The effects of a high-fat diet and bacterial lipopolysaccharide (LPS) induced inflammation on pregnancy and fetal development in mice

by

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Abstract

Inflammation during pregnancy can disturb maternal tolerance of the fetus. In mice, maternal high-fat diet (HFD) induces inflammation without pregnancy complications. I hypothesised that an additional inflammatory insult would exacerbate the immune response, leading to serious complications. To test this, I developed a HFD/LPS model, where female mice were fed a high-fat or low-fat diet prior to mating, and then treated with either bacterial lipopolysaccharide (LPS), an inflammatory stimulant, or a control. Diet, LPS or a diet-LPS interaction had no effect on fetal and placental parameters or maternal levels of TNF-α, an inflammatory marker ($p>0.05$). Furthermore, fetal and placental parameters did not differ between HFD mice that were prone or resistant to weight-gain. While diet or a diet-LPS interaction did not affect pregnancy, LPS treatment alone caused complete fetal loss in some mice ($p<0.05$). These findings suggest that LPS does not exacerbate the inflammatory effects of HFD in pregnant mice.

Keywords: pregnancy; inflammation; high-fat diet; lipopolysaccharide

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Table of Contents

List of Tables

List of Figures

List of Acronyms

ANOVA	Analysis of variance
CAF	Cafeteria diet
CCAC	Canadian Council on Animal Care
ELISA	Enzyme-linked immunosorbent assay
GD	Gestational day
HFD	High-fat diet
IFN	Interferon
IL	Interleukin
IUGR	Intra-uterine growth restriction
LFD	Low-fat diet
LPS	Lipopolysaccharides
LT	Lymphotoxin
NF-κB	Nuclear factor-κappa B
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
UACC	University Animal Care Committee
uNK	Uterine natural killer
WD	Western diet
WGP	Weight-gain prone
WGR	Weight-gain resistant

Chapter 1.

Introduction

1.1. The immune system during pregnancy

1.1.1. Stages of pregnancy

The maternal immune system is carefully regulated during the gestational period, allowing tolerance of the semi-allogeneic fetus, while at the same time protecting the fetus and mother from external pathogens. At the uterus, there are specific immune cell populations that are compositionally and functionally fine-tuned to facilitate events of a normal healthy pregnancy. For most of the gestational period, these immune players work to maintain an anti-inflammatory environment to facilitate fetal growth and development. During this stage, aberrant and unregulated inflammation could be detrimental, and has been implicated in serious pregnancy complications including pre-eclampsia, pre-term labour, spontaneous abortion and stillbirths. These pregnancy complications are prevalent worldwide and contribute significantly to maternal and fetal morbidity and mortality (Regan and Rai, 2000; Sibai, et al. 2005; Khan et al. 2006; Blencowe, et al. 2012; Liu et al, 2016). Given these findings, there has been increasing interest in the topic of maternal immunology during pregnancy.

When the body is subjected to an external insult, for example a pathogen, its first response is inflammation: an infiltration of immune cells and chemical mediators at the site of exposure, to facilitate removal of the external stimulus and promote subsequent healing. During pregnancy, the fetus and placenta express feto-paternal antigens (Petroff 2011), and therefore should be treated as ‘external stimuli’ and rejected by the mother. However, this does not occur. Instead, trophoblast cells, which surround the early blastocyst and continue to form parts of the placenta and fetal membranes, are capable of inducing functional changes in maternal immune cells to benefit fetal development (Mor et al. 2011).

Mor and colleagues (2010; 2011; 2017) proposed that the maternal immune system is not static throughout pregnancy, but rather, changes the nature of its

responses depending on the local environment. Accordingly, they categorized pregnancy into three distinct immunological phases – implantation, fetal growth and development, and parturition. Implantation and parturition are facilitated by pro-inflammatory responses, while the remaining gestational period of fetal growth and development is characterized by an anti-inflammatory uterine environment.

During implantation, the blastocyst attaches to the endometrial lining of the uterus, followed by the formation of the placenta. A crucial event in placental development is the remodeling of the uterine spiral arteries (Pijnenborg et al. 1980; 1983). Trophoblast cells invade and replace the smooth muscle lining of the spiral arteries in the uterine wall, effectively remodeling them to have larger diameters and higher capacitance (Whitley and Cartwright, 2010). This is necessary for the efficient transfer of gases and nutrients between fetus and mother. Insufficient spiral artery remodeling can lead to placental ischemia (inadequate blood supply), which results in smaller placentas with impaired function (Roberts and Escudero, 2012; Roberts 2014). The events of trophoblast invasion and spiral artery remodeling are largely mediated by the production of cytokines, angiogenic growth factors and matrix metalloproteinases by maternal immune cells, namely uterine natural killer cells (uNK) and macrophages (Hanna et al. 2006; Lash et al. 2006; Robson et al. 2012; Wallace et al. 2012; Faas et al. 2014; Fraser et al. 2015). Vascular remodeling also results in considerable amounts of tissue damage and turnover, which is facilitated by the clearing out of dead cells and debris by phagocytic macrophages (Mor and Abrahams 2003; Abrahams et al. 2004).

The period after initial implantation and placentation is characterized by fetal growth and development. An anti-inflammatory environment is crucial during this stage, to prevent the maternal immune system from attacking the semi-allogeneic fetus. This is primarily maintained by a dominance of anti-inflammatory cytokines, which are produced not only by immune cells, but also by cells of the trophoblast, decidua and amnion (Morelli et al. 2015). Bränn et al. (2019) recently confirmed that anti-inflammatory markers in maternal blood plasma were elevated during the gestational period compared to post-partum. During this time there is also an increase in regulatory T cells (T_{regs}), which suppress proliferation and activity of effector T cells (Sasaki et al. 2004; Bettelli et al. 2006; Crome et al. 2010; Saito et al. 2010). Additionally, dendritic cells, which act as antigen presenting cells, show altered interactions with T cells, minimizing exposure of T cells to fetal antigens (Collins et al. 2009). The events of parturition at the end of

pregnancy are facilitated by a pro-inflammatory uterine environment. There is an influx of macrophages into the uterus and an increase in pro-inflammatory mediators, which is associated with regular contractions of the uterus and the expulsion of the fetus and placenta (Shynlova et al. 2008, 2013; Hamilton et al. 2012).

1.1.2. The roles of IL-10 and TNF-α

Cytokines, or cell-signaling proteins, act as important chemical mediators of the immune system, and have important roles to play in immunity during pregnancy. Favorable pregnancy outcomes generally depend on the maintenance of a balance between cytokines that have pro-inflammatory (stimulatory) and anti-inflammatory (immunosuppressive) actions. Interleukin (IL)-10, belonging to the interleukin group of cytokines, is widely studied for its immunosuppressive properties and is fundamental in maintaining the cytokine balance during pregnancy. By regulating the production of pro-inflammatory cytokines, IL-10 is key in maintaining maternal tolerance of the fetus (Robertson et al. 2007; Mobini et al. 2016; Busse et al. 2019). IL-10 can also induce cell surface expression of HLA-G (a major histocompatibility molecule) on trophoblast cells, which protect them from lysis by maternal uNK cells (Soderstrom et al. 1997; Moreau et al. 1999). Additionally, IL-10 can also induce the development of T_{regs} (Mobini et al. 2016). Early in pregnancy, IL-10 may regulate trophoblast invasion and prevent over-invasiveness (Sharma et al. 2016). As pregnancy progresses, increasing levels of this cytokine could gradually decrease the invasiveness of trophoblast cells (Pang et al. 2008).

Interestingly, one of the main factors that can suppress the production of IL-10 and lead to various complications is an increase in the levels of a pro-inflammatory cytokine, tumor necrosis factor (TNF)-α. TNF-α is produced both by immune cells as well as placental and trophoblast cells (Chen et al. 1991; Yang et al. 1993; Steinborn et al. 1996), and plays key roles in initiation of the cytokine cascade, activation of effector immune cells and cell apoptosis (Idriss and Naismith 2000). Interestingly, events of early pregnancy are mediated by this cytokine – blastocyst implantation was found to rely on the production of TNF-α along with IL-1β (Salama et al. 2020). TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) could also be produced by trophoblast cells to aid in apoptosis of smooth muscle cells during spiral artery remodeling (Keogh et al. 2007; Whitley and Cartwright 2009). At the same time, in-vitro studies have shown that TNF-α

can induce apoptosis and decrease proliferation of trophoblast cells, thereby limiting their invasive capacity (Pijnenborg et al. 2000; Reister et al. 2001; Bauer et al. 2004; Kilani et al. 2007; Wen et al. 2018). As pregnancy progresses, unregulated levels of pro-inflammatory cytokines, notably TNF-α, have been implicated in a range of unfavorable events. Clinical cases of spontaneous miscarriage, intra-uterine growth restriction (IUGR), premature rupture of fetal membranes and pre-eclampsia were associated with elevated levels of TNF-α (Holcberg et al. 2001; Arslan et al. 2004; El-far et al. 2009; Azizieh and Raghupathy 2015).

1.2. Triggers of inflammation during fetal growth and development

Unregulated inflammatory responses during the gestational period could lead to unfavourable outcomes, as already discussed. This section will focus on two triggers of such inflammation that have been the subject of considerable study in rodents – a high-fat diet and bacterial lipopolysaccharide (LPS).

1.2.1. High-fat diet

In obese humans, there is an increased risk of pregnancy complications such as spontaneous abortion, preterm birth, stillbirths or neonatal deaths, fetal congenital abnormalities and maternal morbidity and mortality (Galtier-Dereure et al. 2000; Guelinckx et al. 2008; Leddy et al. 2008; Ramachenderan et al. 2008; Fitzsimmons et al. 2009; Marshall and Spong 2012; Stubert et al. 2018). It is thought that chronic inflammation in the obese state could be one of the factors responsible for the development of such complications, however the underlying mechanisms are not clearly understood.

There is contradicting evidence regarding whether obese pregnant women display elevated levels of pro-inflammatory cytokines (Verhaeghe et al. 2005; Stewart et al. 2007; Pendeloski et al. 2017; Zembala-Szczerba et al. 2017). Challier et al. (2008) found that placental macrophages obtained at term from obese subjects were higher in number and produced more IL-1, TNF-α and IL-6. However, these results were not replicated by Laskewitz et al. (2019) who found little difference in placental macrophages in obese subjects and lean controls at term. Circulating populations of natural killer cells,

dendritic cells, monocytes and lymphocytes at term do not appear to be altered by maternal obesity (Sureshchandra et al. 2018). In terms of the effects during the first trimester of pregnancy, Perdu et al. (2016) found that obesity altered the number, function and gene expression of uNK cells. Most notable was the over-expression of decorin, which strongly impaired spiral artery remodeling and placental development. Similar results were found by Castellana et al. (2018), who observed that obesity alters uNK cell function and promotes the release of TNF-α.

It appears that data from human studies do not clearly elucidate the inflammatory mechanisms that might be involved in complications of obese pregnancies. There are also certain limitations of studies of obesity in humans, including how diet and lifestyle may act as confounding variables. Therefore, there has been considerable interest in the development of animal models of diet-induced obesity. In humans, obesity is associated with an increase in dietary fat content (Tucker and Kano 1992; Raatz et al. 2017). Similarly, in mice and rats, there is also a linear relationship between obesity and dietary fat intake (Warwick and Schiffman 1992; Boozer et al. 1995). Studies have therefore used a high-fat diet (HFD) in animals to understand the inflammatory basis of various conditions, including pregnancy complications.

A maternal HFD in mice can cause obesity-related conditions including increased adiposity and adipose tissue inflammation, insulin resistance, hypertension, and changes in liver function (Williams et al. 2014; Summerfield et al. 2018). In terms of placental development, HFD-fed mice show restricted blood vessel remodeling, placental hypoxia and irregular placental morphology (Kim et al. 2014; Gohir et al. 2019). Parker et al. (2014) found a reduction in the number of uNK cells and their expression of IFN-γ, a cytokine which supports spiral artery remodeling. HFD can also elevate maternal plasma concentration of some pro-inflammatory cytokines, such as IL-6 and IFN-γ (Kępczyńska et al. 2013), lower the number of surviving litters (Smoothy et al. 2019) and cause various developmental abnormalities in surviving offspring (Williams et al. 2014). In contrast to these results, some studies found no effect of HFD on inflammatory parameters or fetal survival, size and growth in mice (Ingvorsen et al. 2014; Chin et al. 2017; Baltayeva et al. 2020).

1.2.2. Bacterial Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, can induce strong immune responses in hosts (Alexander and Rietschel 2001). LPS is recognized by the TLR4 receptor of the toll-like receptor (TLR) family, leading to the initiation of signaling pathways including NF-κB, which mediates production of several pro-inflammatory cytokines (Lu et al. 2008; Park and Lee 2013). Studies have demonstrated that the administration of LPS alone, without bacterial infection, can induce inflammatory events including the production of cytokines such as TNF-α and IL-6 (Dentener et al. 1993a; 1993b). LPS from common bacterial species can be used in animal models to produce inflammatory effects that are generally reproducible. As a result, it is widely used to study inflammation.

LPS has specifically been used in animal models to study the effects on pregnancy and fetal development, and there is currently an extensive literature on the topic. Table 1.1 provides a comprehensive view of the effects of bacterial LPS on pregnancy and fetal development in different animal models. Effects vary depending on when the LPS is administered and the dose used. It appears that early in pregnancy LPS results in embryonic resorptions (disintegration of embryonic material before organogenesis), abortion and impaired spiral artery remodelling. LPS administration during mid-gestation may result in fetal resorptions (disintegration of fetal material after organogenesis), growth restriction and various skeletal, physiological, behavioural and neurodevelopmental impairments in offspring. In the later stages of pregnancy, LPS largely induces preterm birth. Smaller doses of LPS generally induce inflammatory effects including elevated pro-inflammatory cytokines and altered uNK cell properties. This is accompanied by various developmental impairments. Larger doses can produce drastic effects such as total fetal loss.

Table 1.1. A review of the effects of bacterial lipopolysaccharide (LPS) administered in varying doses and during different gestational days on pregnancy and fetal development.

Animal Model	Dose (in µg) *	Gestational Day of injection	Reported Effects	Reference
Mice	0.1	8	High rate of fetal resorption.	Gendron et al. 1990
Mice	0.2	6.5	Intra-uterine growth restriction in IL-10 deficient mice, no effect in wild type mice.	Murphy et al. 2005
Mice	0.25	17	50% fetal loss in IL-10 deficient mice.	Robertson et al. 2006
Mice	0.4	9.5	Elevated pro-inflammatory cytokines, fetal resorption, decreased fetal weight, reduced number of viable fetuses.	Baek et al. 2019
Mice	0.5	6.5	Total fetal loss in IL-10 deficient mice, Increase in uNK cell cytotoxicity and invasion into placenta. No effect in wildtype.	Murphy et al. 2005
Mice	~0.5	8-12	Elevated pro-inflammatory cytokines, fetal growth restriction & skeletal malformations.	Zhao et al. 2014
Mice	~0.5	8-12	Elevated placental inflammatory cytokines, increased number of dead fetuses, skeletal malformations in pups.	Fu et al. 2014
Mice	~0.9-14	10	Elevated maternal pro-inflammatory cytokines, reduced anxiety in male pups.	Solati et al. 2015
Mice	1-50	0.5	5 µg minimum dose to induce abortion (inhibit implantation of blastocyst), and also results in leukocyte infiltration, hyperplasia of reproductive organs and increase in uterine pro-inflammatory IL-1α	Deb et al. 2004
Mice	1-20	6.5	Increasing abortion with dose	Clark et al. 2003
Mice	~1.1	9	~50% fetal resorption	Leazer et al. 2003
Mice	~1.25	13-17	Growth restriction in males, reduced testosterone production and spermatogenesis in male pups	Wang et al. 2014
Mice	~1.25	15-17	Growth restriction	Liu et al. 2014

Animal Model	Dose (in µg) *	Gestational Day of injection	Reported Effects	Reference
Mice	2	3	100% fetal loss, altered uNK cell cytoxicity	Qi et al. 2016
Mice	~2.5	15	High rate of fetal death, increase in pro-inflammatory cytokines, morphological abnormalities in placenta	Luna et al.2015
Mice	~2.5	15.5	Increase in proinflammatory cytokine expression, structural changes in placenta, intestinal injury in offspring that lasts into adulthood	Fricke et al. 2018
Mice	~2.5	15-17	Growth restriction, fetal death, placental inflammation; VitD3 inhibits observed effects.	Chen et al. 2015
Mice	~2.5-7.5	16-17	Pups with altered behaviours in adulthood	Chlodzinska et al. 2011
Mice	2.5	17	100% fetal loss in IL-10 deficient mice, 50% fetal loss in wildtype mice	Robertson et al. 2006
Mice	5-10	12	Increasing fetal death with increasing dose	Silver et al. 1995
Mice	~7.5	8	Abnormal object recognition behaviour in adult offspring	Coyle et al. 2009
Mice	~10	8	growth restriction, external malformations	Chua et al. 2006
Mice	~10	14.5	Preterm labour, infiltration of leukocytes in uterus, Increase in expression of pro-inflammatory cytokines	Liu et al. 2016
Mice	10	14.5	~50% live fetuses per pregnancy	Dambaeva et al. 2018
Mice	10	16	Hypotension but no marked inflammatory response	Zollner et al. 2017; 2020
Mice	10	16	Preterm labour, fetal death, leukocyte infiltration in maternal lung and liver, increase in proinflammatory cytokine expression	Edey et al. 2016
Mice	~11	8	External abnormalities in pups	Carey et al. 2003
Mice	~12.5	7	100% embryonic resorption and fetus expulsion	Ogando et al. 2003

Animal Model	Dose (in µg) *	Gestational Day of injection	Reported Effects	Reference
Mice	~12.5	18	Infiltration of leukocytes, abortion, preterm labour, decreased viability of delivered pups	Paintlia et al. 2008
Mice	~25	7	100% embryonic resorption	Aisemberg et al. 2013
Mice	25	12	Fetal resorption, TNF-α in amniotic fluid	Gendron et al. 1990
Mice	25	14.5	Preterm birth, 100% fetal loss	Dambaeva et al. 2018
Mice	~25-50	16-17	stillbirths and fetal resorptions	Chlodzinska et al. 2011
Mice	25	17	100% fetal loss in wildtype mice	Robertson et al. 2006
Mice	25	16 or 17	Increase in proinflammatory cytokine expression, preterm birth, fetal death associated with shorter gestational period (16 days)	Salminen et al. 2008
Mice	50	15	Preterm birth	Shynlova et al. 2014
Mice	50	15 or 18.5	Intra-uterine inflammation evoking cytokine response in placenta, fetus and fetal brain; preterm birth	Elovitz et al. 2011
Rats	~0.08	5	Increased blood pressure of pregnant rats, impaired spiral artery remodeling, fetal growth restriction, elevated inflammatory cytokines.	Xue et al. 2015; Gong et al. 2016
Rats	~0.2	14	Increase in maternal blood pressure, elevated pro-inflammatory cytokines, fetal loss, lower fetal and placental weights.	Zhang et al. 2018
Rats	~15	14	Decrease in number of embryos per dam, increase in pro-inflammatory IL-1β expression	Straley et al. 2014
Rats	~15	16	Reduced placental weight, increase in pro-inflammatory IL-1β expression, decrease in number of embryos per dam	Straley et al. 2014

Animal Model	Dose (in µg) *	Gestational Day of injection	Reported Effects	Reference
Rats	~25	9.5	Placental abnormalities, smaller percentage of live fetuses.	Kirsten et al. 2013
Rats	~30	18	Upregulation of pro-inflammatory cytokines in placenta, maternal serum and amniotic fluid	Gayle et al. 2004
Rats	~43	10	Higher levels of pro-inflammatory cytokines	Er 2013
Rats	~50	14-17	Neurochemical and behavioural alterations in pups	Sharma et al. 2017
Rats	~300	19	Pro-inflammatory mediators in placenta	Dowling et al. 2012
Golden Hamster	~0.6-12	8	Increasing fetal death with dose, low fetal weight and malformations.	Collins et al. 1994
Rabbit	~2.68	8	High rate of fetal resorption	Pitt et al. 1997

* Some studies presented doses in µg per unit of body weight and were converted to approximate values per animal using average body weights.

1.2.3. Placental development in humans and mice

Defects in early placental development can result in pregnancy complications such as pre-eclampsia, preterm-births, stillbirths and fetal growth-restriction (Brosens et al. 2011). Due to the impracticality and ethical issues with studying early placental development in humans (Soncin et al. 2018), rodent models, particularly mice, are commonly used. It is therefore important to understand the similarities and differences between the two species. Humans and mice exhibit hemochorial placentation, which is characterized by close contact between trophoblast cells (which derive from the outer layer of the blastocyst) and maternal blood (Pijnenborg et al.1981; Malassine et al. 2003; Soares et al. 2018). In mice, the trophoblast has three layers separating maternal and fetal blood (2 synctiotrophoblast layers and 1 cytotrophoblast layer), and this is termed hemotrichorial placentation. In humans, there is hemomonochorial placentation, where one trophoblast layer separates maternal and fetal blood. In both species, trophoblast stem cells differentiate into different lineages with different functions (Red-Horse et al. 2004; Senner and Hemberger 2010). Differentiated trophoblast cells are involved in the remodeling of the uterine spiral arteries. However, there are key differences in this process between species, and the types of trophoblast cells that are involved (Silva and Serakides 2016; Hemberger et al. 2020).

In humans, following implantation, trophoblast cells proliferate and differentiate into cytotrophoblasts and synctiotrophoblasts (which form from fused cytotrophoblasts). Cytotrophoblasts give rise to extra-villous trophoblast (EVT's), which, modulated by uNK cells, migrate to the endometrium and myometrium of the uterus where they break down the extra-cellular matrix proteins and invade the uterine spiral arteries, replacing the endothelium and smooth muscle layer (Pijnenborg et al. 1980; 1983; Eastabrook et al. 2008). They also produce and deposit a matrix called fibrinoid during this process. In mice, the remodeling of the uterine spiral arteries is mediated by uNK cells and involves interstitial (between blood vessels) trophoblast invasion, with very limited invasion of the vasculature (Silva and Serakides 2016). Trophoblast invasion in mice does not extend into the myometrium, as it does in humans, and is restricted to the mesometrial decidua (Ain et al. 2003).

In the mouse placenta, maternal and fetal blood flow in a countercurrent fashion, to maximize transfer of nutrients (Adamson et al. 2002). In the human placenta, fetal blood flows through a network of capillaries arranged within the placental villi, which are in contact with maternal blood in the intervillous space (Plitman Mayo 2018). Soncin et al. (2018) found that patterns of gene expression in mouse placentas show some similarities to those of humans, up till gestational week 16. They suggest that the timeline of placental development in mice corresponds to the first half of placental development in humans.

1.3. Study objectives

In mice, maternal HFD can cause inflammatory effects during the gestational period including alterations in the properties of uNK cells and elevation of some pro-inflammatory cytokines (Kępczyńska et al. 2013; Perdu et al. 2014). In spite of these effects, HFD alone does not appear to negatively influence pregnancy outcome (Ingvorsen et al. 2014; Chin et al. 2017; Baltayeva et al. 2020). Therefore, there is no consistent evidence of HFD-induced inflammation leading to adverse pregnancy outcomes. However, it is conceivable that such inflammation may increase susceptibility to other inflammatory insults during pregnancy, and in turn, result in serious complications.

Understanding such an inflammatory interaction would be of clinical interest to humans. Epidemiological studies have found a strong association between maternal obesity and pregnancy complications, including spontaneous abortion, preterm birth and stillbirths (Galtier-Dereure et al. 2000; Guelinckx et al. 2008; Leddy et al. 2008; Ramachenderan et al. 2008; Fitzsimmons et al. 2009; Marshall and Spong 2012; Stubert et al. 2018). However, the underlying mechanisms, including the potential role of obesity-associated chronic inflammation, are poorly understood. Furthermore, many obese women have uncomplicated pregnancies. Therefore, it might be concluded that obesity-induced inflammation may not necessarily cause any negative effects on its own, similar to what is observed in HFD-fed mice. However, it may increase susceptibility to additional inflammatory insults during the gestational period.

I hypothesize that, in HFD-fed mice, an additional inflammatory insult in the form of bacterial LPS, will exacerbate pre-existing inflammation, leading to serious pregnancy

complications. The objective of this study was to test if there is a synergistic interaction effect of maternal HFD and exposure to LPS on pregnancy and fetal outcome in mice. In particular, I measured how this dual treatment impacted:

1. Fetal and placental weights
2. The number of fetal resorptions
3. Maternal serum concentration of TNF-α
4. The incidence of spontaneous abortion

In chapter 2, I discuss my preliminary dose-response experiments to determine the dose of LPS that was subsequently used in further experiments. I then describe my model of diet, LPS and diet-LPS interaction on pregnancy and fetal development. I provide an overview and timeline of the protocol I used to rear mice on special diets (High-fat diet and low-fat diet), inject pregnant mice with LPS or a control, and collect fetal and placental data from these mice. I also describe my attempts to measure a marker of maternal inflammation in mice from my treatment groups – circulating levels of the pro-inflammatory cytokine TNF-α. I then provide the results of my experiments along with statistical analyses and graphical representation. Lastly, I provide a brief conclusion of my main findings. In chapter 3 I provide an in-depth discussion of the main findings of my experiments, their implications, limitations and directions for future work. Lastly, I provide a brief conclusion of my work, and once again emphasize my main findings and their implications.

Chapter 2.

Effects of diet, LPS and diet-LPS interaction on pregnancy

2.1. Introduction

In humans, maternal obesity is associated with adverse pregnancy outcomes, but the underlying mechanisms, including the potential role of obesity-induced chronic inflammation, are largely unknown. Since many obese women have uncomplicated pregnancies, obesity-induced inflammation is unlikely to be solely responsible for any complications, but it might increase susceptibility to other inflammatory insults during pregnancy. Similarly, in mice, maternal HFD causes inflammatory responses during pregnancy (section 1.2.1), but these are not accompanied by any pregnancy complications. I hypothesized that an additional inflammatory insult, in the form of bacterial LPS, would exacerbate HFD-induced inflammation, leading to serious complications. The objective of this study was to test if there was a synergistic interaction effect of diet and LPS, and if this would result in exacerbated inflammation and pregnancy complications in mice.

2.2. Methods

2.2.1. Animals

All experiments were carried out with approval by the University Animal Care Committee (UACC) at Simon Fraser University, in accordance with standards specified by the Canadian Council on Animal Care (CCAC). C57BL/6J strain mice were purchased from The Jackson Laboratory, Bar Harbor, Maine and housed in individual ventilated cages (5 animals per cage; males and females separated) at the Animal Care Facility at Simon Fraser University. The mice were housed under constant and controlled conditions of temperature (20-27°C), humidity (40-50%) and artificial lighting (12-hour light/dark cycle). The mice had free access to food and water.

2.2.2. Determination of LPS dose

The first step to develop the diet-LPS model was to determine the minimum dose of LPS required to induce inflammation, accompanied by pregnancy outcomes that were not severe. A dose that produced no obvious effects on pregnancy would likely produce minimal effects when combined with a high-fat diet and would therefore not be useful. However, if a dose was too high and produced severe effects such as complete abortion, when combined with a high-fat diet, it would not be possible to discern whether severe pregnancy outcomes were due to the diet-LPS interaction or the LPS treatment alone. I decided to use a constant dose per mouse across all my treatment groups, rather than using a dose per unit of body weight. It could be argued that mice fed HFD would weigh more and therefore should be injected with a higher dose as compared to LFD mice. However, increased adiposity and weight gain in HFD mice is due to an increase in the size of adipose cells and not an increase in the number of cells, which are comparable in HFD and LFD mice. Therefore, basing dose on mouse weight would result in HFD mice receiving a higher dose per cell and per lean mass. Furthermore, studies have found that HFD in mice can lead to a condition called metabolic endotoxemia, where LPS derived from gut bacteria is displaced into the bloodstream, leading to an increase in circulating levels of LPS (Nishizawa, 2016; Fuke et al. 2019). The liver plays an important role in clearing out LPS from circulation (Freudenberg and Galanos, 1990; Shao et al. 2007; Yao et al. 2016), and continuous exposure can lead to liver damage and dysfunction, in-turn contributing to the development of other conditions including heart disease and heart failure (Ghosh et al. 2020). Furthermore, HFD in mice also results in steatosis (fat accumulation), cell injury and fibrosis in the liver (Velazquez et al. 2019). Therefore, in addition to the gut bacteria-derived LPS, injecting HFD mice with higher doses of LPS may exacerbate effects on the liver, leading to other health conditions which could confound my results.

The required experimental dose was determined by preliminary dose-response experiments using LPS from *Escherichia coli* (L3129, Sigma-Aldrich, St. Louis, MO) and *Salmonella enterica* (L6511, Sigma-Aldrich, St. Louis, MO). Mice of breeding age were time-mated, with a maximum of 3 females being mated with a single male. Separation of the mating pairs occurred on the morning of the next day (~20 hours later), and this was termed gestational day 0. On gestational day 6, females were weighed, and those with significant weight gain (~1 g) were considered pregnant. On gestational day 7 pregnant

females were weighed by a member of the Animal Care staff, and randomly administered subcutaneous injections of LPS (in saline) or a control 0.85% saline solution. One cohort was treated with LPS from *E. coli* (20µg per mouse) and other preliminary cohorts were treated with LPS from *S. enterica* at doses of either 20 µg or 5 µg per mouse. Although commonly used to infer pregnancy in mice, I did not consider the presence/absence of a vaginal plug after being paired with a male as a criterion for inferring pregnancy. Vaginal plugs can be easily dislodged and are only an indication of copulation, not pregnancy. Mader et al. (2009) found that pregnancy rates in plugged females can range from 33% to 85% in C57BL/6J mice. The same study found that an increase in body weight 7 days after exposure to a male is a more reliable indication of pregnancy.

On gestational day 17, the mice that were treated with LPS or control were sacrificed by carbon dioxide asphyxiation. The mice were dissected for collection of the uteri. The uteri were examined for signs of fetal resorptions and other abnormalities, and the number of fetuses in each litter were recorded. Uteri were immediately placed in a 4% Paraformaldehyde (PFA) solution and stored on ice until being transported to 4°C conditions. The collected uteri were stored in 4% PFA for three days, and then dissected. The collected fetuses and placentas from the uteri were individually weighed using a standard protocol. Based on the results (described below) a dose of 2 µg LPS per mouse was selected for further experiments. The selection of this dose was also based on observations by Lee et al. (2013), who found that 2.5 µg of LPS injected on gestational day 7.5 produced a high rate of fetal resorptions, but not complete fetal loss.

2.2.3. Model of diet-LPS interaction

At wean, females were randomly assigned to one of two diet treatment groups – a high-fat, high-sucrose diet (HFD; 45% kcal fat, 35% carbohydrate (including 17% kcal sucrose), 20% kcal protein, 4.73 kcal/g, D12451, Research Diets, New Brunswick, NJ) or a nutrient-matched, low-fat, no-sucrose diet (LFD; 10% kcal fat, 70% kcal carbohydrate (corn starch and maltodextrin), 20% kcal protein, 3.85 kcal/g, D12450K, Research Diets, New Brunswick, NJ). Diets were chosen based on Chin et al. (2017), who found a significant difference in weight gain and fat mass between mice fed the HFD and LFD, but no effects on fetal outcome. More mice (approximately two-thirds)

were assigned to the HFD, as I planned to analyse differences between mice that gained weight on this diet, and mice that were resistant to gaining weight.

The females were kept on the assigned diets for 13 weeks and weighed weekly to assess weight gain. The amount of food consumed by each female weekly was estimated by measuring the weekly intake of food per cage and dividing this by the number of females in each cage. After 13 weeks, females were time-mated with age-matched males, with a maximum of 3 females being mated with a single male. Separation of the mating pairs occurred on the morning of the next day (~20 hours later), and this was termed gestational day 0. As already discussed, I did not include the presence/absence of a vaginal plug after exposure to a male as a criterion for assessing pregnancy. On gestational day 6, females were weighed, and those with substantial weight gain (~2 g for HFD and ~1 g for LFD mice) were considered pregnant. On gestational day 7, pregnant females were weighed by a member of the Animal Care staff, and randomly administered subcutaneous injections of 2 µg LPS in saline (*Salmonella enterica*; L6511, Sigma-Aldrich, St. Louis, MO) or a control (0.85% saline solution). This resulted in four treatment groups to assess the effects of diet, LPS and a diet-LPS interaction, as shown in figure 2.1. Those females that did not become pregnant were re-mated repeatedly until pregnant. As a result, the females had been on the special diets for 13-27 weeks before becoming pregnant. The number of attempted matings for each female was recorded to assess if diet affected mating success. A few mice (HFD; n=3) that were not thought to be pregnant at gestational day 7, but observed to be pregnant at gestational day 14, were included in data analyses as controls.

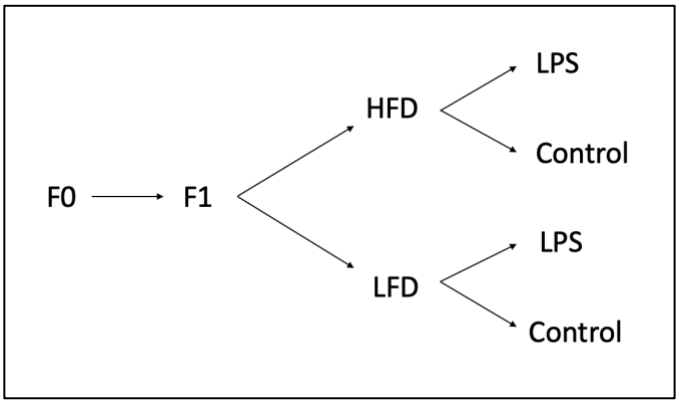

Figure 2.1. Schematic of the high-fat diet (HFD), low-fat diet (LFD) and LPS or control treatment groups. Mice that were purchased (F0) were bred in-house to produce the F1 generation for use in further experiments.

2.2.4. Fetal resorptions, litter size, fetal and placental weights

On gestational day 14, mice from the four treatment groups were sacrificed by isoflurane anesthesia + carbon dioxide asphyxiation. The mice were dissected for collection of the uteri on the same day. The uteri were examined for signs of fetal resorptions and other abnormalities, and the number of fetuses in each litter was recorded. Litter size was recorded based on the number of viable-appearing fetuses only, not including the number of resorbed fetuses. Fetal resorptions were identified as dark masses that were significantly smaller than fetuses from the same litter and appeared relatively undeveloped (lacking discernible body parts). The collected uteri were immediately placed in a 4% PFA (Paraformaldehyde) solution and stored on ice until being transported to 4°C conditions. The collected uteri were stored in 4% PFA for three days for tissue fixation, and then dissected to collect fetuses and placentas. Each uterus was transferred into a large petri dish containing 1% Phosphate-buffered saline (PBS). The conceptuses were each separated from each other, and the amniotic sac surrounding the fetus and placenta was carefully removed. The umbilical cord attaching the fetus and placenta was clipped and excess uterine tissue surrounding the placenta was carefully trimmed. Each fetus and placenta were individually weighed.

2.2.5. Marker of maternal inflammation - TNF-α

Elevated levels of the pro-inflammatory cytokine TNF-α is associated with many negative pregnancy outcomes, as discussed in section 1.1.2. An increase in this cytokine in maternal serum was previously found in pregnant mice fed the same HFD (Baltayeva et al. 2020).

On gestational day 14, at the time of tissue collection, the mice were blood sampled by cardiac puncture. Blood was stored on ice and allowed to clot for ~30 mins. The blood samples were then centrifuged at 2,000 rpm for 10 minutes. The resulting supernatant (serum) was immediately transferred into new tubes and stored at -80°C. Serum samples were measured for TNF-α in duplicate (diluted to 1/2 concentration) using a commercially available ELISA (DY410-05 R&D Systems, Minneapolis, MN). Each step in the assay was carried out according to the manufacturer's instructions. Optical density of the samples was determined immediately using a microplate reader

set to 450 nm with wavelength correction. The standard curve was plotted, and sample concentrations were determined.

2.2.6. Statistical analyses

All data were analysed using JMP® (Version 14.1, SAS Institute Inc., Cary, NC). A one-way analysis of variance (ANOVA) was used to assess differences in mean fetal and placental weights between each cohort that was treated with LPS (20µg *E. coli*, or 20µg *S. enterica*) and their respective controls, using litter size as a covariate. Differences in litter size and number of fetal resorptions per litter between LPS and control mice were assessed using Kruskal-Wallis tests. The effect of LPS on the likelihood of a female showing no signs of pregnancy when collected was assessed using logistic regression.

Differences in weight-gain and food consumed between the HFD and LFD mice were analysed using a one-way analysis of variance (ANOVA). The effect of diet on mating success was assessed using Kruskal-Wallis tests. A two-way analysis of variance (ANOVA) with repeated measures was used to assess the effects of diet, LPS and a diet-LPS interaction on fetal and placental weights, with the dam as a random effect and litter size as a covariate. Since each dam had multiple offspring, the dam was the unit of replication. Litter size was included in the statistical model since fetuses from larger litters are generally smaller in size. Differences in litter size and the number of fetal resorptions per litter between the four treatment groups (HFD-LPS, HFD-control, LFD-LPS, LFD-control) were assessed using Kruskal-Wallis tests. A two-way ANOVA was also used to assess if diet, LPS, or a diet-LPS interaction affected litter size or number of fetal resorptions.

The effects of diet, LPS and a diet-LPS interaction on mean fetal and placental weights, using litter size as a covariate, were assessed in weight-gain prone and weight-gain resistant mice within the HFD group using a two-way ANOVA. Differences in litter size and number of fetal resorptions between weight-gain prone and weight-gain resistant mice were assessed using Kruskal-Wallis tests. A two-way ANOVA was also used to assess if diet, LPS, or a diet-LPS interaction affected litter size or number of fetal resorptions.The effects of diet, LPS and a diet-LPS interaction on the likelihood of a female appearing to be pregnant at GD7 but showing no signs of pregnancy at GD14

(i.e. who had potentially aborted their pregnancies) were analysed using logistic regression.

2.3. Results

2.3.1. Determination of LPS dose

We found no effect of a relatively large dose of *E. coli* LPS (20 µg per mouse) on fetal weight, placental weight, number of fetal resorptions or litter size (*p>0.05*; Figures 2.2 and 2.3). Furthermore, this dose did not appear to cause any fetal loss i.e. situations where a mouse was thought to be pregnant before LPS injection but did not show signs of pregnancy when collected (Figure 2.4). Administering the same dose (20 µg per mouse) of *S. enterica* LPS gave us different results. Overall, there was no effect on fetal size, placental size, litter size or number of fetal resorptions (*p>0.05* Figures 2.5 and 2.6). However, one LPS treated mouse showed 100% fetal resorption. Another mouse treated with this same dose showed 25% fetal resorption, and the other fetuses in the same litter were small for gestational age. Furthermore, it was more likely that a mouse injected with LPS showed no sign of pregnancy when collected compared with controls, possibly indicating that LPS treated mice may have aborted their pregnancies early on (*p<0.05;* Figure 2.7). A lower dose (5 µg per mouse) resulted in 86% fetal resorption in one mouse (data not shown). Based on these findings I chose a lower dose (2 µg per mouse) of *S. enterica* LPS to be used in subsequent experiments. Being lower than the dose capable of inducing a high rate of fetal resorption (i.e. 5 µg per mouse), I believed that a dose of 2 µg per mouse would produce smaller effects on pregnancy and would be suitable for testing if LPS exacerbated the effects of a high-fat diet. The choice of this dose was also based on observations by Lee et al. (2013), who found that 2.5 µg of LPS injected on gestational day 7.5 produced high, but not complete fetal resorption. This study used an intraperitoneal route of LPS administration where absorption is generally faster than a subcutaneous route (Turner et al. 2011). I therefore expected to see similar, but slightly less severe effects.

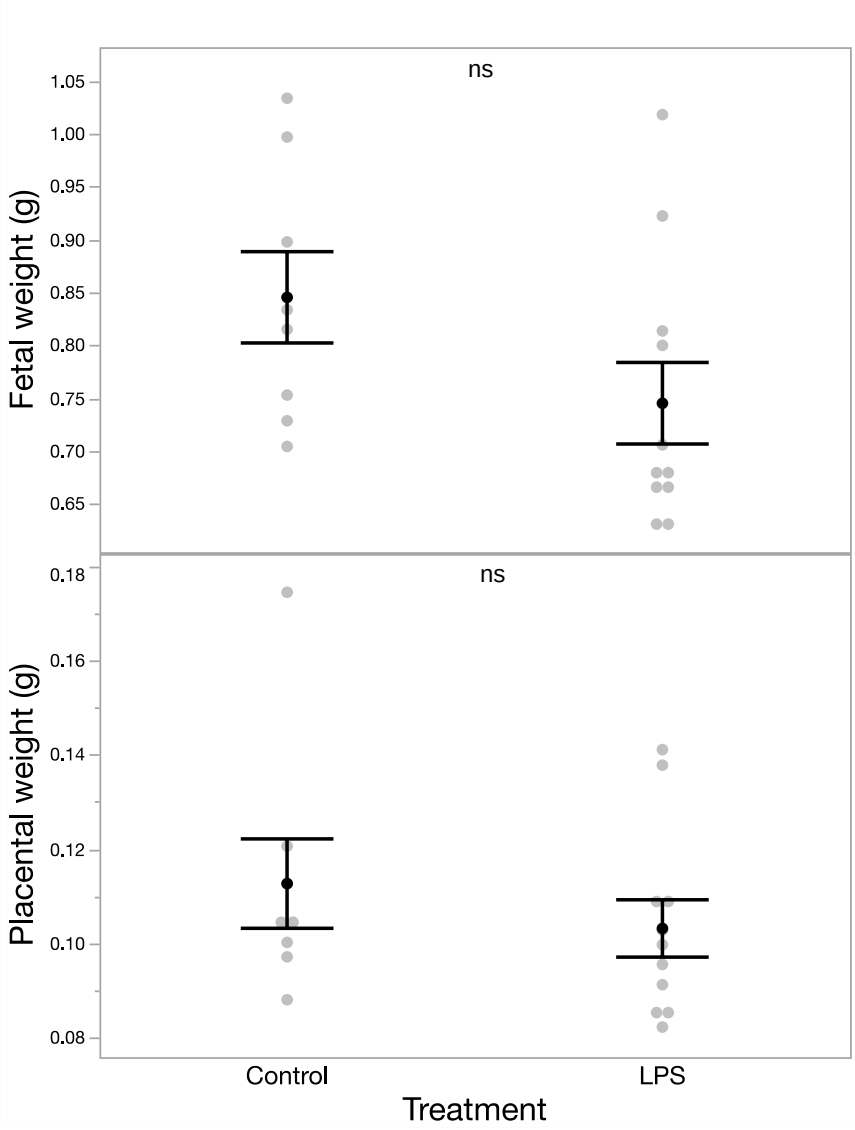

Figure 2.2. **Fetal and placental weights (g) of mice injected with LPS from *E. coli* (20 µg per mouse; n=11) or a control (0.85% saline solution; n=8) on gestational day 7 and collected on gestational day 17. Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).**

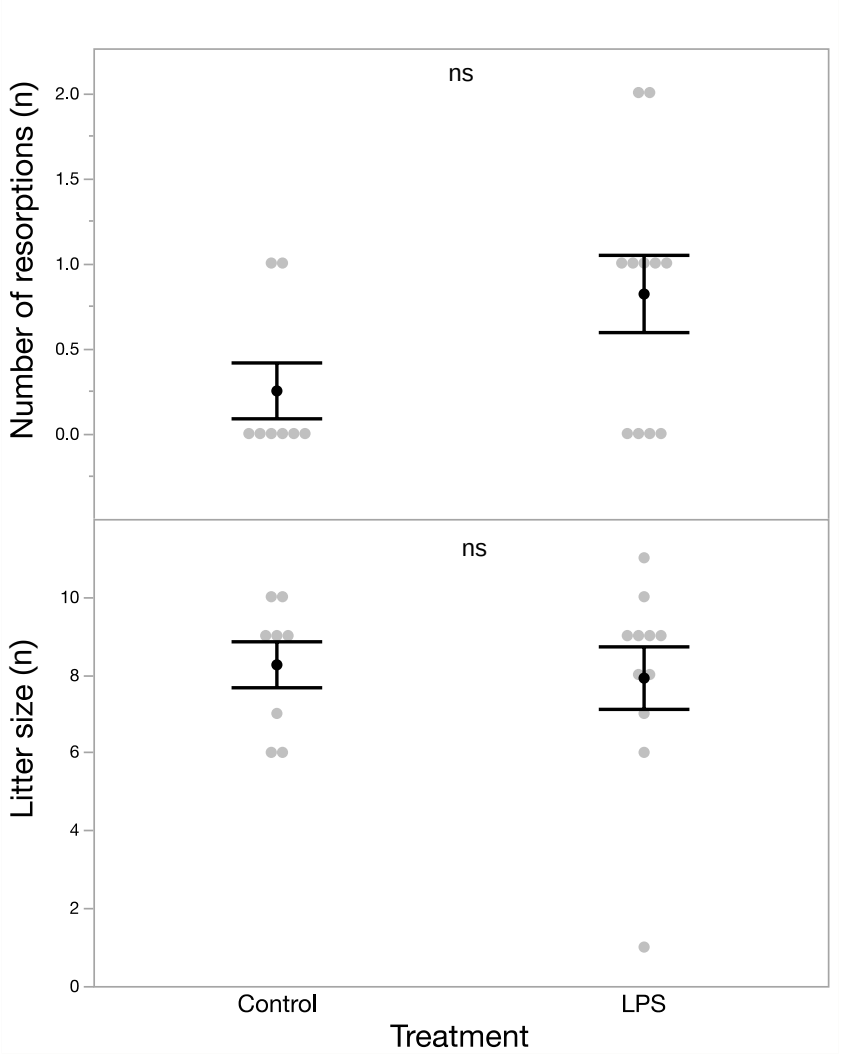

Figure 2.3. **Number of resorptions (n) per litter and litter size (n) of mice injected with LPS from *E. coli* (20 µg per mouse; n=11) or a control (0.85% saline solution; n=8) on gestational day 7 and collected on gestational day 17. Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).**

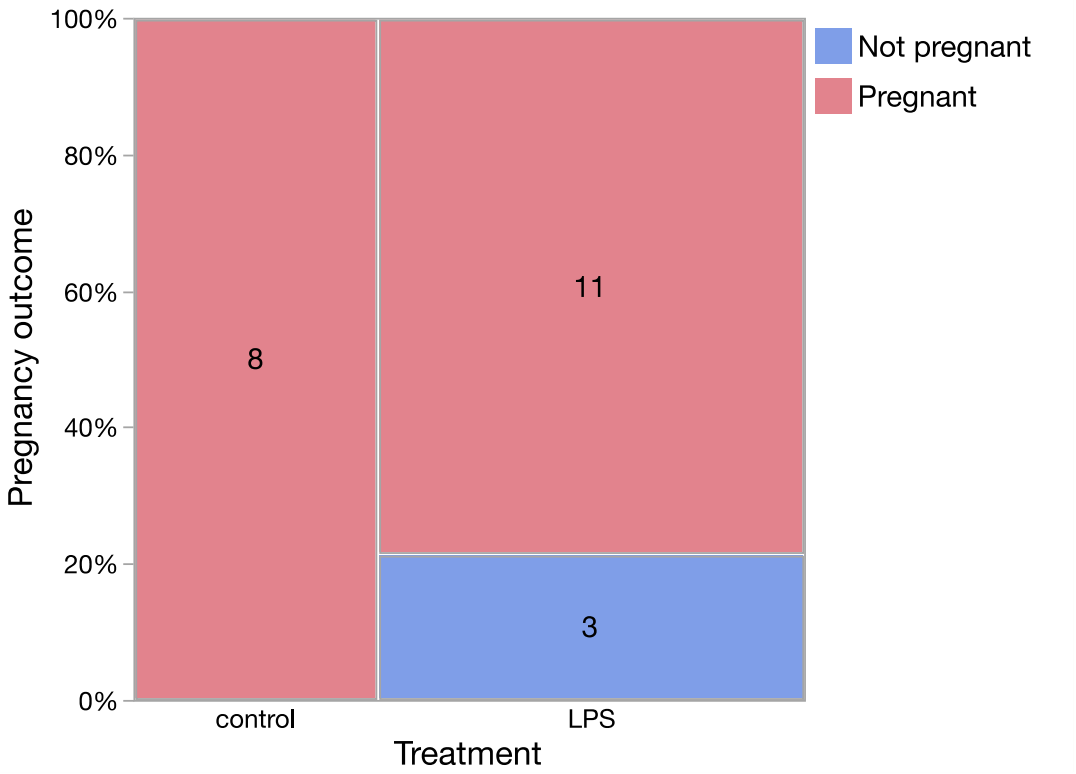

Figure 2.4. **Mosaic plot showing pregnancy outcome of mice treated with LPS (*E. coli*, 20 µg per mouse; n=14) or a control (0.85% saline solution; n=8) on gestational day 7 and collected on gestational day 17. Differences were not statistically significant (*p>0.05*).**

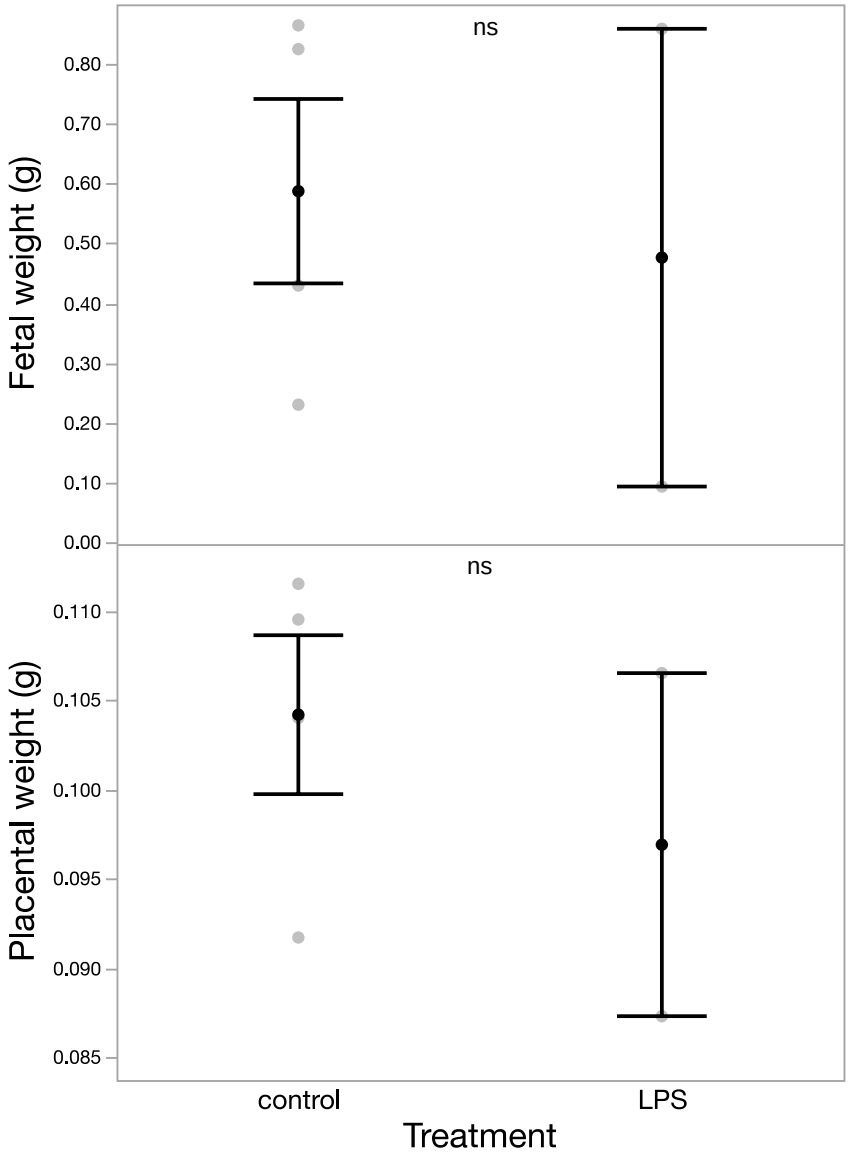

Figure 2.5. **Fetal and placental weights (g) of mice injected with LPS from *S. enterica* (20 µg per mouse; n=4) or a control (0.85% saline solution; n=4) on gestational day 7 and collected on gestational day 17. Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).**

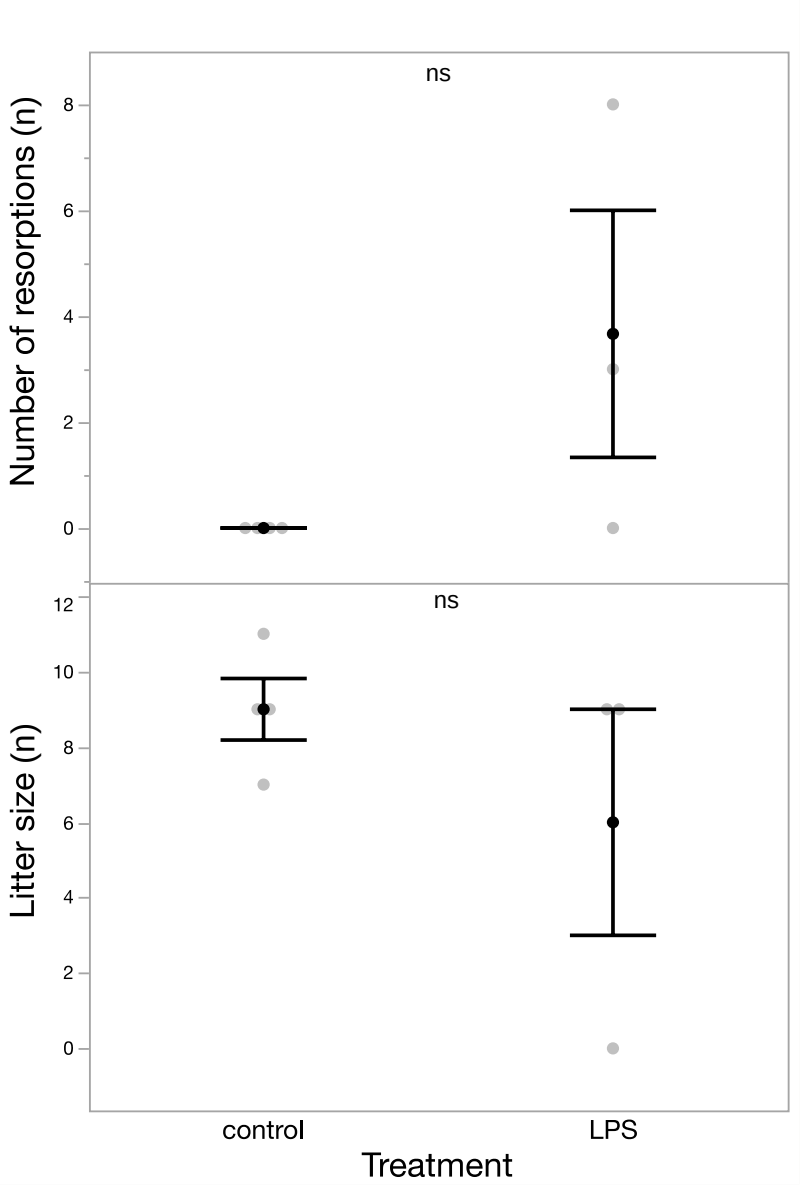

Figure 2.6. **Number of resorptions (n) per litter and litter size (n) of mice injected with LPS from *S. enterica* (20µg per mouse; n=4) or a control (0.85% saline solution; n=4) on gestational day 7 and collected on gestational day 17. Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).**

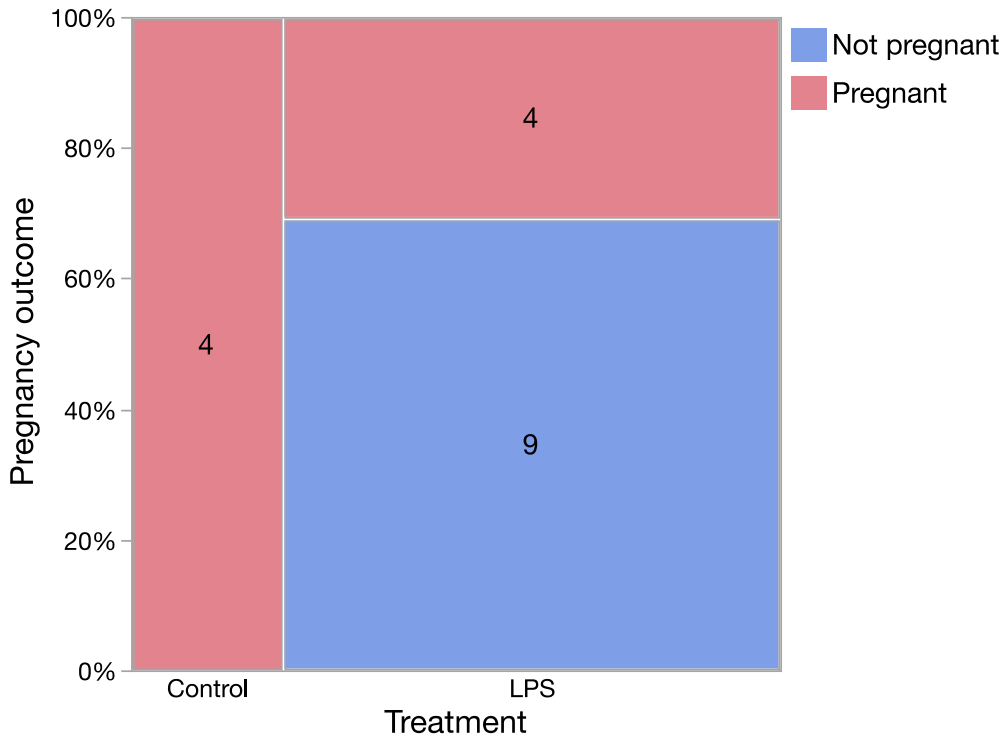

Figure 2.7. Mosaic plot showing pregnancy outcome of mice treated with LPS (*S. enterica*, 20 µg per mouse; n=13) or a control (0.85% saline solution; n=4) on gestational day 7 and collected on gestational day 17. Differences were statistically significant (*p<0.05*).

2.3.2. Maternal traits

On average, HFD mice consumed a lower weight of food but more calories weekly (17.13±0.2 g; 81.02±1.0 kcal) than LFD mice (18.37±0.3 g; 70.74±0.9 kcal; *p<0.001*) (Table 2.1). Mice from the HFD group went on to gain weight and were significantly heavier than mice from the LFD group (24.7±2.7 g vs. 22.9±2.5 g *p<0.05*) at the time of mating. A plot of weight-gain by week prior to pregnancy for mice used in final experiments is shown in Figure 2.8.

Table 2.1. Food consumption, weight gain and reproductive performance of mice on high-fat diet (HFD) and low-fat diet (LFD).

	HFD	LFD
N	49	28
Average food consumed per week	17.13±0.2 g	18.37±0.3 g
Average calories consumed per week	81.02±1.0 kcal	70.74±0.9 kcal
Average weight (at mating)	24.7±2.7 g	22.9±2.5 g
Average weight (at injection)	26.7±3.3 g	24.9±2.0 g
Number of mice that became pregnant*	46	22
Pregnancy rate*	94%	78.6%
Average number of matings** until pregnant*	3.6±0.4	6.5±0.5

* Reproductive parameters are based on pregnancy assessed at gestational day 7.

** Matings represent exposure to a male and are not equivalent to number of copulations.

Diet appeared to affect mating success, as mice from the HFD group took significantly fewer attempts to get pregnant compared with mice from the LFD group (*p<0.001*). From my initial sample (n=77; HFD=49, LFD=28), 68 mice (HFD=46, LFD=22) appeared to be pregnant based on weight gain at gestational day 7 and were injected with LPS or saline. Of these, 34 mice (HFD-LPS=9; HFD-control=14; LFD-LPS=5; LFD-control=6) were pregnant at day 14. Some mice (HFD; n=3) that were not thought to be pregnant at gestational day 7, but were pregnant at day 14, were included in this sample as controls. It was more likely that a mouse treated with LPS had no signs of pregnancy when collected on gestational day 14, compared with control mice (*p<0.05;* Figure 2.9). There was no effect of diet (*p>0.05*) or a diet-LPS interaction (*p>0.05*) on the likelihood of showing no signs of pregnancy at gestational day 14, when thought to be pregnant at gestational day 7. The remainder of the experimental mice were either lost to death by natural causes or culled due to inability to become pregnant after a significant number of attempts.

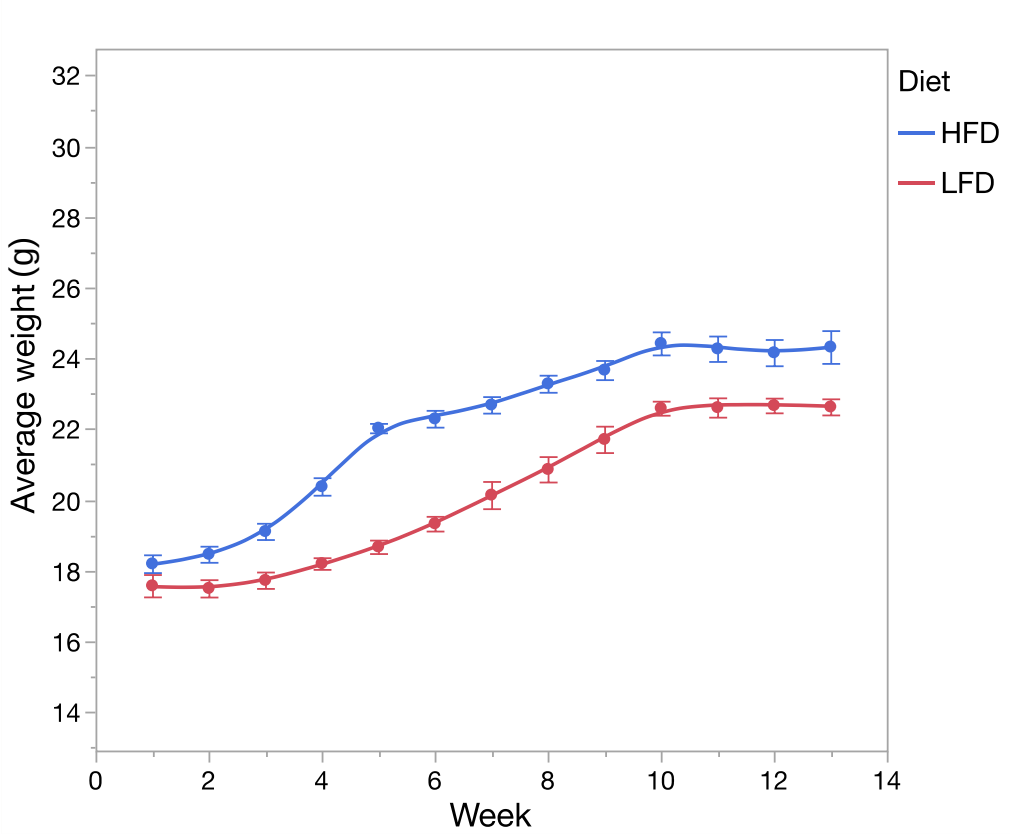

Figure 2.8. **A plot of average weight (g) per week in female mice fed a high-fat diet (HFD; n=49) or low-fat diet (LFD; n=28) prior to pregnancy. Data are presented as mean±SE.**

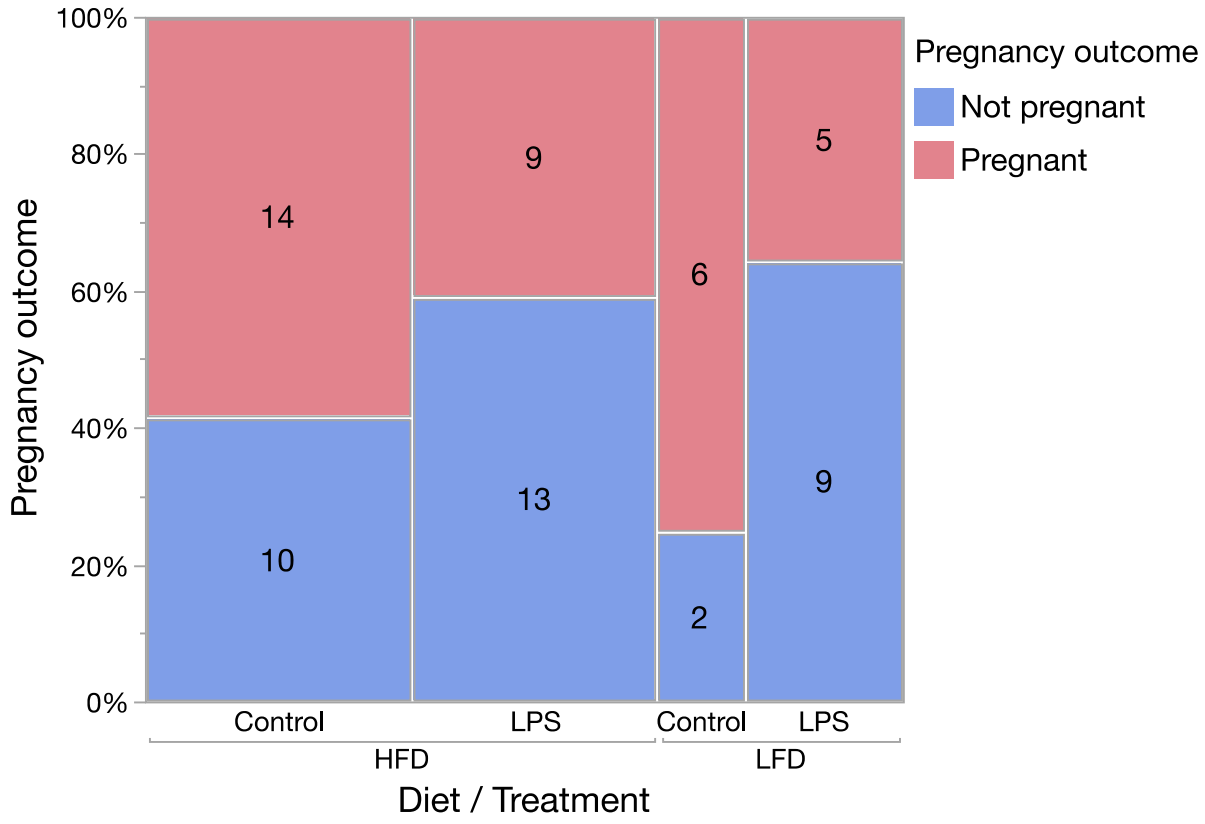

Figure 2.9. **Mosaic plot showing pregnancy outcome of HFD and LFD mice subjected to either LPS (2 µg per mouse) or control (0.85% saline solution) treatments on gestational day 7 and collected on gestational day 14. Differences between LPS and control treated mice were statistically significant (*p<0.05*), but there was no significant effect of diet (*p>0.05*) or a diet-LPS interaction (*p>0.05*).**

2.3.3. Maternal serum concentration of TNF-α

We found no effect of diet, LPS or a diet-LPS interaction on maternal serum levels of TNF-α, although some samples had concentrations below the minimum detection limit of the ELISA (Figure 2.10).

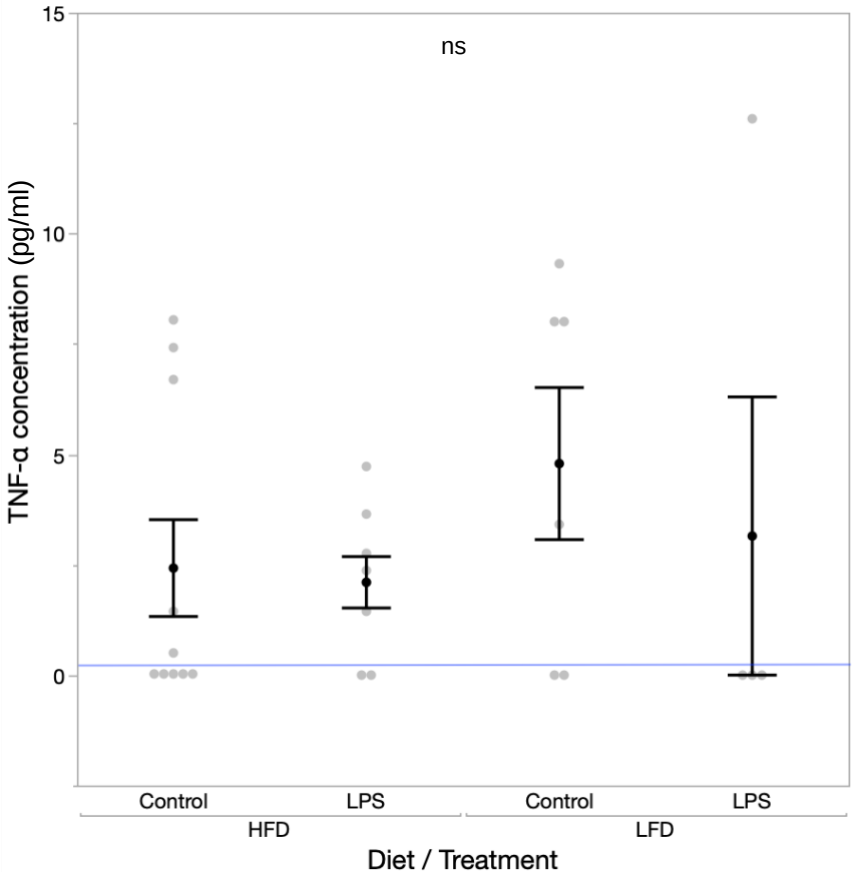

Figure 2.10. Maternal serum concentrations of TNF-α (pg/ml) in mice fed a high-fat diet (HFD) or low-fat diet (LFD) and treated with LPS (2 µg per mouse) or control (0.85% saline solution) on gestational day 7 (HFD-LPS n=9; HFD-control n=14; LFD-LPS n=5; LFD-control n=6). Serum was collected from mice on gestational day 14. Data are presented as mean±SE. The blue line indicates the minimum detection limit of the ELISA (2x the standard deviation of a series of blanks + mean of the blanks, interpolated on standard curve). Differences were not statistically significant (*p>0.05*).

2.3.4. Fetal traits

There was no effect of diet, LPS or a diet-LPS interaction on mean fetal and placental weights (*p>0.05*; Figures 2.11 and 2.12) and litter size did not affect either fetal or placental weights (*p>0.05*). There was also no effect of diet, LPS or a diet-LPS interaction on fetal resorptions or litter size (Figures 2.13 and 2.14). Within the HFD group, some mice were substantially heavier than the LFD mice, while other mice weighed similar to the LFD mice. Accordingly, HFD mice were classified as weight-gain prone (WGP) if their weight at the time of mating was greater than the average weight of a LFD mouse +2 standard deviations (i.e. 25.1 g). HFD mice were classified as weight-gain resistant (WGR) if their weight at the time of mating was less than the average weight of a LFD mouse +2 standard deviations (i.e. 25.1 g). Here, the average weight of a LFD mouse was calculated using LFD mice that became pregnant and were included in data analyses (n=11). This model of classification into weight-gain prone and resistant groups is based on a similar model used by James et al. (2012) and Boi et al. (2016). An analysis of the HFD group (Figure 2.15) showed a trend of both WGP and WGR mice treated with LPS having lower fetal weights compared with controls, however this was not significant (*p>0.05*). Likewise, there were no statistically significant differences in placental weight, number of fetal resorptions or litter size due to diet, LPS or a diet-LPS interaction in these two groups (*p>0.05*; Figure 2.16, 2.17 and 2.18).

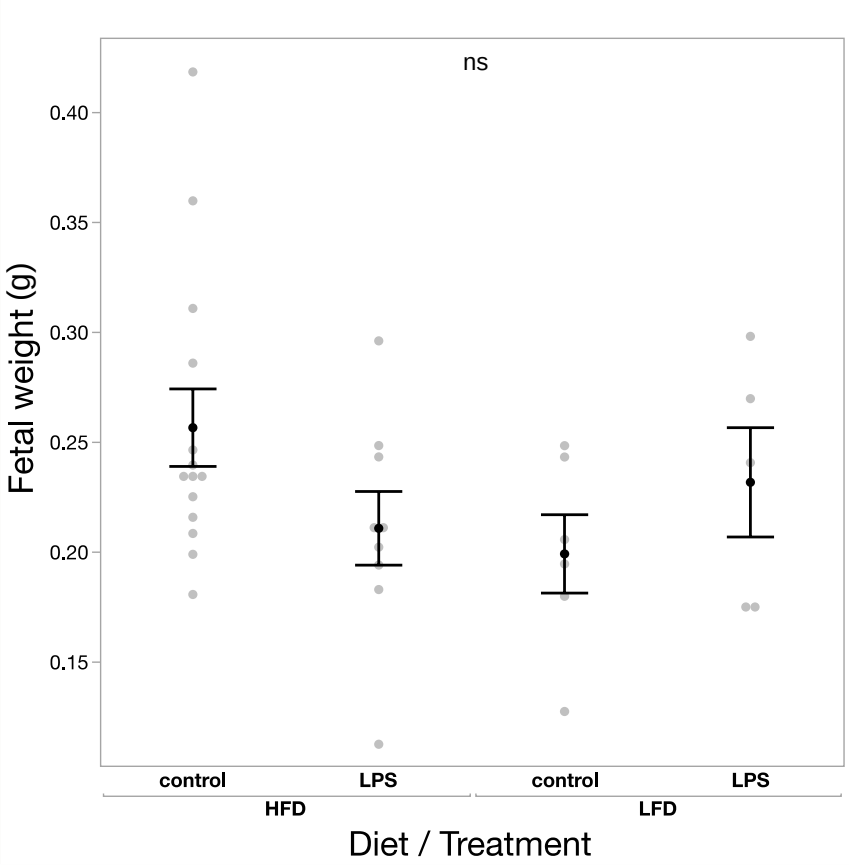

Figure 2.11. Fetal weights (g) of mice fed a high-fat diet (HFD) or low-fat diet (LFD) and treated with LPS (2 µg per mouse) or control (0.85% saline solution) on gestational day 7, and collected on gestational day 14 (HFD-LPS n=9; HFD-control n=14; LFD-LPS n=5; LFD-control n=6). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).

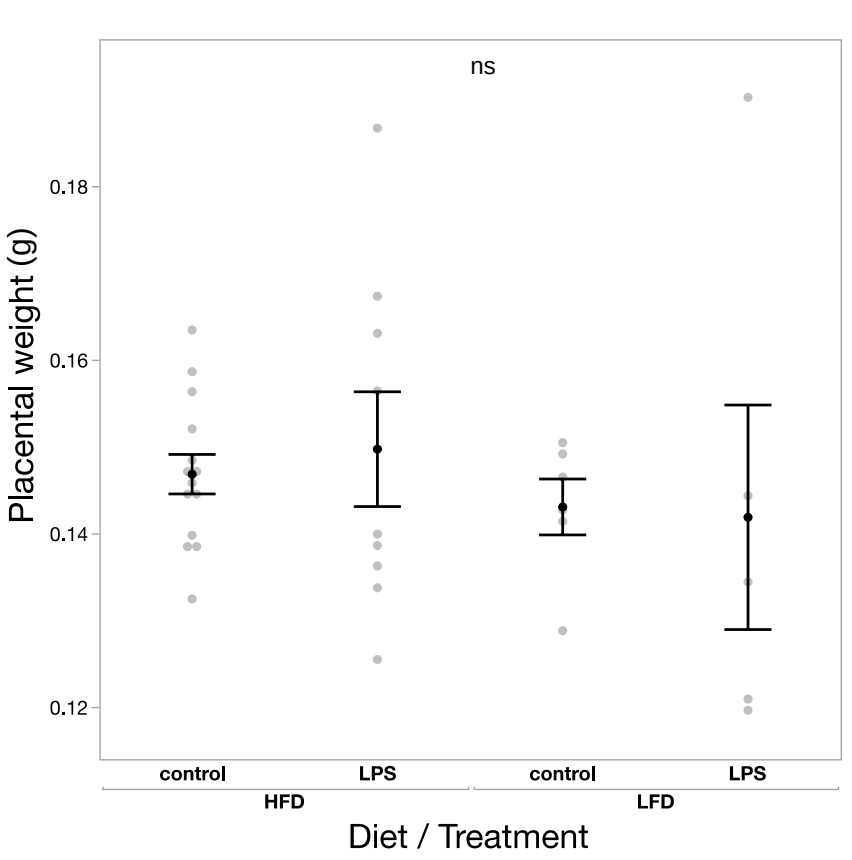

Figure 2.12. Placental weights (g) of mice fed a hIgh-fat diet (HFD) or low-fat diet (LFD) and treated with LPS (2 µg per mouse) or control (0.85% saline solution) on gestational day 7 and collected on gestational day 14. (HFD-LPS n=9; HFD-control n=14; LFD-LPS n=5; LFD-control n=6). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).

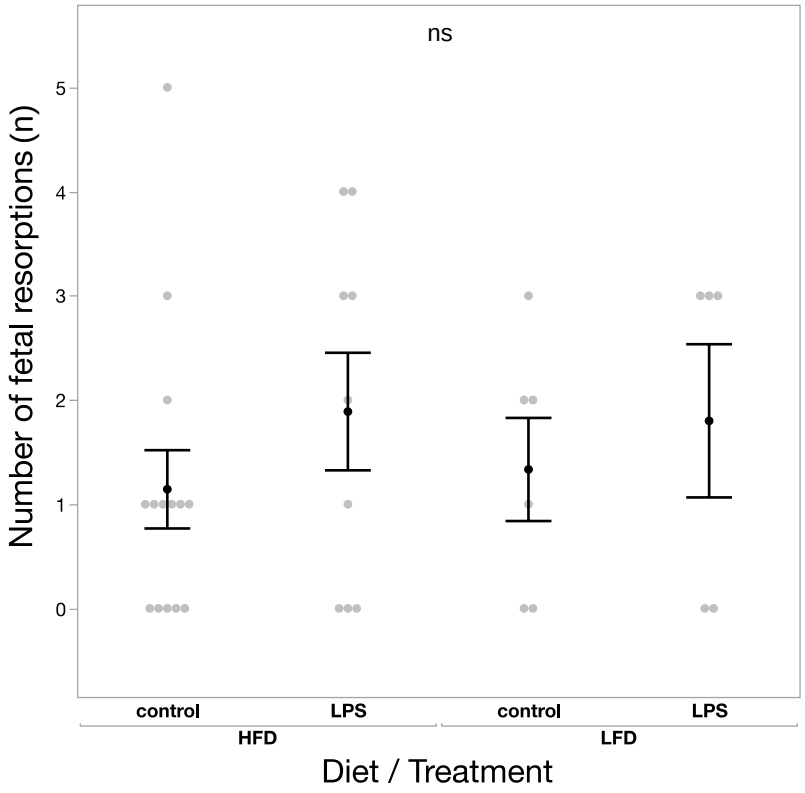

Figure 2.13. Number of resorptions (n) per litter of mice fed a hIgh-fat diet (HFD) or low-fat diet (LFD) and treated with LPS (2 µg per mouse) or control (0.85% saline solution) on gestational day 7 and collected on gestational day 14 (HFD-LPS n=9; HFD-control n=14; LFD-LPS n=5; LFD-control n=6). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).

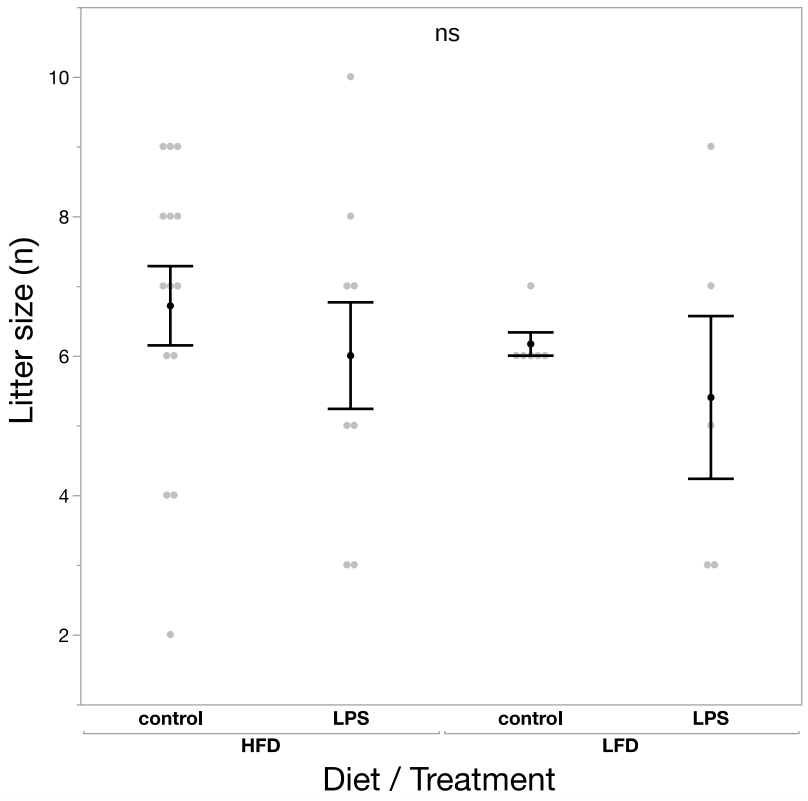

Figure 2.14. **Litter size (n) of mice fed a hIgh-fat diet (HFD) or low-fat diet (LFD) and treated with LPS (2 µg per mouse) or control (0.85% saline solution) on gestational day 7 and collected on gestational day 14 (HFD-LPS n=9; HFD-control n=14; LFD-LPS n=5; LFD-control n=6). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).**

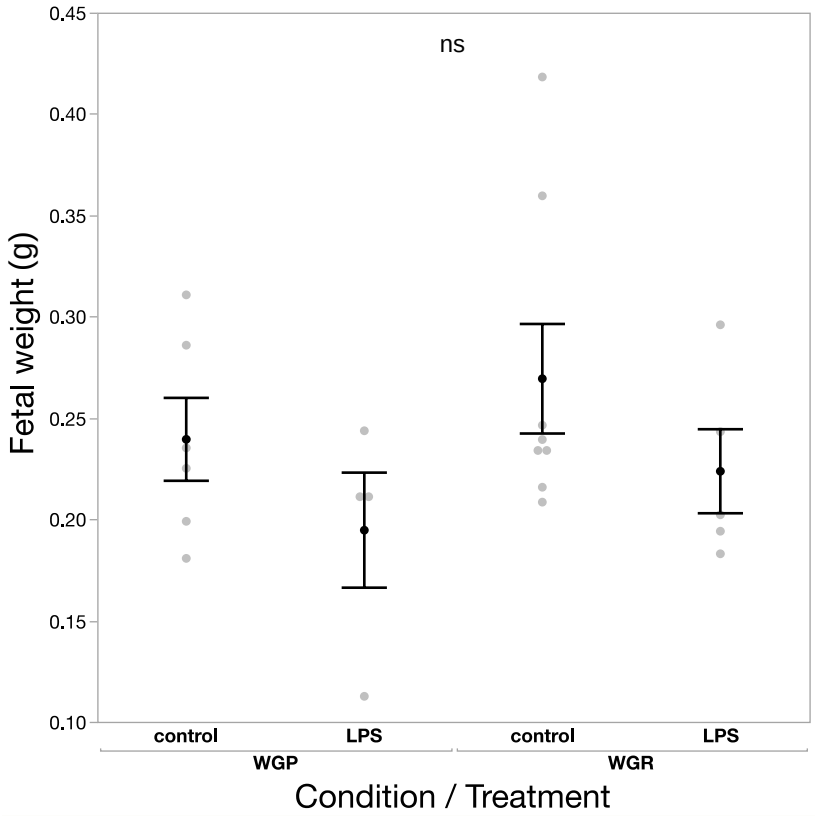

Figure 2.15. **Fetal weights (g) of weight-gain prone (WGP) and weight-gain resistant (WGR) mice fed a high-fat diet and subjected to LPS (2 µg per mouse) or control (0.85% saline solution) treatments on gestational day 7 and collected on gestational day 14 (WGP-LPS n=4; WGP-control n=6; WGR-LPS n=5; WGR-control n=8). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).**

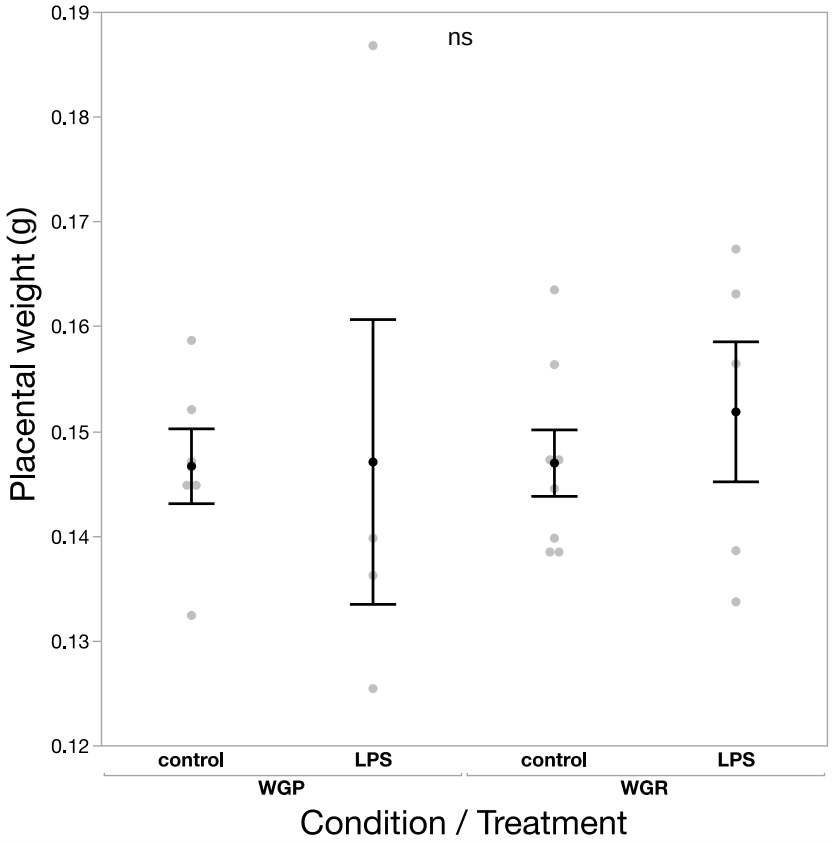

Figure 2.16. Placental weights (g) of weight-gain prone (WGP) and weight-gain resistant (WGR) mice subjected to LPS (2 µg per mouse) or control (0.85% saline solution) treatments on gestational day 7 and collected on gestational day 14 (WGP-LPS n=4; WGP-control n=6; WGR-LPS n=5; WGR-control n=8). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).

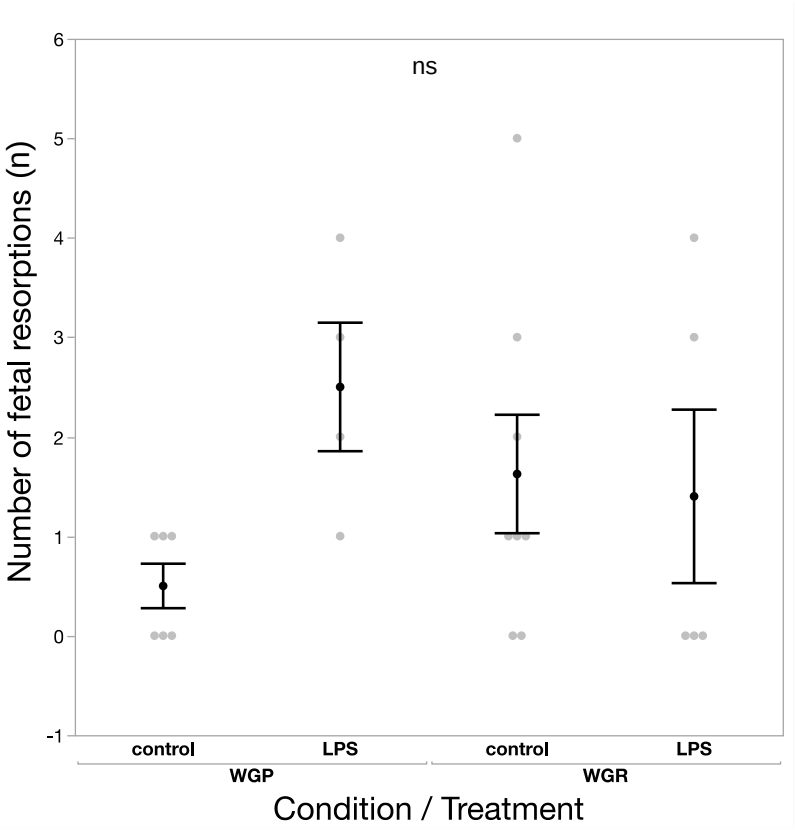

Figure 2.17. Number of resorptions (n) per litter of weight-gain prone (WGP) and weight-gain resistant (WGR) mice subjected to LPS (2 µg per mouse) or control (0.85% saline solution) treatments on gestational day 7 and collected on gestational day 14 (WGP-LPS n=4; WGP-control n=6; WGR-LPS n=5; WGR-control n=8). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).

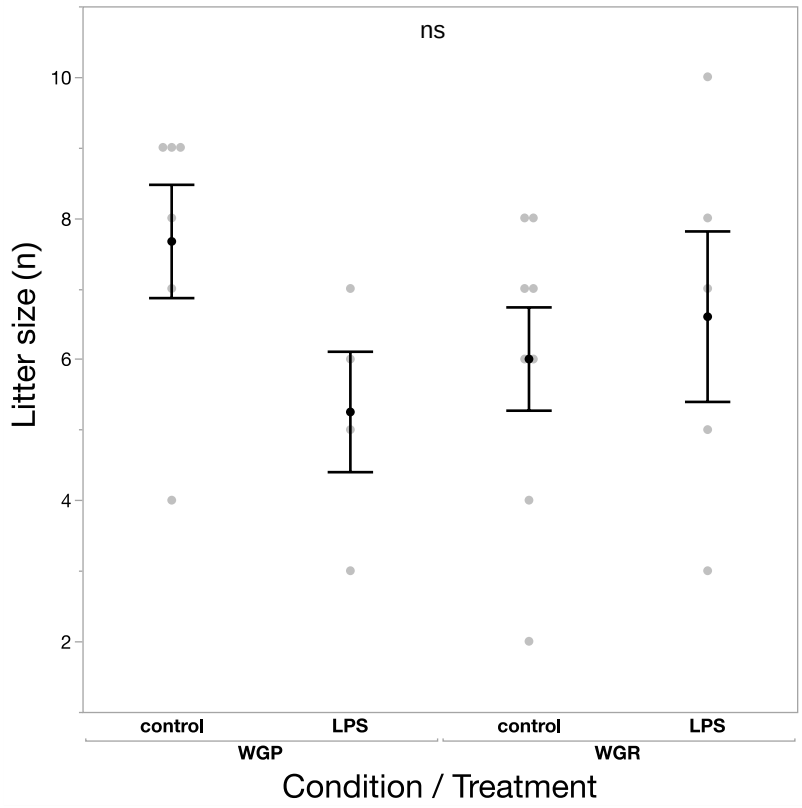

Figure 2.18. Litter size (n) of weight-gain prone (WGP) and weight-gain resistant (WGR) mice subjected to LPS (2 µg per mouse) or control (0.85% saline solution) treatments on gestational day 7 and collected on gestational day 14 (WGP-LPS n=4; WGP-control n=6; WGR-LPS n=5; WGR-control n=8). Data are presented as mean±SE. Differences were not statistically significant (*p>0.05*).

2.4. Conclusion

The first goal of my study was to find a suitable dose of LPS that would evoke small effects on pregnancy, which would not preclude effects being exacerbated by diet in subsequent experiments. The second goal was to establish a diet-LPS model and estimate the effects on pregnancy and fetal development, with focus on fetal weight, placental weight, litter size, fetal resorptions and abortion. Through preliminary dose-response experiments and a review of the literature, I selected 2 µg per mouse of LPS from *S. enterica* to use in my subsequent experiments. As this dose produced significant pregnancy loss (instances where mice thought to be pregnant showed no signs of pregnancy when collected) in subsequent experiments, it was a suitable dose to use.

To address the second goal of my study, I measured parameters of pregnancy and fetal outcome in my four experimental groups (HFD-LPS, HFD-control, LFD-LPS, LFD-control). I found that diet, LPS and a diet-LPS interaction had no effect on fetal weight, placental weight, litter size or the number of fetal resorptions per litter ($p>0.05$). Furthermore, these parameters also did not differ between mice in the HFD group that were categorized as being prone to weight gain or not (WGP vs WGR; $p>0.05$). I also found that diet, LPS or a diet-LPS interaction has no effect on the maternal serum concentration of an inflammatory marker – TNF-α. Irrespective of diet, it was more likely that a mouse injected with LPS on gestational day 7 showed no signs of pregnancy when collected on gestational day 14, compared to controls ($p<0.05$). This indicates that LPS injection may have resulted in abortion or complete fetal loss in some mice. Taken together, the findings of my study show no evidence that bacterial LPS exacerbates inflammation caused by HFD in pregnant mice.

Chapter 3.

Discussion and future directions

3.1. Effects of bacterial lipopolysaccharides (LPS)

3.1.1. Dose and species dependent effects

Severe pregnancy outcomes such as complete fetal loss due to relatively large doses of LPS (generally ≥10 µg per mouse) have been reported multiple times in the literature (Table 1.1). However, I found no effects of a large dose (20 µg per mouse) of LPS from *E. coli* on pregnancy or fetal development. Differences in these observed effects may be due to a difference in the route of LPS administration. Many studies have used intra-peritoneal or intra-venous LPS administration, but I used a subcutaneous route, through which the LPS is absorbed at a slower rate and has more sustained effects (Turner et al. 2011).

It is likely that the species from which the LPS is isolated may also play a role in its effects. The same dose of LPS (20 µg per mouse) from *S. enterica* resulted in more severe effects in some cases, including 100% fetal resorption in one mouse. It also potentially caused abortion in some mice – i.e. situations where a mouse that was thought to be pregnant before LPS injection, did not show any signs of pregnancy when collected. A lower dose from this species (5 µg per mouse) was still capable of inducing 86% fetal resorption in one mouse. This suggests that *S. enterica* LPS might be more potent in inducing immune responses and pregnancy complications in mice compared to *E. coli* LPS.

While considered closely related species, *E. coli* and *S. enterica* show genetic and phenotypic differences, including the molecular structure of LPS (Karibian et al. 1993; Heinrichs et al. 2002; Winfield and Groisman 2004). Therefore, it might be expected that LPS from these species invoke different immune responses and clinical outcomes. Jotwani et al. (1994) found that *S. enterica* was able to induce a much stronger cytokine response in mice compared to another bacterial species (*Bacteroides fragilis*) even with a dose 40 times lower.

Apart from the potency of the immune response, the immune pathway that is invoked by *S. enterica* and *E. coli* LPS also differs. Netea et al. (2001) found that TNF and LT (Lymphotoxin-α) knockout mice showed no effects when injected with *E. coli* LPS, however, showed fatality when injected with *Salmonella* LPS, accompanied by the production of the pro-inflammatory cytokines IL-1 and IFN-γ. Control mice ($TNF^{+/+}LT^{+/+}$) showed fatality with both *E. coli* and *Salmonella* LPS. This suggests that IL-1 and IFN-γ play an important role in the immune response to *Salmonella* LPS, but that TNF and LT may be more important in the case of *E. coli*.

3.1.2. Limitations of the use of LPS

The administration of bacterial lipopolysaccharides to mice during the gestational period can help us understand how an inflammatory insult can affect fetal development and pregnancy outcome. While humans and mice show some similarities in inflammatory responses to endotoxins such as LPS, mice are less sensitive and require a much higher dose to produce similar effects as humans (Copeland et al. 2005). Precautions should also be taken in equating this model of purified LPS administration to bacterial infection. Inoculation of pregnant mice with virulent *S. enterica* has been reported to activate TLR-4 (LPS-binding) receptors, and invoke immune responses including the expression of pro-inflammatory cytokines such as TNF-α and IL-6 and the infiltration of immune cells into the placenta (Chattopadhyay et al. 2010; Pejcic-Karapetrovic et al. 2007; Llana et al. 2014). These effects are accompanied by adverse fetal outcomes including abortion, fetal resorptions, fetal growth-restriction and preterm labour. These results appear to be similar to the effects observed in studies using only LPS administration. However, certain aspects of true infection are not easy to replicate, such as the ability of bacteria to proliferate, localize in placental layers and amniotic fluid, and cause trans-placental fetal infection.

3.2. Effects of HFD on mating-success

We found that mice fed a HFD required fewer mating attempts before becoming pregnant than mice fed a LFD. Additionally, HFD mice appeared to have a slightly greater reproductive success (94% pregnancy rate) than LFD mice (78.6% pregnancy rate; Table 2.1). This is an interesting result and contradicts findings from previous

studies. In general, mice and rats fed a high-fat diet are prone to reproductive impairments including altered estrous cycles and ovulation, irregular production of important reproductive hormones, ovarian cyst formation and infertility (Shaw et al. 1997; Tortoriello et al. 2004; Balasubramanian et al. 2012; Ngadjui et al. 2015; Chakraborty et al. 2016; Skaznik-Wikiel et al. 2016; Volk et al. 2017). Impaired reproductive function was irrespective of whether the animal developed an obese phenotype, that is whether or not the animal was prone to weight gain. Furthermore, these impairments could lower the reproductive rate of HFD animals; Wehmer et al. (1979) found that nearly half of experimental females fed a HFD could not conceive. A reduced conception rate was also observed in other studies (Shaw et al. 1997; Samuelsson et al. 2008). It is not clear why the HFD mice in our experiment had greater reproductive success, when kept under the same conditions and mated with the same males as the LFD mice.

3.3. Effects of HFD on fetal outcome

Based on previous findings (Kępczyńska et al. 2013; Perdu et al. 2014) it might be expected that HFD-fed mice would develop inflammation which would in-turn negatively affect fetal outcome. However, HFD mice in my experiment produced litters that did not differ from those produced by LFD mice in all parameters measured. Placental weights in HFD and LFD litters were similar, suggesting no evidence of altered placental development, which has been reported in HFD mice (Kim et al. 2014; Parker et al. 2014). Similarly, litter size and number of fetal resorptions per litter were not statistically different between HFD and LFD mice, suggesting that diet does not negatively affect fetal survival. These results are consistent with previous studies that used the same diet (Chin et al. 2017; Baltayeva et al. 2020). A limitation of our study however is the inability to assess the viability of offspring once they have been delivered. Even though HFD may not affect in-utero survival, studies have reported high neonatal mortality of delivered pups (Williams et al. 2017; Smoothy et al. 2019).

While there was no significant difference in fetal weight between HFD and LFD mice, there were trends in the data that could be examined. HFD control fetuses tended to be heavier than their LFD counterparts, which is contrary to the expectation that HFD would cause fetal growth-restriction. Instead, our data seem to show that HFD might cause fetal over-growth, however further investigation with larger sample sizes is necessary to confirm this. As reviewed elsewhere, a 45% kcal fat diet, similar to the one

used in this study, does not affect fetal growth in mice (Christians et al. 2019). However, Jones et al. (2009) observed fetal over-growth in mice using a 32% kcal fat diet and reported that this was due to an increase in placental nutrient transfer. Fetal over-growth in HFD mice was also reported in other studies (Rosario et al. 2015, 2016; Nam et al. 2017).

3.4. Effects of diet-LPS interaction on fetal outcome

There was no statistically significant effect of a diet-LPS interaction on any of the parameters measured – fetal and placental weights, fetal resorptions or litter size. However, there was a trend of HFD-mice that were challenged with LPS showing lower fetal weights, irrespective of whether they were weight-gain prone or weight-gain resistant. A larger sample size would be necessary to confirm these effects. These trends are not evident in LFD mice. It therefore appears that HFD mice may be more susceptible to the effects of bacterial LPS, resulting in lower fetal weights.

3.5. LPS mediated fetal loss

The odds that a female who was thought to be pregnant at gestational day 7 but did not show any sign of pregnancy at gestational day 14 were higher in LPS treated mice compared to controls. This suggests that LPS could have caused abortion (expulsion of all embryonic material from the uterus) in some mice; it is unlikely that the aborted material would be detected given that it would likely be eaten by the female. Ogando et al. (2003) reported that an LPS challenge in mice on day 7, at a dose of ~12.5µg per mouse, resulted in similar losses 24 hours after injection, with the implantation sites being completely expelled by the mother and no signs of pregnancy evident a few days later. While this dose was considerably higher than what I used (2µg per mouse), it does illustrate that in mice, early abortion of all embryonic material does not usually leave any evident signs a few days later.

Interestingly, in humans, second-trimester spontaneous abortion is significantly associated with bacterial vaginosis (Nelson et al. 2007; Giakoumelou et al. 2016; Isik et al. 2016). Therefore, Gram-negative bacterial mediated inflammation may have a role to play in spontaneous abortion in humans. However, in a comprehensive study of more than 1900 women, Nelson et al. (2007) found that increased risk of second-trimester

abortion was significantly associated with first-trimester decreases in the Gram-positive *Lactobacillus* in the vaginal flora, and had no association with increases in Gram-negative species such as *Gardnerella* sp. and *Moltibuncus* sp.

3.6. Limitations of the use of a mouse HFD model

Administering HFD to mice is generally a good model to mimic the increased adiposity and conditions like hypertension and hyperinsulinemia observed in obese humans (Lang et al. 2019). However, there are also certain limitations that should be considered when comparing HFD-fed rodents to obese humans. Firstly, the HFD may not be an accurate reflection of an obese person's diet. Many studies have instead used a cafeteria diet (CAF), which includes processed foods and snack items like chips, cookies and crackers, commonly consumed by people (Leigh et al. 2019). Sampey et al. (2011) found that both HFD and CAF diets induced increased adiposity, however the CAF diet provided a more robust model of human obesity and induced higher levels of adipose tissue inflammation compared to the HFD. However, since CAF diets are made up of mixtures of human food with no specific recipe, there is considerable heterogeneity between studies.

More recently, Bortolin et al. (2018) designed the Western diet (WD) to reflect an obese person's diet (high-fat, high-sugar, high-salt, low-fibre) and have a consistent composition. The authors found that this diet effectively induced obesity and obesity-related disorders. The WD led to significantly higher weight gain, fat accumulation and adiposity index in rats, compared to the HFD and CAF. It also caused increased serum TNF-α and leptin compared to the other diets, and more efficiently modeled a pre-diabetic condition by increasing insulin levels and insulin resistance. To my knowledge, a comparison of the effects of HFD, CAF and WD on pregnancy outcome in rodents has not been conducted. Such a study may be useful in choosing the most appropriate diet-based model to study complications observed in obese human pregnancies.

3.7. Concluding remarks

Unregulated inflammation during pregnancy and fetal development can result in undesirable outcomes including spontaneous abortion, pre-term labour, fetal death and various developmental abnormalities in offspring. In this study, I focused on two triggers

of such inflammation during pregnancy in mice - maternal HFD and LPS exposure. I hypothesized that HFD-fed pregnant mice would experience inflammation, which would be exacerbated by an additional LPS challenge, resulting in adverse pregnancy outcomes. To test this, I first conducted a series of preliminary dose-response experiments to determine the dose of LPS that would be appropriate to use. I then set-up a model of diet-LPS interaction and measured various parameters of pregnancy and fetal outcome in my experimental mice.

I found that the effects of LPS on pregnancy can be affected by both the dose, as well as the species from which the LPS is isolated. LPS from *S. enterica* appeared to produce more potent effects compared to LPS from *E. coli*. This can be explained by various differences between the two bacterial species including the molecular structure of LPS, and different immune pathways induced. My results showed that mice from the four treatment groups (HFD-LPS, HFD-control, LFD-LPS, LFD-control) showed no difference in serum concentration of TNF-α, an inflammatory marker that was found to be elevated in mice fed the same HFD by Baltayeva et al. (2020). It was significantly more likely that a mouse injected with LPS showed no signs of pregnancy when collected as compared to controls, irrespective of diet. This suggests that the LPS dose used could result in complete fetal loss (abortion).

There was no effect of diet, LPS or a diet-LPS interaction on fetal weight, placental weight, litter size or number of fetal resorptions per litter. Furthermore, there was also no difference in these parameters between HFD mice that were prone to weight-gain or resistant to weight-gain. While not statistically significant, there were some interesting trends in my data that could be further explored with larger sample sizes. I found that HFD fetuses tended to be heavier, and this has been observed in some other studies. I also found that HFD mice that were challenged with LPS appeared to have lower fetal weights compared to controls, irrespective of whether the mice were prone to weight-gain or not. This trend was not evident in LFD mice.

While not statistically significant, my results show a trend of HFD-LPS challenged mice having smaller fetuses (fetal growth-restriction). In exploring this further, it might be worthwhile to consider other rodent diets that are currently in use, namely CAF and WD. While the HFD has its merits, CAF and WD were found to better model human obesity and its comorbidities, particularly inflammation. In this regard, a comparison of these

diets and their effects specifically on pregnancy and fetal outcome might be useful for future studies.

Taken together, my findings provide no evidence that LPS exacerbates the inflammatory effects of HFD in mice, leading to any complications of pregnancy. In humans, while obesity-induced inflammation might still contribute to the pathophysiology of many pregnancy complications, it is unlikely to be the only factor responsible, and my data support the idea that an additional mild inflammatory insult is unlikely to exacerbate its inflammatory effects.

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