Data are represented as mean fold change relative to control group mean on chow diet. Error bars represent range of fold changes based on SEM of ΔΔCt. Columns with different superscripts are significantly different ($p < 0.05$). * indicates significantly different than control overall, but no difference in pairwise comparisons ($p < 0.05$).

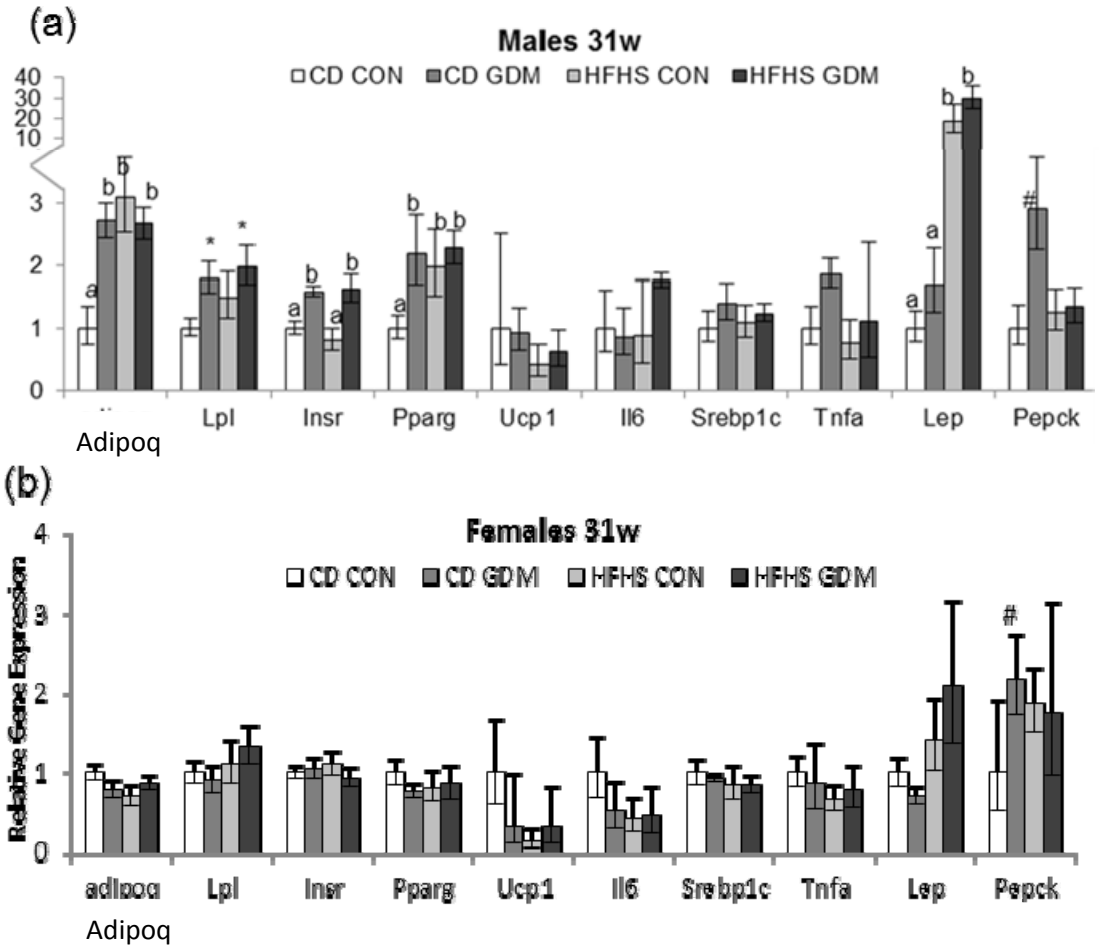

Figure 6: Steady state mRNA levels of genes related to fatty acid synthesis in male (a) and female (b) offspring subcutaneous adipose tissue assessed at 31 weeks of age. Sample size ($offspring_{dam}$): Male CD (19wk $CON8_8$, GDM 4_6, 31wk $CON6_6$, GDM 5_5) Male HFHS ($CON5_5$, GDM 5_6) Female CD (19wk $CON5_5$, GDM 3_3, 31wk $CON6_6$, GDM 6_6) Female HFHS ($CON6_6$, GDM 6_6). Data are represented as mean fold change relative to control group mean on chow diet. Error bars represent range of fold changes based on SEM of ΔΔCt. Columns with different superscripts are significantly different ($p < 0.05$). * indicates significantly different than control ($p < 0.05$). # ($p<0.06$).

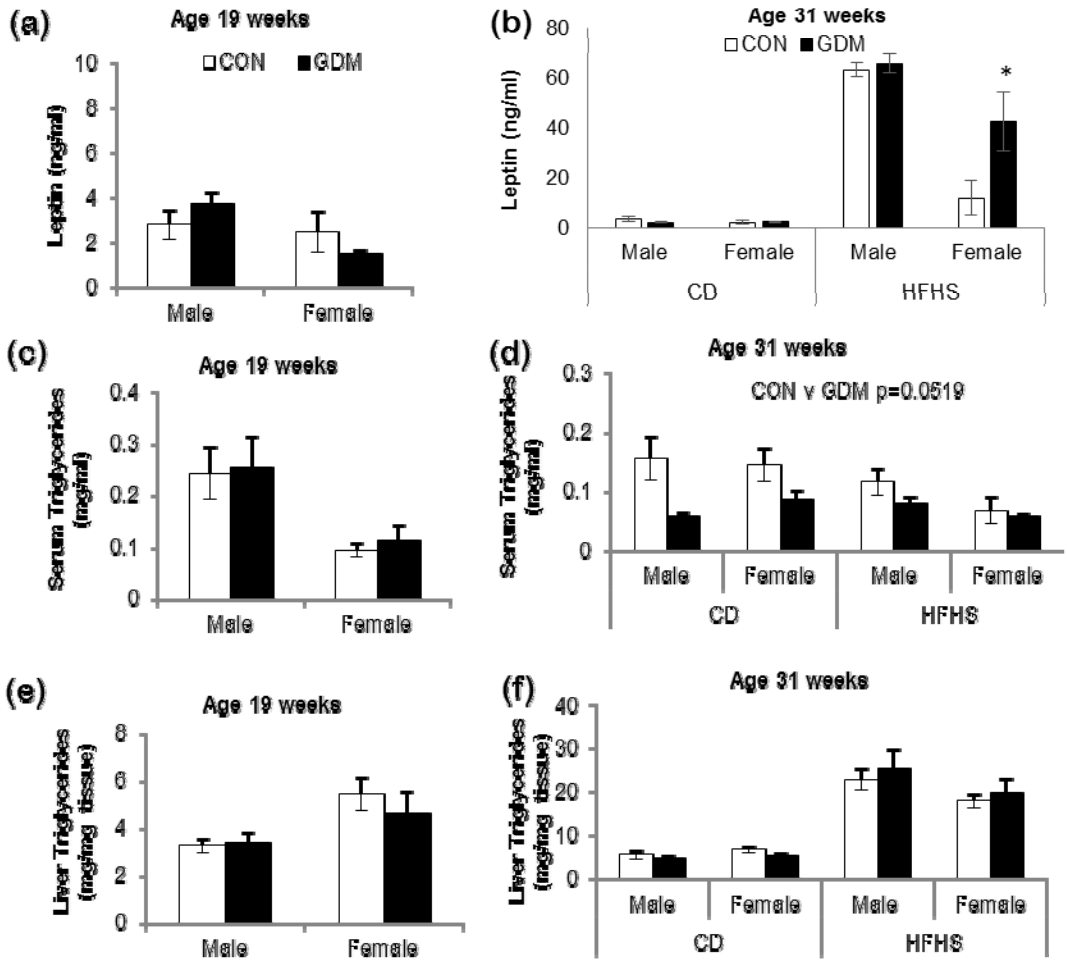

Figure 7: Serum leptin (a,b) and triglycerides (c,d) and liver triglycerides (e,f) were measured in offspring at 19 (a,c,e) and 31 (b,d,f) weeks of age. Sample size ($offspring_{dam}$) Serum leptin: Male CD (19wk $CON6_6$, GDM 4_7, 31wk $CON11_{11}$, GDM 6_6) Male HFHS ($CON11_{11}$, GDM 5_5) Female CD (19wk $CON5_5$, GDM 3_3, 31wk $CON11_{11}$, GDM 7_7) Female HFHS ($CON7_7$, GDM 5_5).
Serum triglycerides: Male CD (19wk $CON10_{10}$, GDM 4_7, 31wk $CON11_{11}$, GDM 6_6) Male HFHS ($CON11_{11}$, GDM 5_5) Female CD (19wk $CON5_5$, GDM 3_3, 31wk $CON11_{11}$, GDM 7_7) Female HFHS ($CON7_7$, GDM 6_6).
Liver triglycerides: Male CD (19wk $CON9_9$, GDM 4_7, 31wk $CON11_{11}$, GDM 6_6) Male HFHS ($CON11_{11}$, GDM 5_5) Female CD (19wk $CON5_5$, GDM 3_3, 31wk $CON12_{12}$, GDM 7_7) Female HFHS ($CON7_7$, GDM 6_6) * indicates significantly different than control ($p < 0.05$). Data are represented as mean ± SEM.

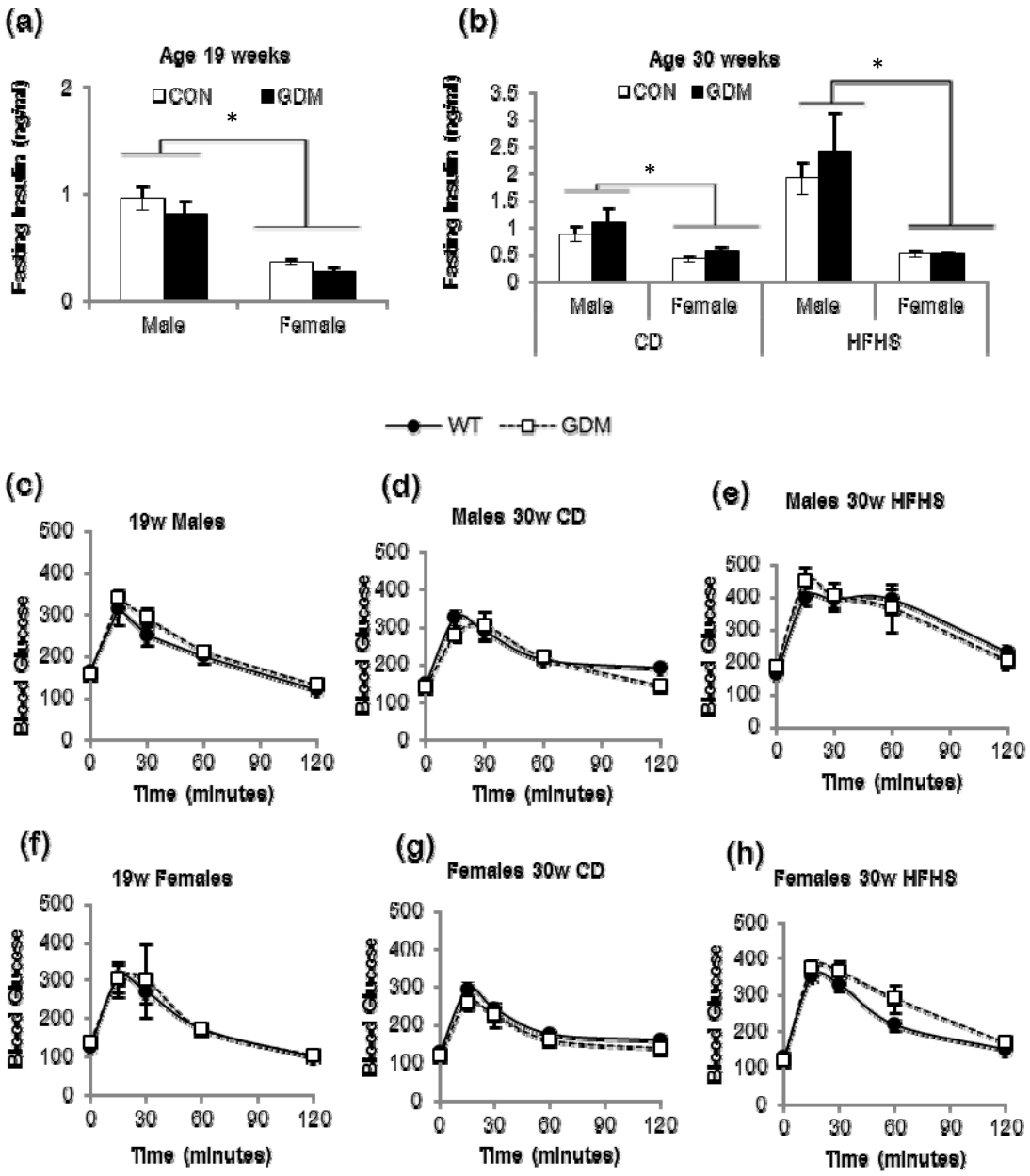

Figure 8: Glucose control in offspring. Serum fasting insulin measures at 19 (a) and 30 (b) weeks in offspring. Glucose tolerance tests in 19 week old male (c) and female (f) offspring, and in 30 week old male (d,e) and female (g,h) offspring on CD and HFHS. Sample size ($offspring_{dam}$):
Serum insulin: Male CD (19wk $CON10_{10}$, GDM 4_7, 31wk $CON11_{11}$, GDM 6_6) Male HFHS ($CON11_{11}$, GDM 5_5) Female CD (19wk $CON5_5$, GDM 3_3, 31wk $CON12_{12}$, GDM 7_7) Female HFHS ($CON7_7$, GDM 5_5).* indicates sex difference ($p < 0.05$). Data are represented as mean ± SEM.

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VITA

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