The influence of maternal diet on intestinal adaptation in the mother and offspring

Stuart Astbury, BSc (Hons), MMedSci

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Abstract

The effect of maternal nutrition on fetal and offspring growth and risk of disease in later life is well established. Animal models have shown that the small intestine can be preferentially affected by growth restriction compared with other organs, yet the effect of maternal diet on gastrointestinal (GI) development has not been well studied. Furthermore, both the maternal GI tract and microbiome undergo significant adaptations during pregnancy in response to the increased nutritional demands of the growing fetus, which may be further modulated by the maternal diet.

Inadequate development of the GI tract may result in an increase in intestinal permeability, as demonstrated in inflammatory bowel diseases. Compromised gut barrier function has been linked to the development of obesity through the passage of endotoxin on Gram-negative bacteria leading to low-level inflammation of the liver and adipose tissue. The aim of this thesis was to use two animal models of maternal dietary manipulation that are representative of suboptimal nutrition characterised by the nutritional excess of macronutrients found in many Western diets. The effect of either fat in the form of palm oil and carbohydrate in the form of fructose were therefore examined with particular focus on the gut. This was undertaken in a pig model to examine the effects around the time of birth and in the juvenile offspring, whilst in a rat model both the mothers and offspring were studied.

Addition of 10% fructose to drinking water in two generations of pregnant Wistar rats induced impaired glucose tolerance and raised serum triglycerides, but only in the second generation. Sequencing of the maternal microbiome in first generation dams through pregnancy revealed significant changes in microbial diversity from pre-mating to late

pregnancy, both between and within dams. Consumption of the fructose diet led to further changes in microbial diversity. Offspring of fructose-fed mothers were growth restricted and had significantly shorter small intestines compared to controls. Changes in gene expression in the ileum and jejunum were indicative of raised intestinal permeability in second-generation dams, and included the epithelial tight junction genes occludin (OCLN), claudin (CLDN) and junctional adhesion molecule (JAM). In both generations the fructose diet significantly upregulated glucose transporters 2 and 5, and sodium-glucose linked transporter 1 in the ileum and jejunum suggesting that a relatively low level supplementation of fructose gradually increases the absorptive capacity of the small intestine for both glucose and fructose. This may explain the significantly impaired glucose tolerance and insulin resistance observed in second-generation offspring.

To study maternal fat supplementation, sows were fed a standard commercial diet supplemented with palm oil (6.6% added to commercial feed) throughout gestation. Median birth weight offspring born to fat supplemented mothers sampled at 7 days demonstrated no growth restriction, but significant downregulation in OCLN, CLDN and JAMA expression, suggesting increased intestinal permeability. No changes in offspring body composition were observed at either time point, and effects on gene expression did not persist up to 6 months of age.

In conclusion, a suboptimal diet through pregnancy in which macronutrient composition is raised has the potential to compromise gut function in the mother and offspring. The magnitude of effect can however be temporary and dependent on the animal model used as well as the macronutrient targeted.

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Declaration

The work presented in this thesis was carried out in the division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham, and the Division of Human Nutrition, Department of Agricultural, Food and Nutritional Science, University of Alberta, between October 2011 and September 2015.

This thesis illustrates my own work, completed under the supervision of Dr Alison Mostyn, Dr Don Sharkey and Professor Michael Symonds at the University of Nottingham, and Dr Rhonda Bell at the University of Alberta. Experimental work carried out by others has been acknowledged. This report is an accurate representation of the work performed and no other study reproducing this work has, to my knowledge, been carried out at the University of Nottingham or University of Alberta.

Stuart Astbury
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List of abbreviations

18s	18s ribosomal RNA
ACC	acetyl-coA carboxylase
AGA	appropriate for gestational age
ANOSIM	analysis of similarity
ANOVA	analysis of variance
APS	ammonium persulphate
ATP	adenosine triphosphate
AUC	area under the curve
BCA	bicinchoninic acid
BD	beta defensin
BLAST	Basic Local Alignment Search Tool
BMI	body mass index
bp	base pairs
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
C/EBPα	CCAAT-enhancer binding protein alpha
CLDN	claudin
C_p	crossing point
C_t	cycle threshold
DNA	deoxyribonucleic acid
dNTP	deoxyribose nucleoside triphosphate
DPX	dibutyl phthalate in xylene
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFr	epidermal growth factor receptor
ELISA	enzyme-linked immune-sorbent assay
EPEC	enteropathogenic *Escherichia coli*
FAS	fatty acid synthase
FITC	fluorescein isothyocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GD	gestational day
GF	germ-free
GI	gastrointestinal
GIT	gastrointestinal tract
GLUT	glucose transporter
GTT	glucose tolerance test
HFCS	high-fructose corn syrup
HRP	horseradish peroxidase
IGF	insulin-like growth factor
IL	interleukin
IPO8	importin-8
IR	insulin resistance
IUGR	intrauterine growth restriction

JAM	junctional adhesion molecule
JNK	C-jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBW	low birth weight
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MLCK	myosin light chain kinase
MUC	mucin
mRNA	messenger ribonucleic acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEC	necrotising enterocolitis
NEFA	non-esterified fatty acid
NF	nuclear factor
NICE	national institute for clinical excellence
OCLN	occludin
OGTT	oral glucose tolerance test
OTU	operational taxonomic unit
PBS	phosphate-buffered saline
PGC1β	peroxisome proliferator-activated receptor γ coactivator-1 β
PMSF	phenylmethanesulfonylfluoride
PPARγ	peroxisome proliferator-activated receptor gamma
PPi	pyrophosphate
PRR	pattern recognition receptors
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPLP0	60s acidic ribosomal protein P0
RT	reverse transcription/reverse transcriptase
SDS	sodium dodecyl sulphate
SGLT	sodium-glucose linked transporter
T1 – T3	first trimester – third trimester
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween (polysorbate) 20
TEMED	tetramethylethylenediamine
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumour necrosis factor
VLDL	very low density lipoprotein
WHO	World Health Organization
ZO	zonulin

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Published work

Work included in this thesis or from related studies has been published or presented during the course of writing. Selected abstracts are included in Appendices A and B.

Articles

Astbury S, Mostyn A, Symonds ME, Bell RC (2015) Nutrient availability, the microbiome and intestinal transport during pregnancy. *Applied Physiology, Nutrition, and Metabolism* (in press, doi: 10.1139/apnm-2015-0117).

Lineker C, Kerr PM, Nguyen P, Bloor I, **Astbury S**, Patel N, Budge H, Hemmings DG, Plane F, Symonds ME, Bell RC (2015) High fructose consumption in pregnancy alters the perinatal environment without increasing metabolic disease in the offspring. *Reproduction, Fertility and Development* (in press, doi: 10.1071/RD15119).

Short communications and conference presentations

Astbury S, Mostyn A, Sharkey D, Symonds ME (2015) Supplementation of the maternal diet with palm oil affects intestinal permeability in offspring at 7 days but not 6 months. *Physiology 2015, Cardiff, UK* (July 2015, poster presentation).

Astbury S, Song A, Nielsen B, Coursen N, Hoedl A, Bell RC (2014) Exposure to a high fructose diet during pregnancy remodels the intestinal and body composition in the near-term pregnant offspring. *Obesity: A Physiological Perspective (Topic Meeting of The Physiological Society), Newcastle, UK* (September 2014, poster presentation).

Song A, **Astbury S**, Coursen N, Hoedl, A, Bell RC (2014) Maternal High-Fructose Intake Induces Adverse Effects on Their Metabolic Profile and that of Their Female Offspring in Pregnancy. *Canadian Diabetes Association/Canadian Society of Endocrinology and Metabolism Annual Meeting 2014, Winnipeg, MB, Canada* (poster presentation by Aleida Song).

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Astbury S, Song A, Nielsen B, Coursen N, Hoedl, A, Bell RC (2013) The effect of maternal diet on offspring gut development. *International Pregnancy Research Symposium, University of Alberta, Edmonton, AB, Canada* (September 2013, oral presentation).

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Chapter 1: Introduction

1.1. Obesity and overweight

Given the increasing prevalence of obesity and its associated comorbidities which include insulin resistance (IR), type 2 diabetes, chronic low-grade inflammation, hypertension, dyslipidaemia, coronary heart disease, stroke and cancer (1), it has now been recognised as a major public health concern. Obesity is defined as the excessive accumulation of body fat; currently overweight is classified by the World Health Organisation (WHO) as a body mass index (BMI) of greater than 25, and obesity a BMI of over 30. As BMI, calculated by weight (in kg) divided by height squared (in metres) does not make any allowances for differing body compositions (e.g. muscle vs fat mass), the National Institute for Clinical Excellence (NICE) recommends that waist circumference be used in conjunction with BMI to define obesity (2). Table 1-1 outlines the WHO classifications for BMI.

Classification	BMI (kg/m²)	
	Principal cut-off points	*Additional cut-off points*
Severe Thinness	<16.00	<16.00
Moderate Thinness	16.00 – 16.99	16.00-16.99
Mild Thinness	17.00 – 18.49	17.00-18.49
Underweight	<18.50	<18.50
Normal Range	18.50 – 24.99	18.50-22.99
Overweight	≥25.00	≥25.00
Pre-obese	25.00 – 29.99	25.00 – 27.49
		27.50 – 29.99
Obese	≥30.00	≥30.00
Obese class I	30.00 – 34.99	30.00 – 32.49
		32.49 – 34.99
Obese class II	35.00 – 39.99	35.00 – 37.49
		37.49 – 39.99
Obese class III	≥40.00	≥40.00

Table 1-1: World Health Organisation classification of adult underweight, overweight and obesity (3).

The proportion of adults worldwide with a BMI of over 25kg/m² has increased between 1980 and 2013 to 36.9% in men and 38.0% in women (4), making obesity one of the leading preventable causes of mortality worldwide. Economically, it is estimated that obesity now has direct costs to the National Health Service of almost £5billion per year, and indirect costs (through loss of earnings and reduced productivity) of almost £2billion (5). In 2013 this was equivalent to over 5% of the total budget for NHS England (£94.2billion) (6).

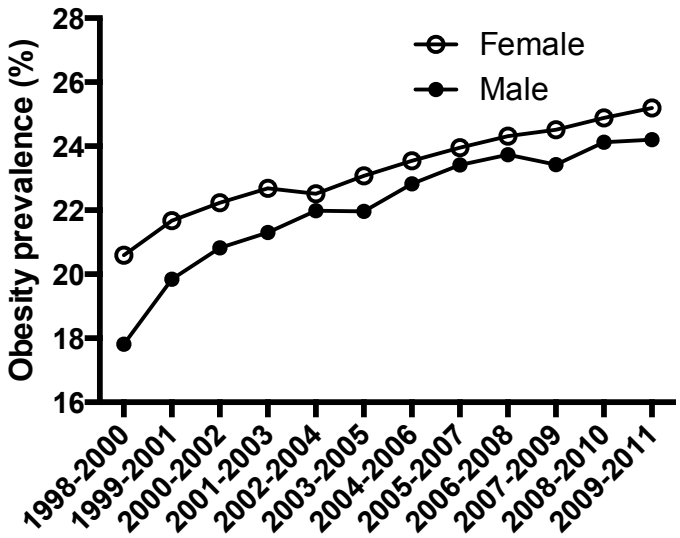


Figure 1-1: Percentage obesity prevalence (BMI >30kg/m^2) in England.

Data points are three year averages. Adapted from (7).

Although the overarching cause of obesity and overweight is understood to be a prolonged imbalance of energy intake and expenditure, the reasons for this imbalance are a complex interaction involving psychology, socioeconomic factors, genetics and the environment (8). The most recent UK-based research suggests that factors such as diet and physical activity have more of a bearing on the development of obesity than any genetic factors (9). However this remains a controversial topic, with the importance of exercise being debated (10,11).

1.2. Insulin resistance and type II diabetes

IR can be defined as a reduced physiological response to the hormone insulin, meaning that glucose is not taken up into tissues for metabolism and remains in the circulation, thereby leading to hyperglycaemia. Initially, increased amounts of insulin will be secreted in response to glucose and maintain plasma glucose levels, known as the compensated phase of insulin resistance. For IR to develop into type II diabetes, pancreatic β-cell activity must be sufficiently diminished that it cannot compensate for the decrease in insulin sensitivity (12). There are a number of potential hypotheses to explain the progression from IR in obesity to type II diabetes. Firstly a decreased release of insulin will lead to disordered glucose regulation due to increased hepatic gluconeogenesis, and a reduction in glucose uptake in tissues sensitive to insulin. Decreased production of insulin may also lead to disrupted adipocyte metabolism, resulting in increased lipolysis and subsequent increase in serum non-esterified fatty acids (NEFA) This simultaneous elevation of both circulating glucose and NEFA have been shown to be detrimental to pancreatic islets and therefore insulin secretion (13,14) and have been proposed to be part of a “feed-forward” mechanism in which the development of type II diabetes is a slow process that can be initiated by obesity (15).

1.3. Developmental origins of health and disease

There is now a significant body of epidemiological evidence linking both fetal and placental size, as well as maternal nutrition, with the development of one or more conditions of the metabolic syndrome in later life (16–19). The most significant of these in the UK are the cohort studies conducted by the epidemiologist David Barker and colleagues, in which reduced placental and birth weights were linked to raised blood pressure in adulthood (20) and low birth weight linked to increased risk of ischaemic heart disease (21). Further follow-up studies then demonstrated an association between low birth weight as a result of failure to thrive *in utero* and cardiovascular disease in later life (22). These effects were also observed after adjusting for postnatal environmental factors, such as socioeconomic status, smoking and obesity in adult life. Finally, the development of disease in later life was also found to be independent of restrictions such as the mother's height and pelvic size (despite the association between these and low birth weight) (23), this would suggest that maternal nutrition, and the effective transport of nutrients to the developing fetus are of more importance and interest than physical restrictions on growth. These findings are also consistent with an equally well-known cohort study, using subjects exposed to the Dutch famine of 1944-1945. Individuals born to mothers exposed to the famine during mid and late gestation displayed a higher risk of cardiovascular disease as well as reduced glucose tolerance (24).

This link between a suboptimal environment *in utero* and disease in later life has been variously termed the 'Barker hypothesis', 'thrifty phenotype' or the developmental origins of health and disease (DOHaD) hypothesis.

1.4. Maternal nutrition

In an effort to understand the above associations, a significant amount of research has been conducted into the effect of maternal diet on fetal development, either via large-scale cohort studies as summarised above, or smaller scale studies using animal models in order to understand the mechanisms behind any associations observed. The use of animal models has enabled the study of a variety of maternal diets, including those that would be unethical to study in humans, such as the effect of a maternal diet deficient in protein on offspring development.

These studies, predominantly conducted in rats and sheep (the use of different animal models will be discussed later), clearly demonstrate a variety of effects caused by suboptimal maternal diets. It has been shown that a maternal diet low in protein will result in raised blood pressure in the offspring, both in rats (25,26), and sheep (27). A maternal high-fat diet will also bring about the same changes, both increasing risk of hypertension in the offspring (28), as well as risk of obesity (29).

However, as is the case with many dietary intervention studies, an awareness of potential confounders is essential. For example in two of the aforementioned studies (25,26), the low protein intervention was supplemented with carbohydrate to make the diets isocaloric.

1.5. Effects of a maternal high-fat diet

Much research has been carried out into the effects of a maternal high-fat diet on offspring development, in part due to its potential relevance to present day "Western diets". It is far more likely for a mother in the West to be consuming a diet with excessive amounts of fat rather than one that is equivalent to the protein restriction used in the aforementioned studies. The established links between increased fat intake and IR (30–32), in addition to pregnancy being an insulin-resistant state (thought to be an evolutionary adaptation to pregnancy in order to increase glucose availability for the developing fetus) (33,34), makes raised fat intake during pregnancy and the effect on the offspring of particular interest.

The study of the effect of a high-fat diet during pregnancy in rats has produced several interesting, although not entirely consistent results. Firstly, hypertension has been observed in the offspring of rats fed lard during pregnancy, with a strong gender bias (hypertension was observed in female offspring, but not male) (35). Offspring of dams fed a high-fat diet throughout pregnancy also display significantly increased amounts of both total and abdominal fat, along with a reduction in insulin sensitivity (measured using an oral glucose challenge) and expression of hepatic insulin signalling genes (36). Lactation has also been shown to be of importance: rats born to fat-fed dams and cross-fostered to control dams show a smaller increase in blood pressure at 180 days of age compared to those born to control dams but cross-fostered to dams fed a high-fat diet (37).

Despite these findings, the results of many of the above studies should be interpreted with caution as discussed above. If a high-fat diet is being used as the intervention, other constituents such as protein are reduced in order to make the intervention and control diets isocaloric. For example, the aforementioned Buckley et al. (36) study increased the fat content of the diet fed to pregnant rat dams to 59% (with 12% in the standard

laboratory chow fed to controls); the diets were made isocaloric by significantly reducing the amount of carbohydrate (20% vs 65% in the control diet). Dietary composition will also influence consumption; rats placed on a high-fat diet may consume less feed, leading to a diet that is high in fat but also low in carbohydrate and protein compared to controls (35). In most cases such effects cannot be avoided, but they do require an awareness of potential confounders, as well as full description of the diets used in a study when interpreting the results.

1.6. Effects of a maternal high-fructose diet

The most common naturally occurring sugars in the conventional human diet are glucose, fructose and sucrose (38). Glucose and fructose are monosaccharides, the most basic unit of carbohydrates which then form the building blocks of more complex carbohydrates. Sucrose is an example of a disaccharide, formed by a molecule of fructose connected to a molecule of glucose via an α-1,4-glycoside bond. In nature as opposed to addition during food manufacture glucose and fructose are most commonly found in fruits and honey, and sucrose is more commonly known as “table sugar” sourced from sugarcane and sugar beets.

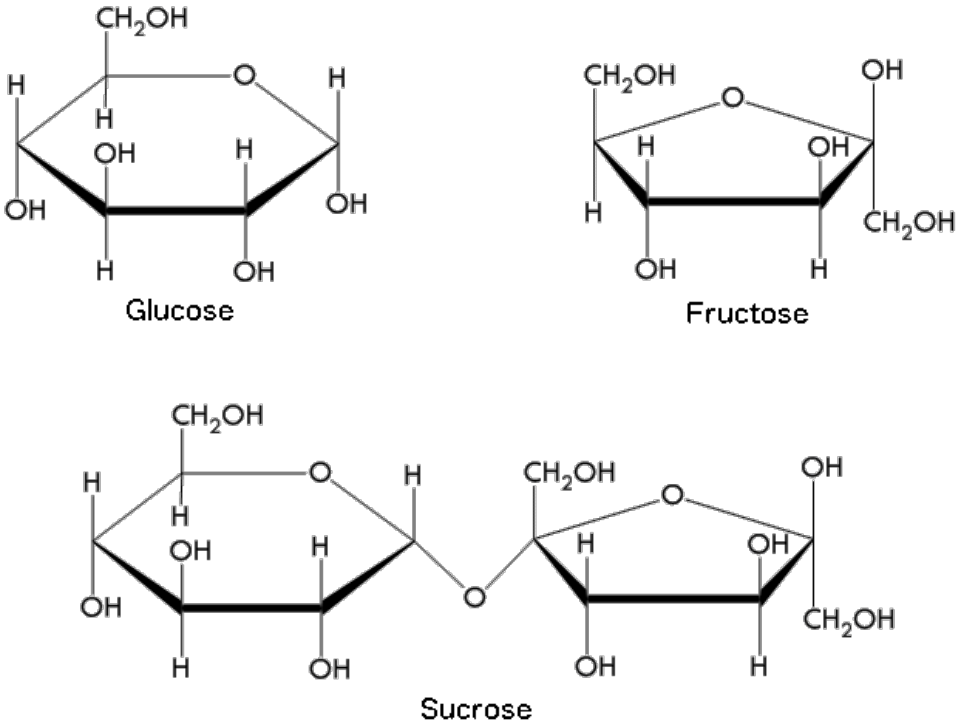


Figure 1-2: Structures of the naturally occurring saccharides glucose, fructose and sucrose.

Intake of dietary fructose has dramatically increased from 1970 onwards, with availability of "added sugars" of which fructose is a major contributor increasing by 25% between 1970 and 2000 in the United States (39). Fructose intake has increased, along with the prevalence in obesity over the last four decades and is now proposed by some to be the predominant carbohydrate responsible for the rise in caloric consumption in the Western diet, both consumed as sucrose (containing 50% fructose) or high fructose corn syrup (HFCS) (40). Although HFCS has received the majority of media attention, fructose alone can cause similar adverse effects, for example the rise in fruit juice consumption has been highlighted as a potential public health concern (41,42).

Due to its increased sweetness, HFCS has become a cheap method of increasing the sweetness of foods and beverages, particularly in countries such as the United States, which produce a relatively small amount of cane sugar due to the climate required, but a large amount of corn. There are

some important international differences here which are relevant to intake of fructose as HFCS – due to a lack of economic regulation in the 1980s, the United States quickly became the world's largest producer, user and exporter of HFCS (43), whereas the European Union placed a strict production quota and import levies on HFCS, in force until 2017 (44).

Whereas phosphofructokinase serves as a rate limiting step in glycolysis with its activity inhibited by adenosine triphosphate (ATP), this step is bypassed in fructolysis allowing effectively unregulated lipogenesis (highlighted in Figure 1-3). Compared with glucose, fructose will not increase insulin and suppress grehlin secretion (39). In a number of adult animal models, feeding of a high-fructose diet will readily induce IR (45,46), hypertension (47) and dyslipidaemia (48).

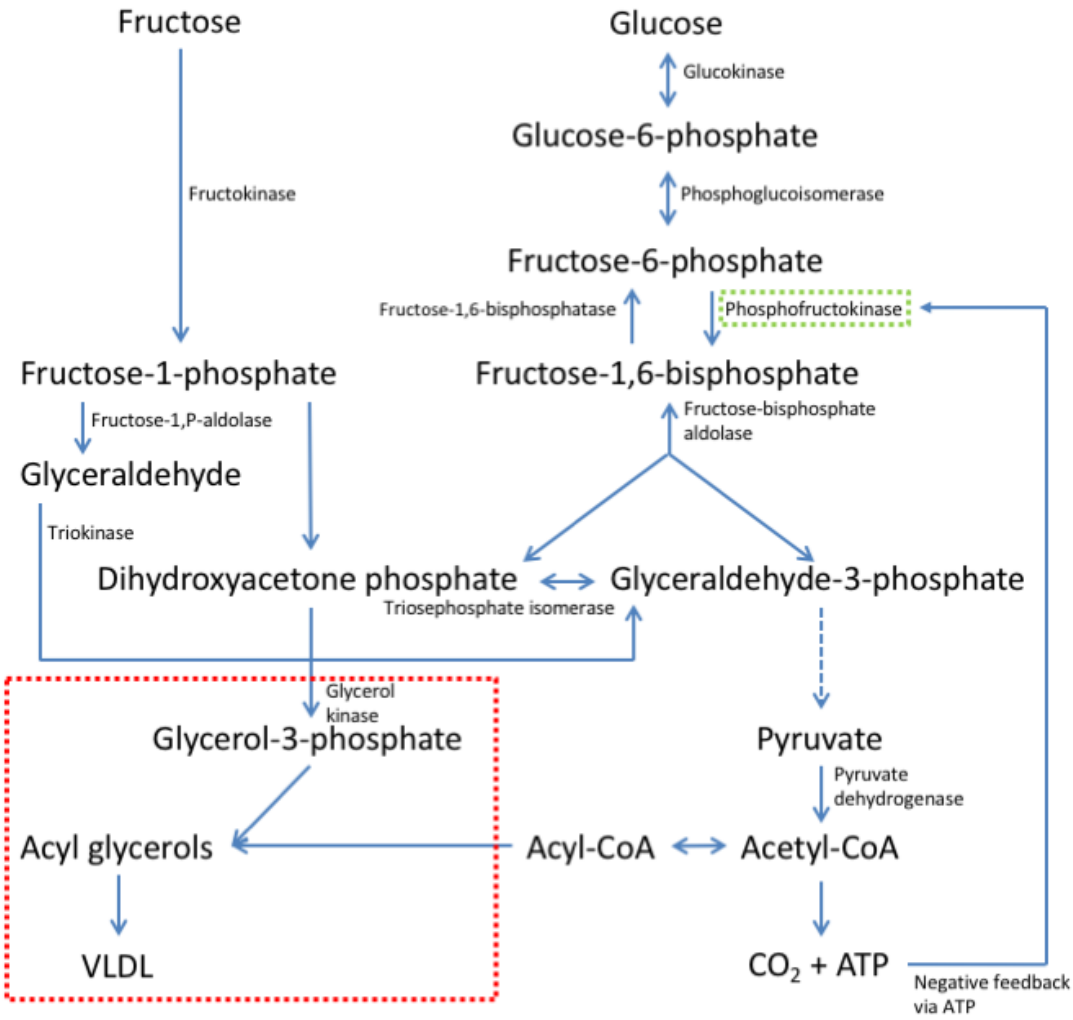


Figure 1-3: Glycolytic and fructolytic pathways.

Excess fructose will be diverted to VLDL production (lipogenesis) with no rate limiting step, highlighted in red, whereas ATP produced via the glycolytic pathway has a negative feedback effect on phosphofructokinase (highlighted in green), limiting the rate of reaction.

This metabolic pathway, as well as the increase in consumption since the 1980s have made fructose of interest with regard to the development of obesity and associated conditions such as hypertension (49) , non-alcoholic fatty liver disease (50) and coronary artery disease (51). Epidemiological studies focusing on children and adolescents have also shown fructose is associated with an increased incidence of obesity, measured from the consumption of sugar sweetened beverages (52) and measured as total fructose from dietary recalls (53). A recent meta-analysis covering 17 studies (~38000 total cases) showed that habitual consumption of sugar sweetened beverages and fruit juices were associated with an increased incidence of type II diabetes, independent of adiposity (42,54).

From a DOHaD perspective, the effect of a maternal diet high in fructose on offspring development is largely unknown. Studies in pregnancy using a rat model have shown fructose will induce increased adiposity, increased serum triglycerides and hyperinsulinaemia in the mother (55) and can significantly reduce the weight of the placenta (an effect restricted to the placentae of female offspring) (56), and can also upregulate genes related to lipogenesis in the fetal offspring liver (57). However research into how this will then impact metabolic health in the offspring is lacking.

1.6.1. Uptake of fructose in the intestine

In the small intestine fructose is primarily absorbed in the jejunum, with glucose transporter 5 (GLUT5) being the transporter responsible for the majority of uptake (58). The remaining fructose that is absorbed will be taken up by either glucose transporter 2 (GLUT2) or sodium-glucose linked transporter 1 (SGLT1) with fructose competing with glucose for uptake (59).

1.6.2. Glucose transporter (GLUT) and sodium-glucose linked transporter (SGLT) proteins

The GLUT and SGLT proteins are located throughout the body, and are responsible for the transport of glucose and fructose into cells. In the intestine the isoforms SGLT1, GLUT2 and GLUT5 are responsible for sugar transport into the enterocytes from the intestinal lumen, as well as into the portal circulation via the enterocyte basement membrane (58,60,61).

Whereas SGLT1 is capable of transporting both glucose and fructose the transporter GLUT5 is highly specific for fructose (58). It has been shown to be located both on the brush border membrane of the small intestine as well as the basement membrane of the enterocytes in the jejunum (62).

Fructose transport (as well as the transport of other carbohydrates) has been shown to be a dynamic process that will respond to the diet. SGLT1 expression has been shown to respond to a high carbohydrate diet within 1 hour (63), and this has been correlated with rate of carbohydrate transport (64). Although the previous two studies were carried out in rats, this hypothesis has also been tested in humans, with samples taken from jejunal biopsies (65). GLUT5 has been shown to respond in a similar way to the diet, with fructose transport increasing 2-fold (measured via J_{max}) within 3 days in the presence of the glucose transport inhibitor phloridzin (66). This has since been shown to be due to an increase in GLUT5 mRNA and protein expression. Feeding high-fructose pellets to rats will increase GLUT5 protein expression 5-fold within 1 day compared to controls, with expression increasing gradually if the fructose supplementation is continued. In the same study the researchers showed that isolating a section of small intestine from the rest (whilst maintaining mesenteric blood supply) prevented this fructose-induced GLUT5 increase, suggesting that fructose has to directly interact with the brush-border membrane to have any regulatory effect (67).

1.7. Intrauterine growth restriction

Offspring birth weight, independent of any dietary interventions also appears to have an effect on the development of diseases associated with the metabolic syndrome in later life. In this case, birth weight is usually reduced due to other factors, with the most common being intrauterine growth restriction (IUGR). In many cases, IUGR is linked closely with maternal diet, and can be caused by maternal malnutrition or smoking (68). However, it can also be caused by preeclampsia, multiple gestation, or infection (69,70). A large amount of research has been carried out in this field, both in the pig (large litter sizes can lead to a natural form of IUGR in some breeds) and the rat (the ease of various interventions such as uterine artery ligation, which can mimic human uteroplacental insufficiency (71,72)).

IUGR induced via ligation of both uterine arteries at gestational day 19 has been shown to lead to type-II diabetes-like symptoms in the rat, with a fasting hyperglycaemia beginning at 7-10 weeks of age and gradually worsening. The same rats also displayed a 50% reduction in insulin sensitivity at 1 week of age, progressing to full type-II diabetes by 26 weeks (72). Similar results have also been found in pigs with natural IUGR (73), with a reduction in insulin sensitivity at 12 months.

1.8. Catch-up growth

While a full description of adequate nutrition in both IUGR and preterm infants is beyond the scope of my thesis, a large amount of research has been conducted into the effects of nutrition on growth and development in IUGR and preterm infants, including development of the gastrointestinal tract. Much of this focuses on 'catch-up growth', an adaptation to the postnatal growth failure observed in both preterm and IUGR infants.

Whilst the cause of growth failure is often obvious in the case of IUGR infants, it is also common in preterm infants, if not universal (74–76).

Several hypotheses have been put forward to explain these altered growth trajectories, in the case of growth-restricted infants the 'thrifty phenotype' hypothesis provides a possible explanation. Fetal adaptation to either inadequate maternal nutrition or placental insufficiency leads to development of peripheral IR, as well as downregulation of insulin-like growth factor (IGF) secretion (77,78). This effectively redistributes the glucose that is available to the vital organs. After birth, once an adequate supply of glucose is restored, this IR continues and is possibly worsened by the effects of IGF. Therefore the adaptations responsible for stimulating catch-up growth are also causing peripheral IR (79). This is consistent with more recent research in both IUGR infants (80,81), as well as preterms (82).

The intestine has been shown to be disproportionately affected by growth restriction in a pig model (83,84), with other organs such as the liver being less significantly affected as a percentage of body weight when comparing IUGR and control (normal weight) piglets. Similar studies have shown the same effects in rat IUGR models(85,86). There has been little work covering the effect of growth restriction on either nutrient transport or intestinal permeability, and whether the changes that have been observed are reversible or will last into adulthood.

1.9. Gastrointestinal anatomy and development

There are established links between both the overall health of the gastrointestinal (GI) tract, the microbiota and the development of obesity (87,88), but precise mechanisms have not been fully characterised. Therefore, from a nutritional programming and DOHaD perspective, the development of the GI tract both *in utero* and in early life may be of some

interest in understanding the developmental origins of obesity. In humans, development of the GI tract from the primitive gut tube is outlined in Table 1-2.

	Characteristics
~4 weeks	Folding of the embryo, part of the yolk sack forms the primitive gut.
~7 weeks	Liver and pancreas form as buds from the gut tube.
~8 weeks	Villi formation, formation of junctional complexes between epithelial cells.
~12 weeks	Organ formation complete, stomach epithelium forms (parietal cells). Measurable enzyme activity. Bile secretion.
16-17 weeks	Fetus begins to swallow amniotic fluid – stimulates mucosal differentiation along GI tract.
36 weeks	Mature motility.

Table 1-2: Timeline of human gastrointestinal tract development, adapted from (89–92).

In terms of the development of nutrient transport systems in the small intestine, active transport of proteins and glucose in the jejunum has been detected from 14 and 18 weeks respectively (92). The ability to absorb fatty acids occurs later at around 24 weeks, as this requires sufficient secretion of bile acids and digestive enzymes (93,94). Figure 1-4 illustrates the anatomy of the fully formed small intestine.

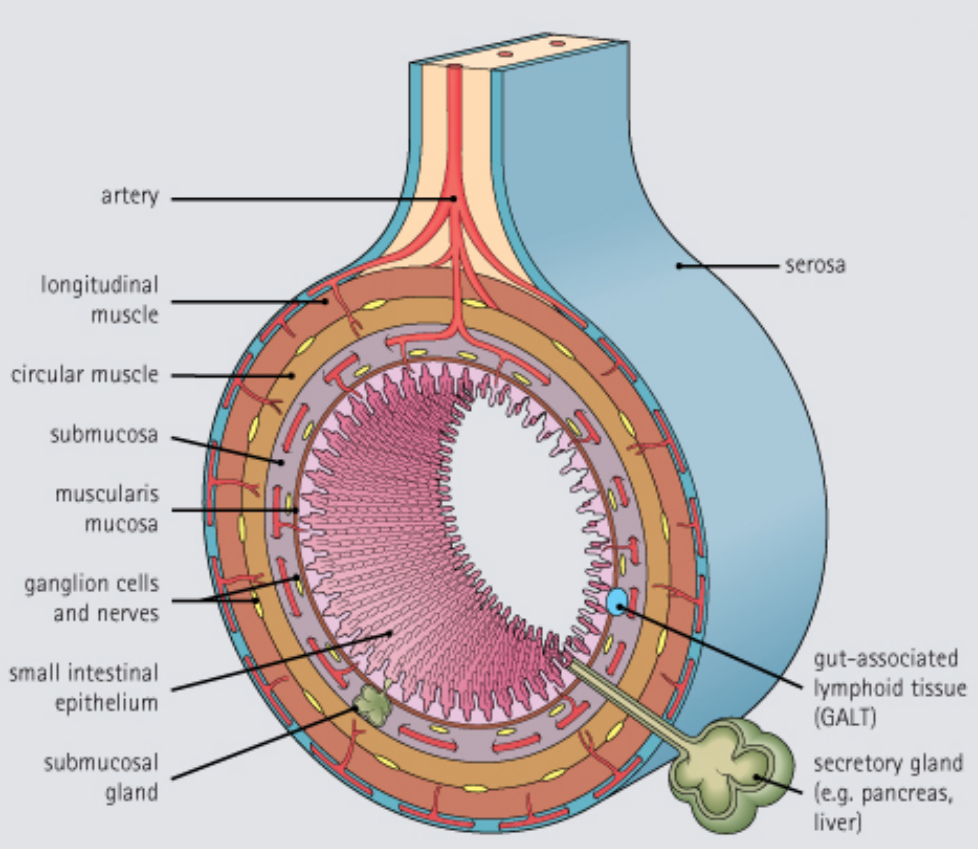


Figure 1-4: Cross-section of the small intestine, reproduced from (95).

Once developed, the intestinal epithelium consists of enterocytes which are specialised simple columnar epithelial cells joined by tight junctions to form a (theoretically) impermeable barrier. Microvilli on the surface of enterocytes increase the surface area available for the absorption of water and ions via facilitated diffusion. At the base of the microvilli, the intestinal crypts secrete enzymes responsible for the breakdown of dietary constituents. Multipotent stem cells, located at the base of the intestinal crypts provide a source of constantly dividing epithelial cells that migrate to the top of the villus, constantly refreshing the intestinal epithelium (92).

Transmembrane proteins along the epithelium of the small intestine will vary depending on the section, for example the highest concentration of glucose and fructose transporter (GLUT/SGLT) proteins will be in the jejunum, where the majority of sugars broken down from dietary carbohydrate are absorbed (93).

1.10. Effects of nutrition and nutritional programming on gut development

The development of the gastrointestinal tract is affected by nutrition both pre- and post-birth. In preparation for the change from parenteral nutrition provided by the placenta to enteral nutrition after birth, the gastrointestinal tract develops rapidly in the weeks preceding birth. This process is initiated earlier in human gestation when the fetus begins swallowing amniotic fluid at around 16 weeks, which stimulates mucosal differentiation of the GI tract (89). Although important for structural development of the GI tract, the role of amniotic fluid in functional development is disputed. Studies in both fetal pigs and lambs, in which oesophageal ligation was used to prevent the swallowing of amniotic fluid have shown that growth of the GI tract is markedly reduced, yet absorption of glucose and amino acids, as well as brush border enzyme activity is unchanged (96,97).

Whilst stimulating mucosal differentiation, amniotic fluid provides little nutrition, providing around 10-20% of fetal energy demands. The minimal effect amniotic fluid has on intestinal function is contrasted sharply with more energy dense substances. For example, infusion of colostrum in fetal pigs at 100 days gestation (2 weeks before term) stimulates maturation of brush border enzymes and increases protein uptake (98). This suggests that the influence of nutrition on the immature GI tract occurs independently of other factors such as increasing cortisol concentration before birth and the change in microbiological environment at birth (89).

Studies using a sheep model have shown that restriction of fetal nutrition both by manipulating the maternal diet and by reducing placental development will preferentially affect GI growth, with a significant decrease in total gut weight as well as the circumference of both small and large intestine (99). Restriction of enteral nutrition in the fetal sheep model using a jejunal ligation at 90 days gestation also brought about

similar developmental changes, with a reduction in villus height and accumulation of glycogen, as well as cellular abnormalities (100). This suggests that enteral nutrition can be necessary for the stimulation of intestinal growth. These findings are similar to research conducted using a pig model (96).

Nutrition immediately following birth has been shown to have significant effects on GI development. The increases in both intestinal weight and DNA content associated with normal growth are not observed in animals prevented from suckling and fed solely with water, as shown in multiple models (101,102). These findings are also consistent with those described in the fetus, with nutrient restriction preferentially affecting GI development whilst overall growth is unchanged. The effects of specific nutritional deficiencies have also been studied with regard to effects on gut development. In rats maternal protein restriction during suckling leads to a marked reduction in villus height in the duodenum and jejunum, but not the ileum. These changes were also reversible once a regular diet was resumed after weaning (103).

There is some controversy as to whether dietary composition, or total energy intake are producing the above effects on gut development. At weaning in pigs, when they are continued on a diet of sow's milk they display almost identical changes in intestinal morphology to those placed on a standard commercial weaning diet (104). Whilst this influence of total energy intake is convincing, the role of individual macronutrients in GI development cannot be ignored, for example glutamine, a conditionally essential amino acid, is thought to be vital for small intestinal development due to it being a primary source of energy for enterocytes. Glutamine supplementation of the postweaning diet prevents the duodenal atrophy observed in pigs consuming diet containing no glutamine (105).

Aside from the above associations, the role of nutritional programming in GI development has been significantly under-researched. In a review of the literature conducted in 2010, Guilloteau *et al.* concluded that "because of the scarcity of data concerning nutritional programming effects on the GIT and due to their potentially important consequences, more research is needed in animal models due to the ethical constraints involved in using human subjects" (106).

1.11. Intestinal permeability in the newborn

Even in a healthy infant born at term, maturation of the gastrointestinal tract is a dynamic process that continues after birth. This maturation will be brought about largely by changes in external stimuli, for example exposure to commensal vaginal bacteria at birth as well as organisms in the surrounding environment will result in establishment of a 'normal flora' in the intestine. This normal flora will continue to develop in early life depending on the environment and circumstances. For example, the normal intestinal flora in infants born by caesarean section varies from those born vaginally (107) and there are significant differences between breastfed and formula-fed infants (108). Therefore, the selective permeability of the intestinal mucosa is important in preventing the passage of bacteria making up the normal flora into the systemic circulation. Whilst beyond the scope of my thesis, it should be noted that the exact mechanisms by which the normal flora avoid initiating an immune response are not fully understood (this also applies to other body surfaces as well as the intestinal tract). A number of hypotheses have been put forward to explain this, involving either the modification of pathogen associated molecular patterns (PAMPs), making them unrecognisable to pattern recognition receptors (PRR), or the induction of 'tolerogenic' signals by organisms of the normal flora (109,110).

Whilst the entire intestinal mucosa is lined with a variety of transport proteins and ion channels allowing passage of a wide range of molecules, it is the epithelial tight junctions which are most important in the mediation of permeability, as they provide a passive mode of transport (111). This is as opposed to the facilitated diffusion observed in the transport of glucose and fatty acids (using glucose transport (GLUT) proteins and monocarboxylate transporters respectively). Tight junctions in the intestinal epithelium were initially thought to serve as impermeable seals between epithelial cells, whilst all transport across the epithelium was via transmembrane transport proteins and ion channels. However, tight junctions have since been shown to allow transport across the epithelium, in a tightly regulated process co-ordinated with other transmembrane proteins (112). The exact mechanisms responsible for tight junction regulation are not fully understood. There are, however, several well established genes and pathways that are of interest. The permeability changes observed in inflammatory bowel diseases such as Crohn's and ulcerative colitis have provided a useful method of linking changes in permeability to possible signalling pathways (113). The importance of maintaining intestinal permeability is perhaps best illustrated by the injection of extracts from bacteria found on the intestinal lumen directly into the colonic wall, this bypass of the epithelial barrier leads to an acute inflammatory response, and is now a commonly used technique in inducing intestinal inflammation in animal models (114).

Studies have also explored the effect of IUGR and preterm birth on intestinal permeability. However, this has mainly consisted of using noninvasive biomarkers such as the measurement of the lactulose:mannitol ratio in urine. Both of these substances will pass through the small intestine and into the urine in a comparable concentration to the administered dose (115,116) These studies have consistently shown an increase in intestinal permeability in babies born before 34 weeks gestation, compared to healthy controls (117,118). However, an understanding of what is causing these changes in

permeability, for example changes in gene expression, was limited chiefly by the difficulty in obtaining human tissue samples, as well as the lack of a well-established animal model (this will be discussed in more depth later).

1.12. Tight junction structure

In the intestine, epithelial tight junctions are located parallel with the base of the microvilli, and along with the adherens junction and desmosome make up the 'junctional complex'. Gap junctions are located below the junctional complex, allowing the passage of molecules between epithelial cells, as illustrated in Figure 1-5.

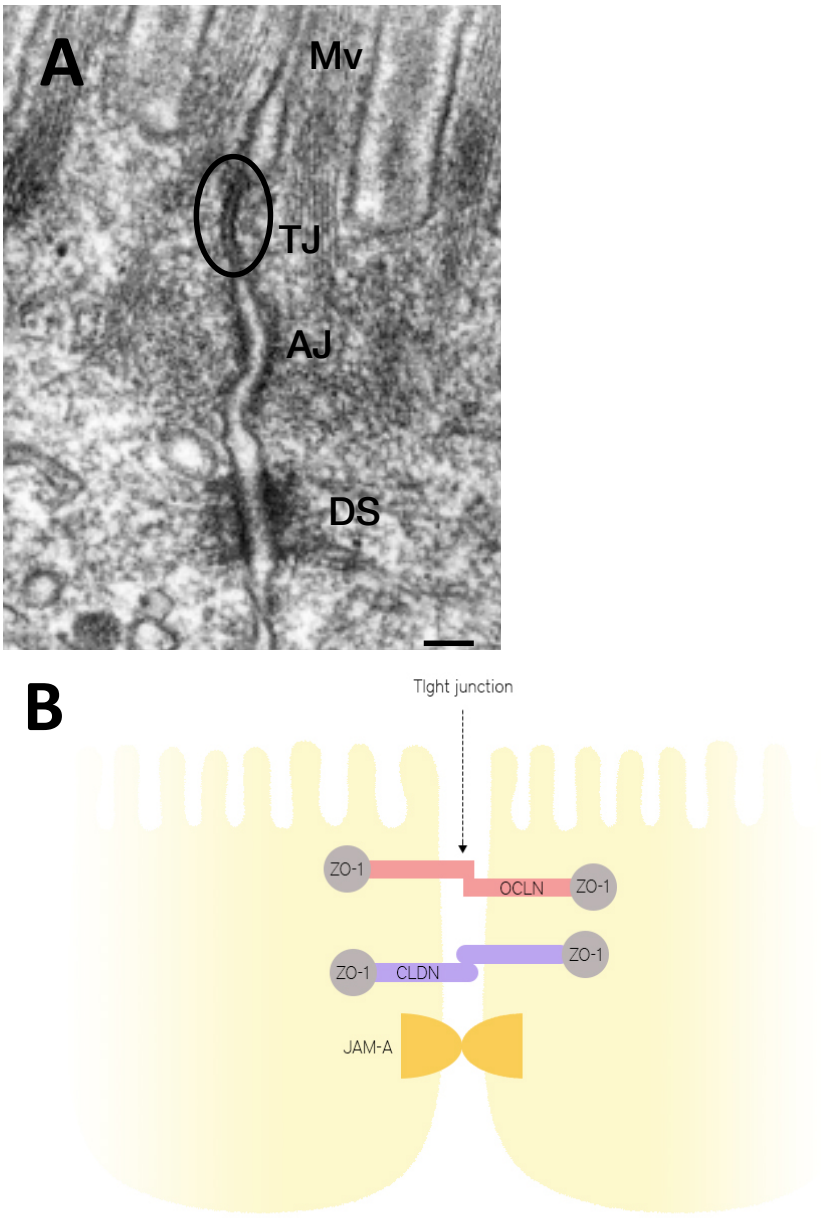


Figure 1-5: (A) Electron micrograph of adjacent enterocytes, with microvilli, tight junction (highlighted), adherens junction and desmosome (119). (B) Diagram showing the constituent proteins in a single epithelial tight junction.

The tight junction itself is made up of several strands, each within the plasma membrane of one cell being associated laterally with a tight junction strand of another cell - thus forming an effective seal between epithelial cells. This structure was elucidated decades ago (120), and it was quickly realised that far from being an impenetrable barrier between epithelial cells, tight junctions provide a tightly regulated means of passive

diffusion across the epithelium. These strands were not characterised until much later, and it can be argued that there is still no complete outline of their structure and function. Two possible ideas for their structure have been put forward, a lipid and a protein model. The lipid model proposes that tight junction strands are formed of cylindrical micelles which are sandwiched between the external membranes of adjacent epithelial cells, these micelles form in a way similar to that of the lipid bilayer during cell membrane fusion (121). Relatively recent advances in molecular biology, as well as the lack of any recent research has meant the lipid model is now largely disregarded, with the characterisation of several tight junction specific proteins providing a far more convincing model.

Identification of proteins specific to tight junctions began in the early 90s, with the characterisation of occludin, first in tissue isolated from chicken liver (122), followed by a mammal homologue (123). Expression of occludin has been shown (using immunohistochemistry) to be varied across different epithelial tissues, and has been proposed as a potential marker of permeability. Occludin expression in tight junctions of neuronal tissue is higher than in any other tissue type, where more variability is observed. This is one possible explanation for the extremely tight regulation of permeability across the blood brain barrier, as well as the more variable permeability of the gastrointestinal tract (119,124).

1.12.1. Occludin (OCLN)

Despite the relatively lower expression of occludin in the gastrointestinal tract, the study of its effects on intestinal permeability, as well as the potential for nutritional manipulation have yielded interesting results. A study looking at the effect of supplementing a standard *ad libitum* diet with zinc on intestinal permeability in pigs found that an increased intestinal permeability (as measured by urinary excretion of lactulose and mannitol) was correlated with an almost 3-fold increase in occludin

expression (125). Human cell culture studies have also been conducted into the effect of enteropathogenic *Escherichia coli* (EPEC) on occludin. It was found that in addition to inducing cytoskeletal contraction via phosphorylation of myosin light chain kinases (MLCK), EPEC will also phosphorylate occludin, dissociating it from the tight junction (126). This leads to the increase in intestinal permeability (as measured by transepithelial electrical resistance) observed in EPEC infection, and is not an observed effect of non-pathogenic *E. coli* strains (127). Clearly there is some inconsistency here, with both up and downregulation of tight junction proteins apparently leading to an increase in intestinal permeability. This highlights the need for further research linking these proteins and tight junction permeability.

1.12.2. Claudin (CLDN)

The gene coding for the transmembrane protein claudin was discovered shortly after the characterisation of occludin (128), and was found to be structurally similar (both proteins have four transmembrane domains, and cytoplasmic 'tails' of varying length), but without any sequence similarities. Initially 2 isoforms, claudin-1 and claudin-2 were characterised. This has now expanded to a large family of proteins, with over 20 isoforms currently documented displaying varying tissue specificity. Looking at the gastrointestinal tract specifically, most members of the claudin protein family are represented, with expression varying between isoforms. More interestingly, expression patterns have been shown to vary during postnatal development (using a mouse model) (129). In humans, dysregulation of claudin proteins has been associated with the progression of a variety of epithelial cancers. In particular, upregulation of claudin-1 has been shown to be a marker of colorectal cancer progression, which is also associated with an increase in intestinal permeability (130,131).

1.12.3. Zonulin-1 (ZO-1)

Initially discovered as a receptor for the zonula occludens toxin secreted by *Vibrio cholerae,* zonulin-1 has since been found to mediate intestinal permeability under a variety of different conditions (132). Since its discovery almost a decade ago, altered expression of zonulin has been found to be caused by exposure to a variety of enterobacteria (both pathogenic and non-pathogenic) in cell culture, leading to reduced barrier function (133). Increased expression has also been found in the acute phase of coeliac disease, along with the characteristic increase in intestinal permeability (134). Besides research into the role of ZO-1 in various intestinal pathologies, very little research has been conducted into comparative expression in term and preterm infants. The increase in expression between fetal and adult intestine is well characterised however (132), therefore changes directly after birth could be of interest.

1.12.4. Junctional adhesion molecule (JAM)

The last of the proteins found across all types of epithelial tight junction to be discovered was the junctional adhesion molecule (JAM) (135). Several isoforms of JAM have since been characterised in humans, with some being unique to endothelial cells, others present in all epithelial cells. As the name suggests, JAM are responsible for the cell/cell adhesion of epithelial cells, with a single transmembrane domain consisting of two long immunoglobulin-like loops (as illustrated in the diagram above) (119). Although JAM has been shown to have a broadly similar transmembrane loop structure to occludin and claudin, it behaves differently to both proteins. Claudin and occludin are only observed when part of the tight junction complex, JAM appears to act independently of this. Cell culture studies have shown that in cells not presenting organised tight junctions under an electron microscope, transfected JAM will still

migrate to cell-cell contacts. This suggests that JAM may be most important during the formation of tight junctions (135).

As with the previously mentioned tight junction proteins, research has been conducted into the possible effect of altered JAM expression on intestinal permeability, although an investigation linking JAM to intestinal development is lacking. Knockout mice have shown that reduction in a JAM isoform, JAM-A is concurrent with reduced intestinal barrier function, as well as reduced cell division and increased apoptosis (136,137). However, such results should be interpreted with care; while being widely expressed in the intestine JAM-A is also expressed in a wide range of other locations, including endothelial cells, antigen presenting cells and neutrophils. Therefore it cannot be asserted that a reduction in JAM-A expression causes an increase in intestinal permeability, any changes seen may simply be caused by a change in circulating neutrophils, even a simple change in epithelial cell shape (138).

1.13. The role of intestinal permeability in systemic inflammation and obesity onset

As previously discussed, changes in intestinal permeability have been associated with inflammatory bowel disease. Crohn's disease has also been associated with 'creeping fat', in which mesenteric adipose tissue becomes inflamed and begins to spread around the wall of the intestine (139). This link between increased intestinal permeability and adipose tissue inflammation also suggests possible links between dysregulated gut development and the inflammation of adipose tissue, and hence the increased risk of developing obesity related comorbidities (87).

Central to this hypothesis is the idea that an increase in intestinal permeability will allow the passage of large molecules and bacteria which, although part of the normal flora are usually contained within the GI tract.

The passage of lipopolysaccharide (LPS), the endotoxin present on the cell membrane of a number of Gram-negative bacteria, into the systemic circulation has been well researched. LPS is found on several Gram-negative strains that are part of the normal gut flora, in particular *Bacteroides*, *Fusobacterium*, and *Escherichia* (140). Under normal conditions, any Gram-negative bacteria that do cross the intestinal epithelium will diffuse directly into the portal circulation where LPS is internalised by the specialised macrophages of the liver (Kupffer cells). However, this system is easily overwhelmed. This has been demonstrated in pigs, with selective breeding for leaner animals leading to genotypes which are more susceptible to immunologic challenge. These pigs then display metabolic endotoxaemia on infection (141). Endotoxaemia is also observed in patients with inflammatory bowel disease, who have shown circulating endotoxin levels around 50% higher than healthy controls (142).

There are convincing links between endotoxaemia and the development of IR and obesity. Using a mouse model, it has been shown that an induced endotoxaemia (via infusion of LPS) leads to fasted hyperglycaemia and hyperinsulinaemia as well as an increase in adipose tissue mass, liver mass and total body weight (143). Mice placed on a high-fat diet for 4 weeks also displayed a 2 to 3-fold increase in serum LPS. Similar findings have also been replicated in humans, with serum LPS increasing moderately directly following the consumption of a high fat meal (144). This suggests that regular consumption of a high-fat diet will contribute to the underlying low-grade inflammation observed in obesity, partly derived from endotoxins derived from the gut. Sprague-Dawley rats, when fed a high-fat diet will also display an increase in ileal TLR4 expression, as well as an increase in serum LPS and intestinal permeability (145).

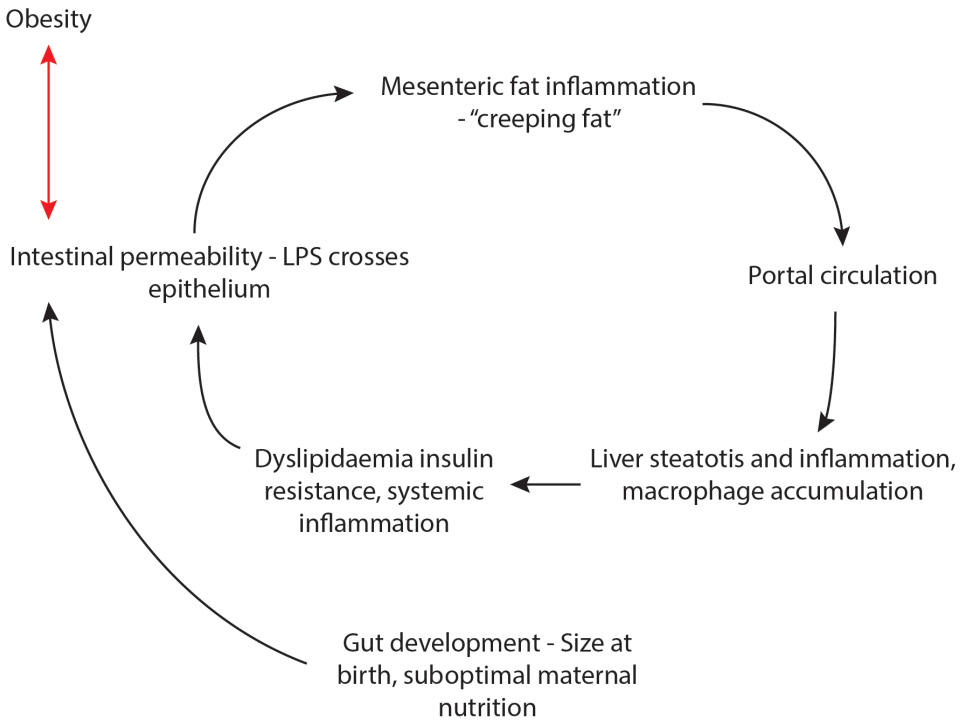


Figure 1-6: Potential interactions between gut development, portal circulation and the onset of obesity. Adapted from (87).

1.14. Molecular mechanisms of intestinal growth, repair and inflammation

1.14.1. Epidermal growth factor & epidermal growth factor receptor

In the GI tract, epidermal growth factor (EGF) plays a role in cell survival and proliferation, making it vital for both intestinal development in early life as well as part of adaptation to injury (146–148). The developing fetus is first exposed to EGF in amniotic fluid (149), and following birth colostrum and breast milk are the main intestinal sources of EGF (148,150), with concentration declining ~1 month in to lactation (151).

The extremely delayed development of the gastrointestinal tract (along with other epithelial surfaces) observed in epidermal growth factor receptor (EGFr) knockout mice (152) has led to EGFr expression as well as its ligand EGF being studied in GI development, particularly in the first

month of life (153). EGFr$^{-/-}$ mice display multiorgan failure, significant undernourishment despite an *ad libitum* diet, intestinal distension and haemorrhage, and death by 8 days of age (152). However, it is also important to be aware of potential confounders when using global $^{-/-}$ knockouts rather than tissue specific as a gene will be deleted in the tissue being observed but also in all other tissue types, introducing many potential confounders.

There are well-established connections between both growth restricted infants born at term and appropriate for gestational age (AGA) preterm infants, and an increased incidence of intestinal inflammation and infection (154). EGF and EGFr may play a role in this process, as supplementation of EGF in a neonatal rat model leads to a significant reduction in NEC vs a rat milk substitute containing no EGF (155). These findings have been replicated in a neonatal pig model, with those fed formula milk having a far higher incidence of NEC compared with controls fed colostrum. Formula fed pigs also displayed shorter villi, reduced enzyme activity and reduced nutrient absorption relative to controls (156). This suggests that both EGF and EGFr are potentially of interest when studying both the effect of diet and size at birth on the developing intestine.

1.14.2. Toll-like receptor mediated inflammation

The expression of toll-like receptors in both the small and large intestine is particularly important when studying inflammation and permeability. This is due to the interactions between various TLR subtypes and LPS present on the membrane of Gram-negative bacteria making up part of the intestinal normal flora (157,158). Forming a significant part of the innate immune system, TLRs are present on a wide variety of cell types, with expression varying depending on the subtype. Several subtypes have been shown to be nutritionally regulated (159) and linked to the development

of IR (160). TLR subtypes 2, 4 and 5 are highly expressed on the intestinal epithelium and submucosa, with TLR2 and 4 both being shown to be upregulated in the small intestine during 'flare ups' of both Crohn's disease and ulcerative colitis (161).

Research in a mouse model has shown that TLR2$^{-/-}$ mice display reduced plasma glucose and insulin when fed rodent chow supplemented with fat. The TLR2$^{-/-}$ mice also showed significantly reduced adiposity compared to their wild-type counterparts when fed the high fat diet, whilst lean body mass was unchanged (162). This demonstrates an interesting link between TLR expression and the response to nutritional intake, as well as a link between TLR mediated inflammation and the development of the metabolic syndrome. Finally, liver expression of TLR4 is of interest as a method of measuring bacteria crossing the intestinal epithelium into the portal circulation (40,87), as discussed above (section 1.13).

1.14.3. Tumour necrosis factor alpha

The role of tumour necrosis factor alpha (TNF-α), as well as its interaction with the nuclear factor kappa light chain enhancer of activated B cells (NFκb) and mitogen activated protein kinase (MAPK) pathways is relevant when studying intestinal development, due to its importance in mediating inflammation throughout the body (163,164). TNF-α has also been established as a mediator of intestinal permeability, mainly through studies of inflammatory bowel disease. Use of a mouse model of colitis (induced via 2,4,6-trinitrobenzene sulfonic acid, TNBS) has demonstrated that TNF$^{-/-}$ mice do not exhibit chronic inflammation when treated with TNBS whereas wild-type mice do (165). In the same study, cells taken from anti-TNF-α treated mice also showed a significant downregulation of inflammatory cytokines. This suggests that TNF-α expression is a useful biomarker for measuring the extent of intestinal inflammation.

In terms of intestinal permeability, TNF-α has been shown to affect the regulation of zonula occludens 1 (ZO-1), part of the epithelial tight junction complex, by the NF-κB pathway. Much of this research has been conducted using Caco-2, a commonly used cell line derived from human colorectal adenocarcinoma epithelial cells, which can be stimulated to differentiate into cells resembling small intestinal enterocytes. As such it is now a widely used model of intestinal permeability (166). Western blot and immunohistochemichal analysis have both demonstrated that TNF-α downregulates ZO-1 expression (167). The effects of this potential increase in intestinal permeability mediated by changes in tight junctions will be discussed later. Finally cA2, an anti-TNFα antibody has shown some promise in small-scale clinical trials when used as a treatment for Crohn's disease, albeit as a short-term treatment and with no apparent dose-response relationship (109).

1.14.4. Beta defensin

Beta defensins are secreted from a variety of epithelial cell types, including enterocytes. They are either expressed as part of the innate immune system (hBD1), or are induced by proinflammatory cytokines such as TNF-α or IL-1β (hBD2-4) (168). They display antimicrobial activity, with hBD2 especially displaying antimicrobial effects against *E. coli* (169,170). At low concentrations, hBD2 also displays a bacteriostatic effect, therefore it may provide a mechanism of maintaining the intestinal normal flora (169).

The expression of beta defensins therefore provides an appropriate marker of the ability of the intestine to respond to bacterial infection as well as inflammation. hBD2 has been shown to be upregulated in various inflammatory bowel diseases (while hBD1 expression remains constant) (171). A polymorphism in the gene coding for hBD2 has also been shown to predispose to Crohn's disease (172). However, the role of beta

defensins in intestinal development, or any possible effect of nutrition on their expression, has not been researched.

1.15. Gastrointestinal and liver interactions and the role of fructose

Fructose is potentially of interest with regard to the processes outlined in Figure 1-6. Excess consumption of dietary fructose has been associated with the development of non-alcoholic fatty liver disease (NAFLD) (40,173), and there are also links between NAFLD and a decrease in gut barrier function (174). Once taken up in the intestine, as discussed in section 1.6.1, fructose is efficiently taken up by the liver once it has passed into the portal circulation and follows the metabolic pathway covered in section 1.6. *De novo* lipogenesis caused by the metabolic pathway of fructose may not be sufficient to induce steatosis alone, and requires either a deficiency of fatty acid export out of the hepatocyte or an abnormal increase in β-oxidation within the liver (174). Therefore other explanations are required to explain this involvement of fructose in steatosis and eventually NAFLD. Fructose has been shown to increase the expression of sterol regulatory element binding protein 1c (SREBP1) and lipogenic enzymes (acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS)) in rat liver after 8 weeks feeding of a high fructose diet (175). Knockdown in rats of peroxisome proliferator-activated receptor γ coactivator-1 β (PGC1β), a transcriptional coactivator of SREBP1, will alleviate the hepatic lipogenesis and triglyceride synthesis induced by a high fructose diet, compared to controls with functioning PGC1β (176). Taken together these findings suggest an involvement of fructose in steatosis beyond a simple induction of lipogenesis by the fructolytic pathway, however there is still a lack of a true mechanism to explain this.

The intestine also has a role in NAFLD, and was first discovered when nonalcoholic steatohepatitis (NASH; a precursor to NAFLD) was shown to be a common complaint in jejunal bypass patients (177). In some cases the

NASH would progressively worsen and require liver transplant, and recur again in the transplanted liver (178). This has been hypothesised to be due to increased bacterial overgrowth and an increase in intestinal permeability, with raised plasma endotoxin being demonstrated to be a hallmark of NAFLD from its early stages onwards (179,180). The fact that administration of the prebiotic oligofructose (which is thought to inhibit the availability of carbohydrates in the intestine (181)), causes a reduction in fructose induced liver-damage in rats, suggests that bacterial overgrowth is an important component of NAFLD (174,181). However a direct mechanism or role for fructose to affect intestinal permeability, which as shown in sections 1.11 to 1.13 is well established as a factor in the onset of the metabolic syndrome, is not fully understood.

1.16. The role of the gut microbiota in obesity

As discussed, the normal gastrointestinal flora develops from birth, and is influenced by various stimuli during development. The importance of this flora has led to it increasingly being referred to as a metabolic "organ" which can break down dietary components such as plant polysaccharides into absorbable products, a process which would not otherwise be possible in the human GI tract (108).

It has been proposed that the microbiota also has a role in energy balance, and therefore may have an influence on the development of obesity. Studies comparing mice raised in a germ-free (GF) environment (housed in gnotobiotic isolators, and fed autoclaved standard laboratory chow) with conventionally raised control mice showed significant increases in body fat, VO_2 max and chow consumption in the controls at 8-10 weeks (182). A third group in the same study, GF mice colonised with gut flora taken from controls at 8 weeks, displayed the same characteristics as controls. This suggests that the gut flora does have a causative role to play

in the onset of obesity, but a study such as this makes it impossible to focus on any one organism or mechanism.

The fact that the majority of organisms making up the gut flora are anaerobes makes culturing specific samples impossible (108), which has led to a focus on microbial gene expression. In humans that the relative abundance of *Bacteroidetes* and *Firmicutes* (measured by 16s ribosomal RNA in faecal samples) will change along with body weight as shown when obese subjects are placed on a low calorie diet (88). When followed over a one-year period, with faecal samples taken at 12, 26 and 52 weeks, subjects displayed an increase in *Bacteroidetes* and a reduction in *Firmicutes*, the change in *Bacteroidetes* abundance was also significantly correlated with the change in body weight over the duration of the study.

A study comparing the gut microbiome (the totality of microbes and their associated genomes) of *ob/ob* mice with wild-type controls produced results consistent with the studies above (183). The study demonstrated that the *ob/ob* microbiome had a significantly greater capacity for breaking down dietary components, shown via an upregulation in genes related to glucose, starch and lipid metabolism. This suggests that the microbiome can be manipulated by the diet, and that the microbiome may affect energy balance and could be considered a separate risk factor for the development of obesity. The above study and others (184,185) have also shown marked differences in microbial diversity between lean and obese study participants or animals. Obesity and conditions such as inflammatory bowel disease are both associated with a reduced microbial diversity (88,184).

Diversity is measured within an individual (alpha diversity, for example, the number of different types organisms within an individual's intestinal microbiome) and between individuals (beta diversity). Patterns of alpha and beta diversity differ significantly when making comparisons from the same habitat. For example, a high alpha diversity indicates a diverse

microbiome but this can be concomitant with a low beta diversity, with members of a defined population all sharing similar organisms. Currently a functional explanation for changes in diversity has not been identified, with a need for studies taking into account short and long term diet, diurnal rhythms, mode of delivery at birth and host genetics (186).

1.17. Gastrointestinal adaptations in pregnancy

Gross changes of the GI tract using weight as an indicator of overall size have been assessed in pregnant and lactating rats in studies dating back to the 1930s (187), with more detailed documentation beginning in the 1960s. Relative to the non-pregnant state, increases in weight of both the stomach and the small intestine have been measured consistently during lactation but not pregnancy (188–190). One study in sheep reported a 45% increase in small intestine weight ($p<0.05$ vs age matched non-pregnant controls) in the third trimester (191). Although intestinal weight does not appear to change in pregnant rats, surface area increases through pregnancy. Villus height is increased in the duodenum by mid-pregnancy in rats (192) and this is accompanied by gastric dilatation commencing at the beginning of the third week of gestation. These changes persist into lactation (189,193). The surface area of the small intestine was increased by the end of lactation in rats as serosal circumference and villus height were raised in the jejunum and ileum. Similar observations have been reported in pregnant sheep (194) and lactating mice (188). The mechanisms proposed to mediate these gross anatomical changes include changing nutrient intake and hormonal adaptations. High concentrations of thyroxine, and IR/hyperglycaemia occur in pregnancy (195) which have both been linked with intestinal hyperplasia/hypertrophy, but these associations have only been described in non-pregnant models (196,197).

Pregnancy related changes in intestinal permeability to larger molecules such as bacteria are of interest especially given changes in gut microbiota

during pregnancy (198,199) and the recent description of a unique microbiome in the placenta (200). The effect of pregnancy on epithelial tight junctions has not been studied. There is overlap here with research into the role of the gut microbiota in pregnancy, and there are suggestions that LPS in the microbiota, released into the circulation due to poor gut barrier function is one of the effects of the increased catecholamines released as part of the maternal stress response (201).

1.18. The gut microbiota in pregnancy

The current evidence base for the changing gut microbiome has compared the effect of different diets and physiological states including pregnancy. Although this field is very new, it appears that the gut microbiome does change with pregnancy (199), and it has been hypothesized that inappropriate adaptation of the gut microbiome may contribute to the development of pregnancy complications including pre-eclampsia, intrauterine growth restriction and miscarriage (202). Some, of these changes are comparable with the aforementioned changes observed in an "obese" microbiome. Pregnancy is associated with a significant reduction in alpha diversity and an increase in beta diversity by the third trimester (199). This is due to an increase in Proteobacteria, similar to the pattern of dysbioses observed in obesity and chronic inflammatory states (184,203). In contrast, changes in the ratio of *Bacteroidetes* to *Firmicutes* is associated with obesity (88) but remains unchanged in pregnancy relative to the non-pregnant state.

Similarities between the pregnant and obese microbiome are of interest since both conditions are associated with increased fat mass, although the relative distribution of this additional fat is different between pregnant and obese individuals (204). Representation analysis using the biological pathways database Kyoto Encyclopedia of Genes and Genomes (KEGG, (205)) suggests that a microbiota associated with obesity develops which

is capable of increasing the amount of energy available from otherwise indigestible dietary polysaccharides (184,185). Species of *Bacteroidetes* in particular contain a number of glycoside hydrolases which are able to ferment sugars which would otherwise pass through the large intestine and remain undigested (206), resulting in increased energy availability during pregnancy. These could help in meeting maternal energy demands and supplying energy to the fetus, and suggest that during pregnancy the microbiome could be modulated to increase energy available for absorption. However it is currently unclear whether they are solely due to the changing microbial environment, or are modulated by other intestinal adaptations that are known to occur in pregnancy.

1.19. Summary

The research outlined in this chapter demonstrates that both nutrition and size at birth will influence growth trajectory of the offspring, and the incidence of disease in later life. Although it has been underexplored there is some evidence to suggest that organs such as the GI tract are affected both by maternal diet and diet in early life either through lactation or at weaning. The aim of this thesis is therefore to use two animal models – a rat model of a high fructose diet in pregnancy and a porcine model of a high fat diet during pregnancy, to study how the supplementation of these macronutrients can affect the gastrointestinal tract both in pregnancy and in the offspring. A number of molecular biology techniques will be utilised to measure changes in a variety of markers of intestinal inflammation and function, these will be discussed in chapter 2. Detailed hypotheses of how these techniques will be used to test the effect of macronutrient supplementation on GI development and adaptation are explained in chapters 3, 4 and 5.

1.20. Studying the developmental origins of health and disease: animal models

As described above, the early epidemiological studies of the effects of maternal nutrition and overall health on offspring development have led to further research focused on explaining physiologically these effects that are observed in data from a large population. However, conducting research of this nature poses an obvious ethical problem. For example, epidemiological data has shown a convincing link between intrauterine growth restriction and the onset of obesity, yet any sort of controlled trial of this effect in humans would be impossible to justify and conduct ethically. Consequently, the use of several different animal models has now become commonplace in the study of the developmental origins of disease, with each model varying in its suitability depending on what is being studied. The most widely used of these models will be discussed below, along with the advantages and disadvantages of each.

1.20.1. Rodents

The small size of mice and rats, as well as the wide availability of transgenic strains and fully sequenced genomes have made them the 'go-to' animal for a huge range of medical research. In 2010, mice and rats made up ~95% of all animals used in scientific research in the US, with pigs and sheep combined making up less than 1% (207).
While humans, sheep and pigs are born relatively mature, mice and rats are altricial, therefore significant 'windows' of development are more difficult to compare between species (208). In rodents at birth the intestine is relatively undeveloped, with an extremely quick adaptation that occurs around weaning (around 18-22 days). This contrasts with the more gradual development in humans, maturational changes are still observed after birth and weaning but are far less rapid (89). However, the fact that rodent organs are still immature at birth means that they may be

manipulated (for example by nutrition) in ways that would not be possible, or impractical when the animal is *in utero*. However, this also means that the timing of interventions is extremely important if the findings are to be of any relevance to human development.

1.20.2. Pigs

The pig is a relatively uncommon animal model, making up 0.2% of the ~1.3million animals used in research in the US in 2010 (207). However the pig has a number of advantages that make it particularly appropriate for use as a model of both fetal development and postnatal growth. Pigs exhibit the most severe naturally occurring growth restriction among domestic mammals, with size at birth greatly influenced by both position in the uterus and placental size (209). The "runts" in a given litter can therefore be used as a model for the postnatal growth of an IUGR infant. However, this can also lead to a problem with study design, with an average litter size of 11 and a maximum of ~20 piglets born at a time, care must be taken when selecting animals for study. This problem with animal selection, and the importance of fully describing the reasons and statistical analyses have led to some controversies in the literature, mainly focused on the variability introduced through selecting animals from multiple large litters for comparison (210,211).

The pig is born with a GI tract that is at a similar stage of development to a human infant, with rapid increases in maturity observed both directly before and after birth, and at weaning (3-6 weeks) (89). This can be contrasted with rats, which are born with an immature GI tract that matures very rapidly at weaning (18-22d). These similarities in gut development, along with the fact that pigs will readily become hypertensive and insulin resistant, makes them a useful model for the interactions between gut development and the onset of the metabolic syndrome. The fact that the pig has similar adipose tissue biology to man

(excluding the presence of brown fat in early life), as well as proportionally similar organ weights also make it a useful model of obesity (212). However, the sheer cost and time involved in conducting a pig study has meant the model is underused compared to rodents.
The commonly used animal models for DOHaD research are summarised in Table 1-3.

	Average offspring number	Average birth weight	Gestation	GI tract	Advantages	Disadvantages
Man	1	3.4kg (UK average)	~280 days	Monogastric	-	-
Rodent	11, (wide variation in both mice and rats depending on strain)	0.5-4.0g (variation depending on litter size and strain)	~20 days (wide variation depending on strain)	Monogastric	Relatively low cost. Availability of transgenic strains. Structurally similar GI tract. Can induce metabolic syndrome. Short gestation and lifespan.	Altricial. Large litters. Short gestation and lifespan.
Pig	11 (litter bearing, variation depending on breed)	0.6-3kg (variation depending on litter size)	~110 days	Monogastric	Similar GI tract development to human. Large litters provide model of growth restriction. Can induce metabolic syndrome. Long gestation.	Large litters. No brown adipose tissue. High cost, long study duration.

Table 1-3: Summary of comparisons between DOHaD animal models and man. Adapted from (208).

Chapter 2: Materials and methods

This chapter will explain the animal models used in both studies included in my thesis and how they were implemented, and a description of the methods used in analysing the tissues obtained and their scientific basis. Differences in methods between the Schothorst study conducted at the University of Nottingham (chapter 5), and the SM study conducted at the University of Alberta (chapters 3 and 4) are highlighted.

2.1. University of Nottingham – procedures and legislation

All animal procedures described in section 2.3 and chapter 5 were carried out by the scientific staff at Schothorst Feed Research, the Netherlands and were approved by the Ethics Committee for Animal Experiments of the Animal Sciences group at Wageningen Research Centre, the Netherlands.

All laboratory work described in chapter 5 was carried out in the Division of Child Health, Obstetrics and Gynaecology, School of Medicine, and the School of Veterinary Medicine and Science, University of Nottingham. All chemicals, reagents and laboratory procedures were assessed and implemented in compliance with the UK Health and Safety Executive's Control of Substances Hazardous to Health (COSHH, SI No. 1657, 1988) and Risk Assessment guidelines. Methods were followed as recommended by the relevant manufacturers or optimised by myself, Mr Mark Pope or Mrs Victoria Wilson.

All materials were purchased from Sigma-Aldrich (Gillingham, Dorset, UK) unless otherwise specified.

2.2. University of Alberta – procedures and legislation

Animal and laboratory procedures presented in section 2.4 and chapters 3 and 4 were carried out at the Division of Human Nutrition, Department of Agricultural, Food and Nutritional Science, University of Alberta. Extraction and preparatory work for DNA sequencing (section 2.20) was carried out in the Division of Animal Science, University of Alberta. All animal procedures were approved by the Animal Care and Use Committee of the Faculty of Agricultural, Life and Environmental Sciences at the University of Alberta, in accordance with regulations set by the Canadian Council on Animal Care (CCAC). All chemicals, reagents and laboratory procedures were assessed and implemented in compliance with Health Canada's Workplace Hazardous Materials Information System (WHMIS) 1988.

2.3. Schothorst study design

2.3.1. Animal handling and diets

The work covered in chapter 4 used tissue from the already completed Schothorst study (213,214). Yorkshire x Landrace sows of parity 2 to 6 (mean 3.8) were equally assigned to the two treatment groups. Each week a single sow from each group was artificially inseminated with pooled semen from multiple sires, as per normal practice in the pig industry. Pregnancy was confirmed when sows did not return to oestrus within 24 days of insemination. Sows that failed to become pregnant at the first attempt were removed from the study and replaced with another sow in the same treatment group, this process continued until there were 8 pregnant sows in each group. During gestation and lactation sows were monitored daily for illness, with medical treatments applied as needed by a veterinarian and noted.

The diets assigned to the two treatment groups are shown in Table 2-1, and full dietary composition is included in appendix C. Diets were assigned on the first day of breeding, and sows were fed according to an increased feeding scheme during gestation, providing 25.1MJ/day from day 0-70, and 32.6MJ/day from day 70-110. Diets were manufactured according to UK National Research Council guidelines on energy and nutrient requirements for sows in gestation and lactation (215). The fat supplemented diet was produced by supplementing standard commercial pig feed with palm oil, reducing starch content and feed allowance to maintain an equal energy intake between groups.

	Feed quantity during gestation (kg/day)			**Diet composition (MJ/kg)**		
Diet	**Day 0-40**	**Day 40-70**	**Day 70-110**	**Starch**	**Fat**	**Protein**
Control (n=8)	2.89	2.89	3.75	5.37	0.93	2.09
Fat supplemented (n=8)	2.51	2.51	3.26	2.74	3.29	2.07

Table 2-1: Composition and quantity of diets in the Schothorst study, full diet composition is included in appendix C.

Diets were continued until day 110, at which point both groups were switched to the control diet, with feed intake being reduced in both groups shortly before farrowing and increased into lactation as is normal practice in the pig industry.

Day	Feed allowance (kg/day)
111-113	3.2
114-115	2.5
Farrowing	2.0
1	2.5
2	3.0
3	3.5
4	4.0
5	4.5
6	5.0
7	5.5
8	5.5
9-10	6.0
11-28	*Ad libitum*

Table 2-2: Feed allowance of sows from day 110 until the end of lactation. Nutritional composition of the lactation diet is included in appendix C.

Sows were moved to farrowing crates ~1 week before the expected farrowing date, all sows farrowed naturally and piglets were allowed to suckle *ad libitum*. In litters greater than 13 piglets, additional piglets were removed before day 3 of lactation due to the maximum number of functional teats. To prepare for weaning, creep feed was fed to the piglets at two weeks of age, and at 28 days (the mean length of lactation) piglets were switched to a commercial post-weaning diet. At 5-6 weeks post weaning all piglets were switched to a grower diet, followed by a commercial diet for finishing pigs. This diet continued until 6 months of age. Nutrient composition of the above diets is included in appendix C.

2.3.2. Physiological measurements

Maternal blood samples were taken at -4, 40, 70 and 108 days of gestation, directly before morning feeding. All samples were stored in EDTA tubes and immediately centrifuged at 1800g for 15 minutes. The plasma was then removed and stored at -20°C. Plasma glucose, triglycerides, sodium, chloride and potassium were measured using an autoanalyser (RX Imola, Randox Laboratories Ltd., Co. Antrim, UK). Insulin samples were analysed

using a porcine specific ELISA kit (Porcine Insulin ELISA, Mercodia AB, Uppsala, Sweden).

Sows fed both diets were tested for glucose tolerance near the end of gestation, at 108 days. A catheter was placed in the sow's ear the day before sampling, and samples taken every minute beginning at 4 minutes prior to the administration of glucose. Glucose solution was infused intravenously (dextrose monohydrate, 495.4g/L, AVEBE, Veendam, the Netherlands) for 5 minutes. Following the glucose dose, samples were taken 15 times in 1 hour, with the catheter being flushed with saline between samples.

Milk composition was analysed by employees at Schothorst Feed Research, the Netherlands. 10IU/ml oxytocin (Eurovet Animal Health, Bladel, the Netherlands) was injected in to the ear on day 2 of lactation to stimulate let down of milk. Fat, protein and lactose concentrations were determined by infrared analysis, using a Fourier Transform InfraRed (FTIR) interferometer (MilkoScan, Foss Electric, Hillerød, Denmark).

At 6 months of age, the back fat thickness of piglets that were not selected for tissue sampling was carried out using the P2 method (back fat measured at 5cm from the midline over the last rib). Measurements were made using a Lean-Meater (Renco Corp. Minneapolis, MN, USA).

Piglets selected for dissection at 7 days were weighed; otherwise weights were taken at weaning (28 days), 63 days and 6 months.

2.3.3. Dissection

Piglets were selected for sampling at 7 days and 6 months of age; in both cases the lowest birth weight piglet in each litter was selected regardless of sex, followed by selecting a piglet of the same sex if possible, of a median weight. 7 day old piglets were not fasted before blood sampling and dissection as they were still suckling, 6 month old piglets were fasted for 12 hours before sampling.

Piglets selected for sampling were first sedated with a ketamine-xylazine combination (ketamine 10%, Alfasan International BV, Woerden, the Netherlands). Piglets were then weighed and a blood sample was taken intracardially immediately prior to euthanasia with T-61 (Intervet, Boxmeer, the Netherlands). Blood samples were stored in EDTA tubes prior to centrifugation at 1800g for 15 minutes at 4°C. Plasma was then removed from the samples and stored at -20°C in sterile Eppendorf tubes. At dissection all major organs, tissues and glands were removed and weighed, and immediately snap-frozen in liquid nitrogen before storage at -80°C.

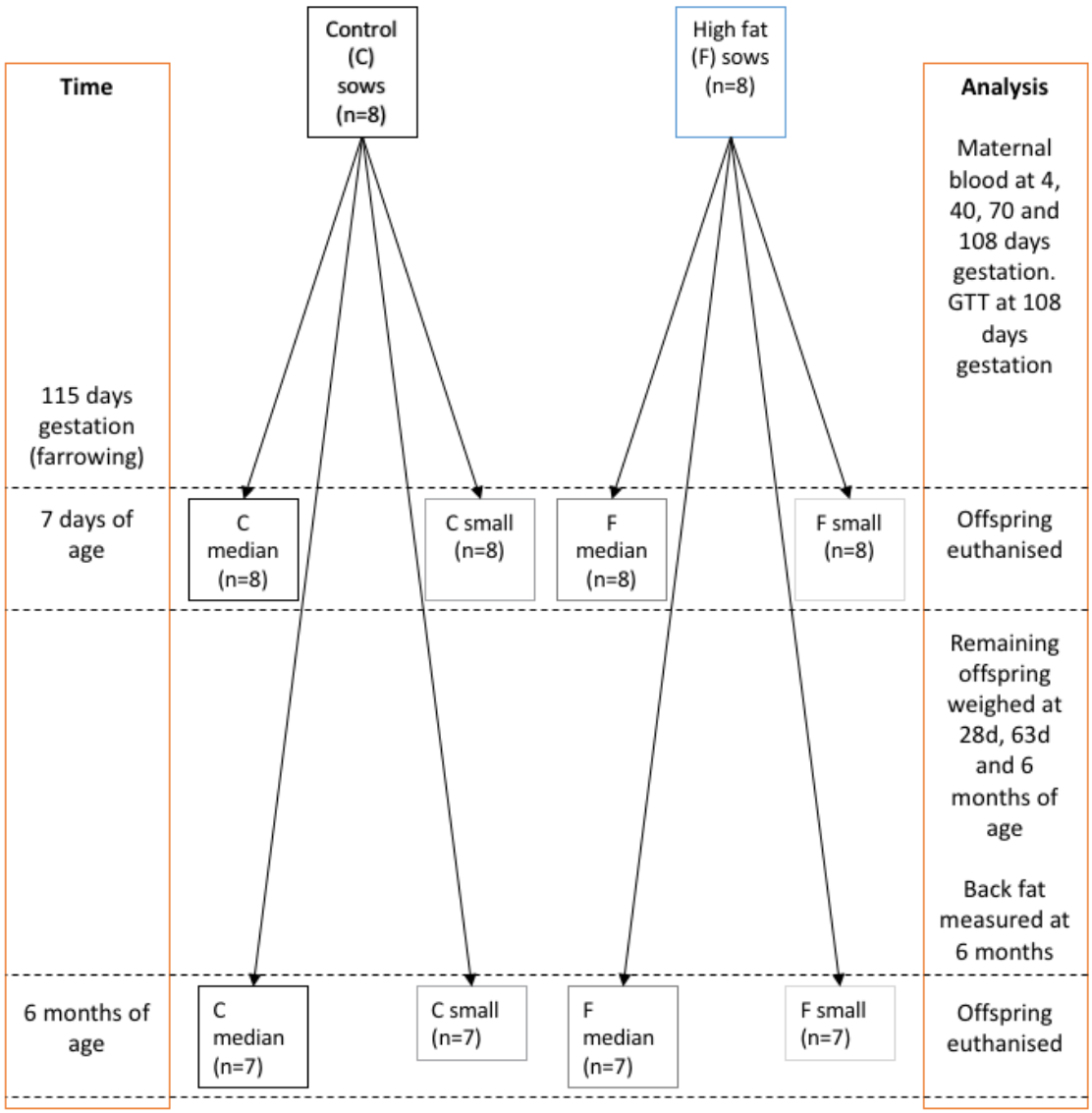


Figure 2-1: Outline of the Schothorst study.

2.4. Sweet Moms study design

2.4.1. Animal handling and diets

This study was carried out as the animal component of the Sweet Moms (SM) study in the Division of Human Nutrition, University of Alberta. Wistar rats (Charles River, Montreal, QC, Canada) were obtained at 6 weeks of age. Animals were given 2 weeks to acclimate to the animal facility at the University of Alberta, during which time they were given *ad libitum* access to distilled water and laboratory chow (LabDiet 5001, Purina, Mississauga, ON, Canada) conforming to the American Institute of Nutrition (AIN) 93 guidelines on laboratory rodent diets (216). At 8 weeks of age female rats were randomly assigned to control (n=15) or fructose (n=17) groups. All rats were fed chow and C1 rats were provided with distilled water for drinking, and F1 rats distilled water containing 10% D-fructose (Sigma-Aldrich). In both groups water and chow were fed *ad libitum*. At 11 weeks of age female rats were pair housed with male Wistar rats for mating, successful mating was confirmed by vaginal lavage with saline followed by confirmation of the presence of semen under a microscope at the end of week 10. Females were then pair-housed throughout pregnancy. When dams reached gestational day 20 (~13 weeks of age), dams in each group were randomly selected for dissection and tissue sampling, these formed the groups C1 (n=9) and F1 n=6).

The remaining first generation dams were allowed to litter out and suckle normally, with litters culled to 10-12 rats/dam. For the first week of suckling all dams were switched to the control diet, before being switched back to their respective diets. At the end of lactation, two female pups from each litter were randomly selected for the second generation study and placed on the same diet as their dam at 8 weeks of age. These rats were then mated as above at 11 weeks, followed by euthanasia at 21 days of gestation. These dams formed the groups C2 (n=10) and F2 (n=10).

Smaller groups of non-pregnant females were also divided into diet groups (C1, n=4, F1 n=4, C2 n=5, F2 n=5), these rats were subject to the same sampling as the pregnant rats and euthanised at the same time points.

2.4.2. Physiological and body composition measurements

In both generations, rats were weighed weekly. 24-hour food and water intake was measured at 5 time points through the study: pre-diet (7 weeks of age), pre-mating (11 weeks), early pregnancy (12 weeks, GD 4-7), mid pregnancy (13 weeks, GD 14-17), late pregnancy (14 weeks, GD 19-20). For these 24-hour periods rats were housed individually. At the same time points monitoring blood samples were also taken at ~8am for plasma glucose, insulin and triglyceride. Whole blood was collected from the tail vein, and stored in K_2 EDTA microtainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Tubes were immediately centrifuged at 1800g to separate the plasma, which was then stored in sterile 0.5ml Eppendorf tubes at -20°C for further analysis.

Oral glucose tolerance tests (OGTT) were carried out on rats at GD19. Rats were fasted overnight and a blood sample was collected from the tail vein at ~8am the following morning, oral glucose (3g glucose/kg body weight in distilled water) was then administered by gavage. Blood samples were then taken at 15, 30, 45, 60 and 90-minute intervals following the glucose dose. Blood samples were stored as described above. Area under the curve (AUC) measurements were calculated using the trapezoidal rule.

2.4.3. Dissection

Immediately prior to dissection at GD 21, percentage lean and fat mass was measured in pregnant rats using magnetic resonance imaging (EchoMRI Whole Body Composition Analyzer, Echo Medical Systems,

Houston, TX, USA). Following this, rats were euthanised using CO_2. Body weights were measured immediately after euthanasia and a blood sample was taken by cardiac puncture. The pancreas, liver, adipose tissue depots (uterine, retroperitoneal and brown/interscapular) and small intestine were removed and snap-frozen in liquid nitrogen. Before freezing the small intestine was flushed using cold 10% PBS and separated in to ileum and jejunum sections. Separate sections of ileum and jejunum were also stored in Z-Fix (buffered zinc formalin, Anatech, Battle Creek, MI, USA) for histological analysis. Fetuses were counted and individually weighed, as were placentae for calculation of fetal:placental weight ratios. All snap-frozen tissue was stored at -80°C for later analysis.

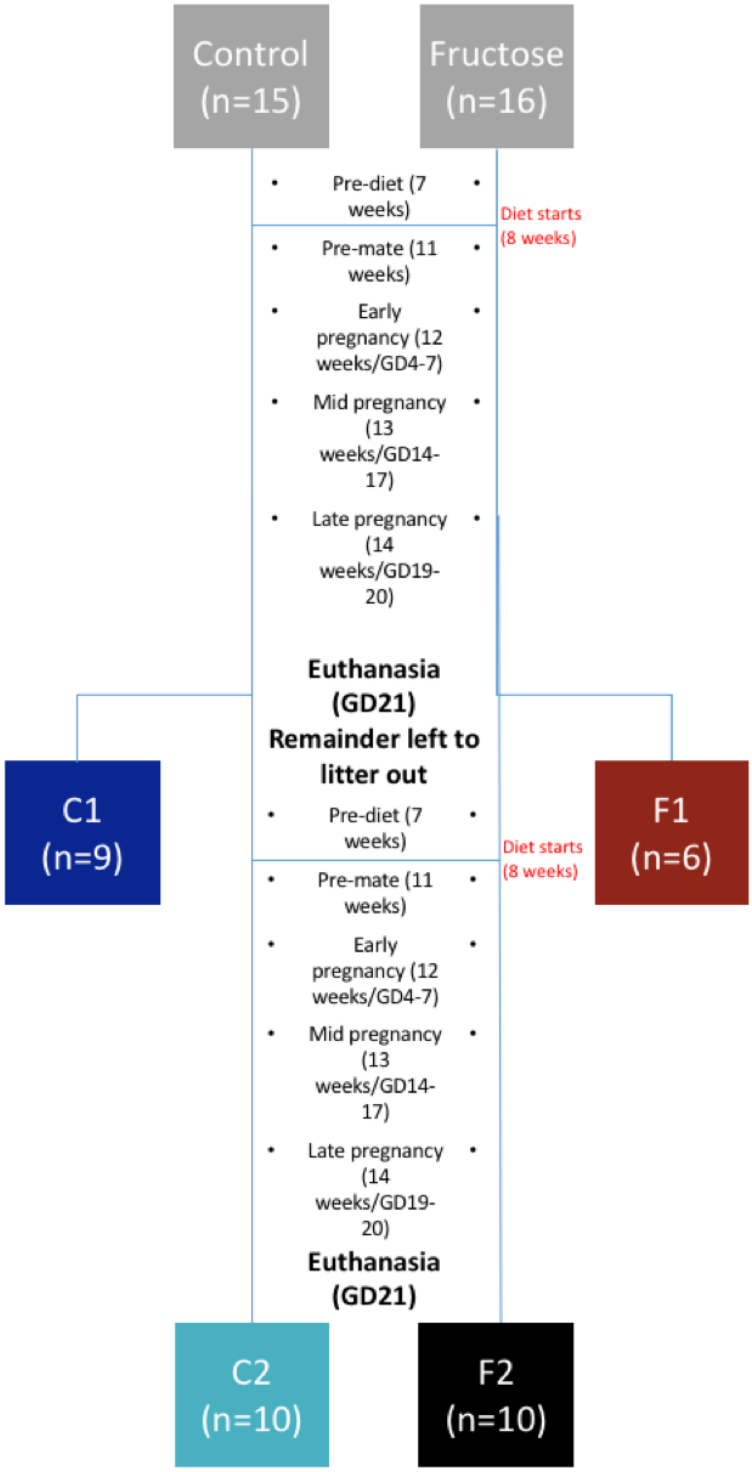


Figure 2-2: Outline of the SM study showing blood collection and euthanasia time points.

2.5. Tissue analysis

Prior to working with animal tissue and before carrying out any molecular techniques, bench surfaces and instruments were cleaned with RNAse Zap (Ambion, Foster City, CA, USA) and 70% ethanol. Where required, experimental work was performed on ice using autoclaved equipment and filtered pipette tips to prevent sample carryover between tips. All tissue samples were stored at -80°C until needed.

2.6. Ribonucleic acid extraction

Total ribonucleic acid (RNA) extraction can be achieved using the phenol/chloroform method (217), now most commonly carried out using commercially available RNA extraction kits. By lysing samples in a guanidine thiocyanate and phenol solution (most commonly available as TRIzol), the proteins are lysed and any RNAses present are inhibited (218). Adding chloroform to this solution and centrifuging will result in separation into three phases: a lower phase of dissolved protein, an interphase of DNA precipitate and a clear liquid upper phase of RNA. This RNA phase can be carefully removed and treated with ethanol to make it suitable for use in RNA binding columns containing silica membrane filters. Samples are passed through these columns with a number of guanidium and ethanol based washes to remove any contaminants that may be present. Finally sterile nuclease-free water can be used to elute the RNA from the column.

Once extracted it is important to ascertain the purity and concentration of each extracted RNA sample, as proteins can be introduced at any point of the extraction procedure and will not be removed by the washing steps. These effects can be detrimental in downstream applications of RNA such as quantitative polymerase chain reaction (qPCR) (219). This purity is most commonly measured using spectrophotometry; as nucleic acids

absorb light at 260nm the relative wavelength absorption of a sample and therefore the RNA concentration can be measured. Proteins absorb light at 280nm and therefore the 260/280 ratio can be used as a measure of protein contamination of a sample (220); with a 260:280 of between 1.8 and 2.0 being recommended (221).

2.6.1. RNA extraction procedure

Small intestinal samples of ~100-150mg were homogenised in 1ml TRI Reagent at 3000rpm using a Dispomix Drive and tubes (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting homogenate was transferred to sterile 1.5ml Eppendorf tubes and 200µl of chloroform (Fisher Scientific, Leicestershire, UK) was added to each sample. These tubes were then mixed briefly and left for 10 minutes at room temperature to begin phase separation. Following this room temperature incubation the samples were centrifuged at 12000g for 15 minutes at 4°C, the top aqueous layer of RNA (~600µl) could then be removed and transferred to another sterile Eppendorf tube. An equal amount of ethanol was then added to each sample.

The resulting RNA/ethanol mixture was then purified using the RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK). Each sample was transferred to a spin column and centrifuged at 8000g for 15 seconds, the RNA is retained in the spin column and the flow-through can be discarded. This spin step was then repeated with RW1 buffer, and a 2-minute spin after the addition of RPE buffer, discarding the flow-through after each spin. Each column was then transferred to a new Eppendorf tube and spun again for a further minute, again at 8000g, to reduce the chance of RPE buffer contaminating the extracted sample. Columns were transferred to another clean Eppendorf tube and 30µl of nuclease free water (Qiagen Ltd.) was added before spinning for 1 minute at 8000g, the flow-through was then applied to the column and spun again to increase the concentration of RNA.

Following RNA extraction, samples were placed on ice and RNA concentration and purity was measured via Nanodrop (Thermo Scientific, Waltham, MA, USA). Extraction was repeated for samples with a 260:280 outside of the recommended 1.8-2.0 range. RNA samples were then diluted to 1µg/µl for reverse transcription to complementary DNA (cDNA). All RNA samples were stored at -80°C.

2.7. Polymerase chain reaction

The polymerase chain reaction (PCR), allows the amplification and subsequent analysis of a known DNA sequence, using a pair of complementary oligonucleotide primers. When the target DNA is denatured, the double helix strand separates and the primers bind to the 3' of each single strand. The addition of a DNA polymerase enzyme (most commonly sourced from *Thermus aquaticus* bacteria) enables DNA replication beginning at the primer binding sites and by variations in the reaction temperature, the exponential amplification of the template sequence.

The specificity of PCR depends largely on the design of the 18-30 base pair primers which need to be targeted to the DNA sequence of interest. Poorly designed primers will result in the incorrect product being amplified or primer pairs binding to each other to form primer dimers – both of these will interfere with downstream applications. Table 2-3 contains the recommended parameters for primer design (222).

Parameter	Optimal value(s)
Primer length	18-30 bases
Melting temperature (T_m)	55-72°C
Percentage guanine/cytosine	40-60%
No self-complementarity	≤3 continuous complementary bases
No-complementarity to opposite primer	≤3 continuous complementary bases
Distance between primers on target sequence	<2000 bases
T_m difference between primers	≤5°C
Identical continous bases	≤4

Table 2-3: Recommended parameters for PCR primer design, adapted from (222).

2.8. Reverse transcription PCR

As explained above, PCR requires double stranded DNA to amplify, therefore the single stranded RNA generated from tissue extraction must be transcribed to complementary DNA (cDNA) for further analysis. By incubating the RNA sample with random PCR primers, dNTPs and a reverse transcriptase enzyme, a double stranded cDNA product complementary to the template RNA molecule can be generated. This cDNA is then suitable for standard PCR as described above, or other analytical techniques such as quantitative PCR.

2.9. Quantitative PCR

Whilst standard PCR followed by gel electrophoresis on the DNA product can be used to detect the presence or absence of a particular sequence within a DNA sample, it is not possible to detect more subtle changes in gene expression. The advent of quantitative or real-time PCR in the 1990s (223) enables reactions to be tracked as they progress, removing any further steps such as agarose gels and Northern blotting and thus reducing time and cost, as well as the potential for contamination (224). In qPCR the DNA sequence is amplified as in a classical PCR reaction, along with a fluorescent dye that binds to double stranded DNA, therefore the level of

fluorescence can be used to measure the concentration of DNA present. As these dyes will emit a baseline level of fluorescence, the instrument used for detection is calibrated with a threshold – the PCR cycle at which the reaction crosses this threshold of fluorescence is known as the "cycle threshold" (C_t) or "crossing point" (C_p). The cycle threshold of each sample can then be used to compare the amount of DNA present in the initial template, and eventually the level of expression of a given gene.

As described above, the amplification in a PCR reaction is exponential, therefore the output of the qPCR instrument will display these increases in fluorescence as the reaction progresses as sigmoidal curves, as shown in Figure 2-3.

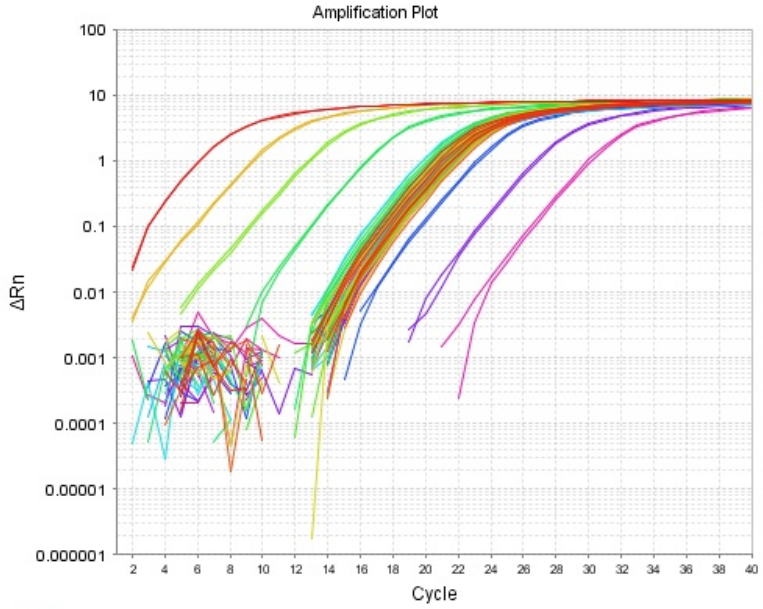


Figure 2-3: Example amplification plot generated from a qPCR instrument.

The fluorescent signal crosses the cycle threshold (Ct) and increases exponentially until the curves plateau as reagents in the reaction are depleted.

By using these C_t values, along with serial dilutions of a known sample of DNA, the concentration of unknown samples can be interpolated using a standard curve. The log concentrations (in ng/µl) of these serial dilutions are plotted against the cycle number at which the C_t is reached, and a straight line equation is calculated (Figure 2-4). The efficiency of the PCR

reaction can be calculated from this (where efficiency = $10^{(-1/gradient)}$-1) (225), which is derived from the equation describing PCR amplification (226).

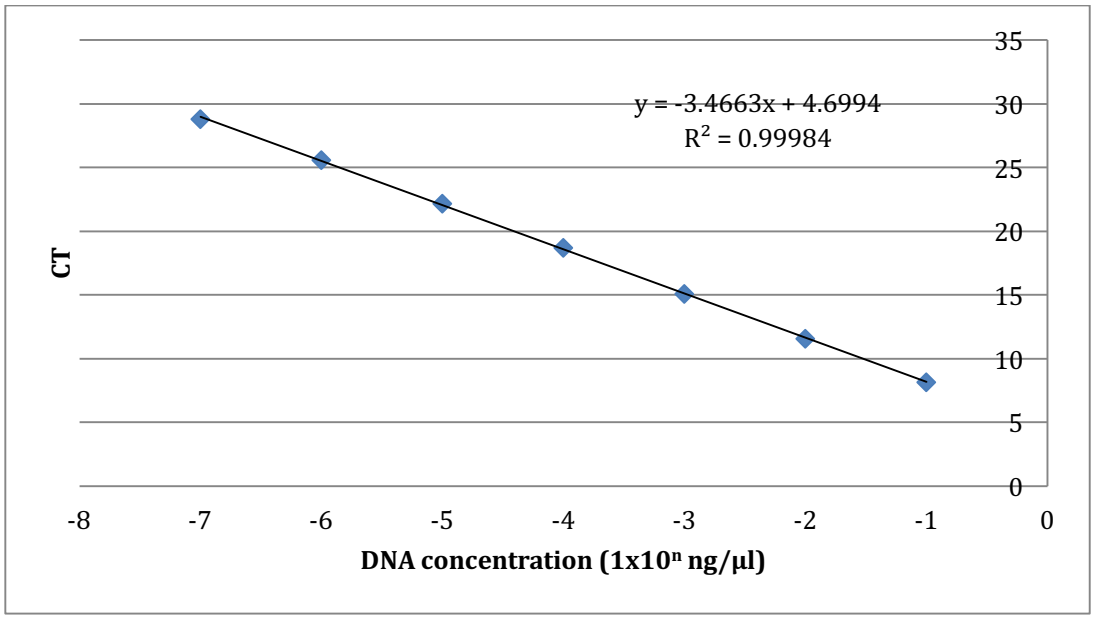


Figure 2-4: The data from Figure 2-3 plotted as a standard curve, with the associated R_2, gradient and intercept.

As it is impossible to ensure that each sample measured contains exactly the same concentration of starting RNA, it is necessary to normalise the samples to the expression of a gene or genes which are expressed at a consistently high level regardless of experimental conditions. The expression of the gene of interest can then be compared against the expression of the chosen reference gene in the same sample to quantify gene expression. This calculation requires the use of a simple mathematical model, known as the $2^{-\Delta\Delta CT}$ method (227). This model uses a ratio of change in cycle threshold between the reference gene and the gene of interest, where ΔC_t = average C_t of target gene – average C_t of reference gene of the same sample. This method assumes that the qPCR reaction is progressing at 100% efficiency, meaning that the cDNA strands are replicated entirely in each PCR cycle. As explained above the efficiency can be verified using a standard curve. Recommended efficiencies for the qPCR reaction are between 95 and 105% (225).

In the case of qPCR reactions using non-specific fluorescent dyes such as SYBR Green (as opposed to probes such as TaqMan), further steps must be taken to verify that the fluorescence produced is due to the dye binding to the intended DNA product and not another sequence (such as a contaminant or primer dimer). Effective primer design and good laboratory practice can go some way toward achieving this, and adding a melt curve to the end of a qPCR reaction enables qualitative analysis of the PCR product. As DNA products of different sizes will have different melting temperatures, gradually increasing the temperature of the reaction and measuring the subsequent drop in fluorescence can confirm the presence of a single DNA product.

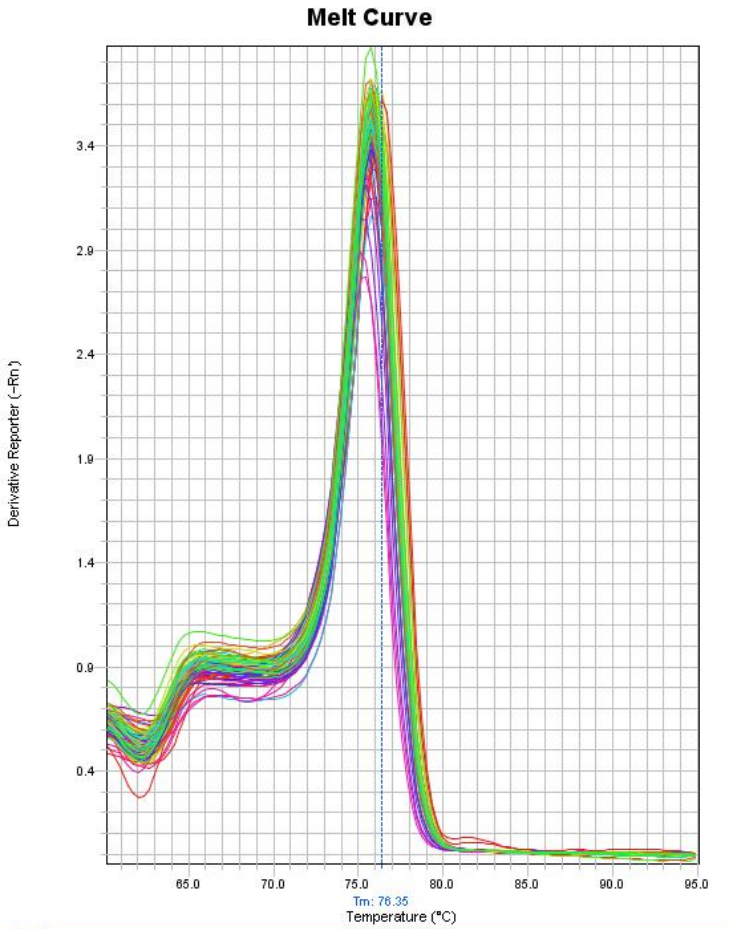


Figure 2-5: Example melt curve showing a single DNA product.

A single peak on the melt curve confirms that there is a single DNA product present. For further confirmation this product can then be sequenced, and the sequenced matched to the relevant genome using the Basic Local Alignment Search Tool (BLAST) (228).

2.10. Reference genes in qPCR

For the $2^{-\Delta\Delta CT}$ method to be used effectively, it is vital that the reference genes selected are stable between groups and ideally have been validated previously in the relevant tissue type (229). It is also recommended that several reference genes be tested, and the geometric mean of the most stable genes be used (229,230). For samples taken from the Schothorst study (contained in chapter 3 of this thesis), reference genes were chosen based on those already used in porcine tissue (231), as well as the commonly used ribosomal proteins 60s acidic ribosomal protein P0 (RPLP0) and 18s ribosomal RNA (18s). For samples used in the SM study, the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RPLP0 were used based on their previous use in the literature (232–234)

The web-based tool RefFinder (available at http://fulxie.0fees.us/?type=reference) was used to assess the stability of the groups of reference genes chosen: RPLP0, 18s and importin 8 (IPO8) in the Schothorst study and GAPDH and RPLP0 in the SM study. RefFinder combines several widely used algorithms: BestKeeper (235), NormFinder (236), Genorm (230), and the comparative ΔCT method (237) to determine the most stable reference gene or the most stable combination of genes. Based on these results the geometric mean of RPLP0 and 18s was used as the reference gene value for all $2^{-\Delta\Delta CT}$ equations for samples in the Schothorst study. The geometric mean of GAPDH and RPLP0 was used as the reference value for samples in the SM study.

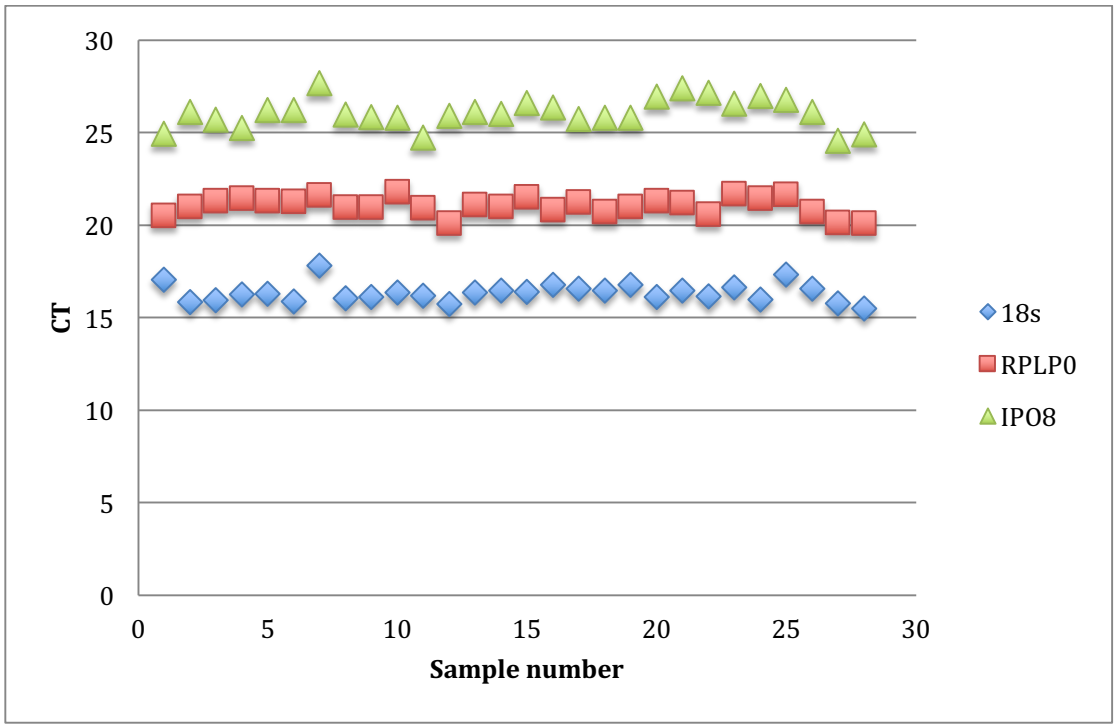


Figure 2-6: Example comparison of CT values for reference genes 18s, RPLP0 and IPO8 between all samples in the Schothorst study.

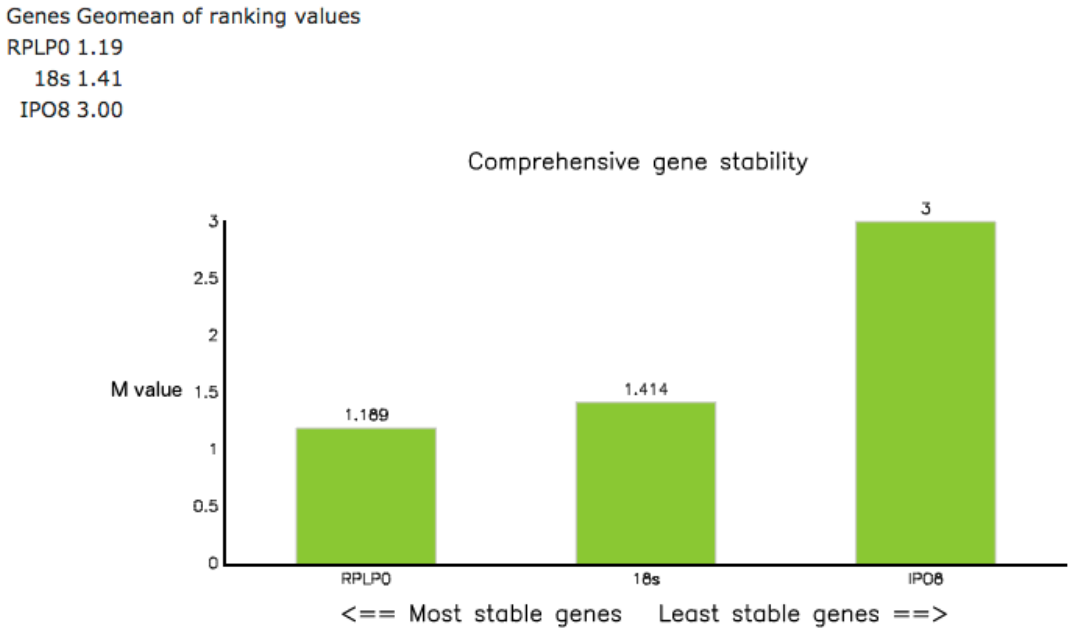


Figure 2-7: RefFinder output using the CT values contained in Figure 2-6.

The average stability (M) value is calculated from the coefficient of variation for each gene. A lower M value represents a higher stability (230).

2.11. Primer design for qPCR

Primers used in my thesis are either taken from the literature and will be cited, or self-designed using the web-based tool Primer3 Plus (238), sourcing the target sequences from the ENSEMBL genome database (239). Self-designed primers were designed according to the parameters in table Table 2-3, and all sequences were designed to span introns of the target sequence, to remove the possibility of amplifying any genomic DNA. All primers were tested using agarose gel electrophoresis (described in section 2.13) before qPCR to ensure the primer is producing a single product of the correct size and not forming any larger primer dimers.

Primers for the Schothorst study were purchased from Sigma-Aldrich as desalted powder at a scale of 25nmol, these were then made up to a 100µM stock solution using nuclease-free water and stored at -20°C. Primers for the SM study were purchased from Integrated DNA Technologies (Coralville, IA, USA) with the same specifications. All primer sequences were tested before purchase using BLAST (228) to ensure that only the correct sequence was targeted. Table 2-4 - Table 2-6 detail all sequences used for PCR amplification.

Gene	Species	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Product size (bp)
18s ribosomal RNA (18s)	Pig	GATGCGGCGGCGTTATTC	CTCCTGGTGGTGCCCTCC	125
60s acidic ribosomal protein P0 (RPLP0)	Pig	CAGCAAGTGGGAAGGTGTAATC	CCCATTCTATCATCAACGGGTA	75
Importin-8 (IPO8)	Pig	GCCCTTGCTCTTCAGTCATT	GTGCAACAGCTCCTGCATAA	93
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Rat	TGGAGTCTACTGGCGTCTT	TGTCATATTTCTCGTGGTTCA	138
60s acidic ribosomal protein P0 (RPLP0)	Rat	GATGCCCAGGGAAGACAG	CACAATGAAGCATTTTGGGTAG	91

Table 2-4: Primers for reference genes used in the Schothorst and SM studies.

Gene	Species	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Product size (bp)
Occludin (OCLN) (125)	Pig	ATCAACAAAGGCAACTCT	GCAGCAGCCATGTACTCT	157
Claudin-3 (CLDN-3) (240)	Pig	TATCATCCTGGCCGTGCTA	CATCATCCACGCAGTTGGT	71
Zonulin-1 (ZO-1) (125)	Pig	GAGTTTGATAGTGGCGTT	GTGGGAGGATGCTGTTGT	298
Epidermal growth factor receptor (EGFr)	Pig	CCTTGGGAACTTGGAGATCA	GGTTTTATTGGCCCCGTAGT	208
Toll-like receptor 4 (TLR4)	Pig	CGTGGGTGTCCCAGCACTTC	CGGCATGACTCCTCAGAAACT	90
Interleukin 1β (IL-1β)	Pig	AGTGGAGAAGCCGATGAAGA	ACGTTGGCATCACAGACAAA	138
Tumour necrosis factor α (TNF-α)	Pig	GCCTCTTCTCCTTCCTCCTG	CGGGCTTATCTGAGGTTTGA	162
Mucin 1 (MUC1) (240)	Pig	CCCTGGCCATCATCTATGTC	TGCCCACAGTTCTTTCGTC	64
Porcine beta defensin 2 (pBD2) (240)	Pig	GCTGACTGTCTGCCTCCTCT	CCAAGACCCGTAAGCAGGT	63

Table 2-5: Primers used in the Schothorst study.

Gene	Species	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Product size (bp)
Intestine				
Zonulin-1 (ZO-1)	Rat	GCATGTAGACCCAGCAAAGG	GGTTTTGTCTCATCATTTCCTCA	65
Claudin-3 (CLDN-3)	Rat	GGACCCAGTTCACCTTTCC	GCCTCTAATCCCTCATTTCACA	188
Occludin (OCLN)	Rat	ATCTAGAGCCTGGAGCAACG	GTCAAGGCTCCCAAGACAAG	63
Junctional adhesion molecule A (JAMA)	Rat	GCCTATAGCCGTGGATACTTTG	ACGAGGTCTGTTTGAATTCTCC	112
Glucose transporter 2 (GLUT2)	Rat	AAAGCCCCAGATACCTTTACCT	TGCCCCTTAGTCTTTTCAAGC	75
Glucose transporter 5 (GLUT5)	Rat	CTTCGGAGTGTCTTGGAAGC	GGCAGGGACTCCAGTCAG	69
Sodium-glucose linked transporter 1 (SGLT1)	Rat	GAAGGGTGCATCGGAGAAG	CAATCAGCACGAGGATGAAC	62
Liver				
Toll-like receptor 4 (TLR4)	Rat	GGATGATGCCTCTCTTGCAT	TGATCCATGCATTGGTAGGTAA	127
Cluster of differentiation 68 (CD68)	Rat	TGAGGTTCCCTGTGTGTCTG	CTTCCGGTGGTTGTAGGTGT	127
Tumour necrosis factor α (TNF-α)	Rat	TGAACTTCGGGGTGATCG	GGGCTTGTCACTCGAGTTTT	122
C-jun N-terminal kinase (JNK1)	Rat	GCAGCCGTCTCCTTTAGGT	CATTGACAGACGGCGAAGA	71

Table 2-6: Primers used in the SM study.

2.12. PCR procedures

2.12.1. Reverse transcription PCR procedure

0.2ml Eppendorf tubes were labelled for each sample, tubes were also prepared containing the template RNA with no RT enzyme to ensure no DNA contaminants were present in the reaction mixture. A negative RNA control, containing the RT reaction mixture with nuclease free water in place of the RNA sample was also included. Reaction mixtures were set up as follows, following the recommended instructions included with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA):

20µl reaction volume containing:

- 10µl 2x RT buffer (Applied Biosystems)
- 1µl 20x RT enzyme mix (Applied Biosystems)
- 6µl template RNA, diluted to 2µg/µl with nuclease free water
- 3µl nuclease free water (Qiagen N.V.)

Tubes were then centrifuged for 1 minute at 5000g and incubated in a Techne TouchGene thermocycler (Techne Ltd., Cambridge, UK) using the following run method:

- 37°C for 60 minutes
- 95°C for 5 minutes
- Held at 4°C

Following the RT reaction, cDNA samples were diluted 1:10 with nuclease free water, and the resulting 200µl solution stored at -20°C.

2.12.2. Classical PCR procedure

To ensure that all primers were optimised, and to produce DNA of a known concentration for the qPCR standard curves, all samples were amplified using classical PCR. Reactions were carried out as follows in 0.2ml Eppendorf tubes:

20µl reaction volume containing:

- 10µl *Taq* polymerase (One*Taq* Hot Start Quick Load 2x Master Mix, New England Biolabs, Ipswich, MA, USA)
- 4µl forward primer (1:40 dilution from 100µM stock)
- 4µl reverse primer (1:40 dilution)
- 2µl cDNA sample

Negative templates containing water either in the place of cDNA or primer pairs were also prepared to confirm the efficiency of the reverse transcription reaction and ensure that no DNA contaminants were present. Tubes were centrifuged for 1 minute at 10000g and the reaction was then carried out in the TouchGene thermocycler (Tenche Ltd.). Table 2-7 contains the run method.

Step	Temperature (°C)	Duration (mins)
Enzyme activation	94	0.5
Denaturation*	94	0.5
Annealing*	60	0.5
Extension*	68	1
Final extension	68	5
Hold	4	∞

Table 2-7: Run method for classical PCR.

***Steps are cycled 40 times before the final extension step.**

2.13. Agarose gel electrophoresis and DNA extraction

DNA samples can be analysed and resolved by passing them through an agarose gel matrix. By applying an electric charge to the agarose gel the DNA can be separated according to size, as DNA is negatively charged it will migrate towards the positive electrode with smaller DNA fragments progressing more quickly than larger ones (241).

DNA fragments that have been passed through an agarose gel can be visualised by adding ethidium bromide (a dye which will bind to double stranded DNA and fluoresce under ultraviolet light), and using a "ladder" – a sample of DNA fragments of known molecular weights. Running out samples on an agarose gel therefore allows the weight of DNA fragments to be determined, these can then be matched with the expected product sizes to give an indication of primer efficiency. Secondary products and primer dimers will also be shown on agarose gels and indicate that the primers are not suitable for use in qPCR and require further optimisation. Fragments of the correct size can then be cut out of the agarose gel and extracted using a commercially available DNA extraction kit.

2.13.1. Agarose gel electrophoresis and DNA extraction procedure

2% w/v of electrophoresis grade agarose powder (Invitrogen Life Technologies, Waltham, MA, USA) was dissolved in 50ml of 1x TAE buffer (tris(hydroxymethyl)aminomethane (tris) base, glacial acetic acid and EDTA buffer), and heated until dissolved. Following heating 5µl of 10mg/ml ethidium bromide was added and the mixture was left to cool in a gel cast along with a comb to form wells to which DNA samples could be added. Once the gel was set the comb was removed and the 20µl PCR products were added, along with 5µl of DNA ladder (100bp Blue eXtendec, Bioron, Ludwigshafen, Germany) in the leftmost and rightmost wells of the gel. The gel was then run in an electrophoresis tank (Medical Engineering

Unit, School of Life Sciences, University of Nottingham) in 1x TAE buffer at 100v for 50 minutes. PCR products were then visualised under a UV trans-illuminator CCD camera (LAS-1000 v1.01, Fujifilm Europe GmbH, Düsseldorf, Germany). Relevant samples were then excised from the gel under UV light, using a scalpel.

DNA extraction from excised gel fragments was carried out using the QIAquick™ gel extraction kit (Qiagen). Dissected gel bands were weighed and dissolved in 300µl of QG buffer per 100mg of gel, and incubated at 50°C for 10 minutes, with frequent shaking until the agarose gel was completely dissolved. 100µl of isopropanol per 100mg of agarose was then added and the mixture shaken. This mixture of agarose and buffer was then applied to the QIAquick spin column and centrifuged at 10000g for 1 minute, the DNA binds to the membrane within the column and the QG and isopropanol flow-through was discarded. To remove any leftover traces of agarose, 500µl of buffer QG was applied to the column and spun at 10000g for 1 minute and the flow-through discarded. 700µl of PE buffer was then added to the column and allowed to stand for 5 minutes before being centrifuged at 10000g for 1 minute, discarding the flow-through. The spin column was then transferred to a new sterile 1.5ml Eppendorf tube and centrifuged again at 10000g for 1 minute to remove any residual buffer in the column. DNA was then eluted by adding 30µl of elution (EB) buffer to the column and centrifuging for 1 minute at 10000g. DNA samples were then quantified via Nanodrop, and any samples produced using previously untested PCR primers were sequenced at the DNA Sequencing Facility, Medical School, University of Nottingham. All samples were diluted to 1ng/µl and stored at -20°C.

2.14. qPCR procedure

The following process applies to all qPCR carried out, including reference genes. DNA extractions carried out in section 2.13.1 were used to generate standard curves for all qPCR reactions, serial dilutions from 0.1ng/µl to $1x10^{-8}$ng/µl were produced from the 1ng/µl samples extracted from agarose gels and nuclease free water. Reagents were made up using Fast SYBR Green mastermix (Applied Biosytems, and primer pairs in the following quantities:

- 5µl Fast SYBR Green mastermix
- 1µl forward primer (1:40 dilution of 100µM stock)
- 1µl reverse primer (1:40 dilution)
- 3µl unknown cDNA, standard, or no template (H_2O control).

All samples, controls and standards were pipetted in duplicate. These were pipetted on to 96 well qPCR plates (MicroAmp Fast Optical, Applied Biosystems, CA, USA), and qPCR reactions run in the StepOne Plus Real Time PCR system (Applied Biosystems). The run method for all reactions was as follows:

Step	Temperature	Duration
Denaturation	95°C	20 seconds
Denaturation*	94°C	3 seconds
Annealing/Extension*	60°C	30 seconds
Melt curve	0.3°C increments to 95°C	-

Table 2-8: Run method for qPCR.

***Steps repeated for 40 cycles.**

Reaction data was recorded using the StepOne v2.2 (Applied Biosystems) software, and exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA) for fold change calculations using the $2^{-\Delta\Delta CT}$ method.

2.15. Histology

Histology is the microscopic study of sectioned tissues to allow visualisation of the cellular composition, a process that can be aided by a variety of stains. Tissue must be "fixed" prior to any histology to prevent any degradation of the sample – this is most commonly achieved via storing the tissue in formaldehyde immediately following dissection. Formaldehyde will irreversibly cross-link proteins within the sample to preserve it (242).

Once fixed, the tissue must also be encased within a matrix within which the tissue can be embedded – paraffin wax is most commonly used for samples to be analysed with light microscopy, it will permeate through the tissue without altering any of the structural components. The formaldehyde fixed tissue must first be dehydrated, usually using ethanol, as paraffin is immiscible in water, and following cleaning with xylene the sample can be embedded and sectioned using a microtome.

2.15.1. Histological processing – University of Alberta

At dissection intestinal samples were placed directly into histological cassettes (30x27x5mm, Histosette II, Simport, Saint-Mathieu-de-Beloeil, QC, Canada) and submerged in Z-Fix (buffered zinc formalin, Anatech, Battle Creek, MI, USA). Samples were processed in a Leica TP1020 tissue processor (Leica Microsystems, Concord, ON, Canada). Processing consisted of six stages of dehydration with increasing concentrations of ethanol, followed by three stages of xylene cleaning.

Tissue embedding in paraffin was carried out using a Leica EG1160 (Leica Microsystems). Transverse sections of intestine were cut and laid flat to enable the clearest visualisation of the villi. Following embedding, tissue blocks were sectioned on a rotary microtome (Leica RM2125 RT, Leica

Microsystems) at 5µm thickness, with 2 sections included on each slide and 5 slides prepared for each sample. Following sectioning slides were dried at 60°C overnight.

2.15.2. Haematoxylin and eosin staining

Staining with haematoxylin and eosin is one of the most widely used techniques in histology, enabling differentiation of nuclei and extracellular proteins to determine the structure of a sample. The method uses haemalum, a complex of aluminium ions and the dye haematein which will bind to DNA, and a "counterstain" of eosin which will bind to positively charged (acidophilic) structures such as both intracellular and extracellular proteins (243).

All staining procedures were carried out by staff at the Histology Core, Alberta Diabetes Institute, University of Alberta. Once mounted on slides, samples were stained with haematoxylin and eosin following the manufacturer's instructions (aqueous instant dyes, Shandon, Thermo Fisher Scientific). Samples were dewaxed using two washes of xylene for 10 minutes each, followed by rehydration using progressively decreasing concentrations of ethanol (100% ethanol x2, 5 minutes each, 95% for 2 minutes and 75% for 2 minutes). After briefly washing with distilled water, samples were stained in Harris's haematoxylin for 4 minutes and washed under running tap water for 5 minutes. To prevent oversaturation of tissues with the haematoxylin stain, the samples were briefly washed with an acid alcohol step (1% hydrochloric acid in 70% ethanol) which was then washed with tap water. Samples were then counterstained in 1% eosin for 5 minutes, and again washed with tap water for 90 seconds. Finally samples were dehydrated using increasing concentrations of ethanol (95% for 2 minutes and 100% for 2 minutes) before being cleaned with 2 washes of xylene for 2 minutes each. Slides were then immediately

covered using dibutyl phthalate in xylene (DPX) to adhere cover slips to each slide, and left to dry.

2.15.3. Histological measurements

All slides were imaged using a AxioImager.Z1 microscope (Zeiss Ltd., Toronto, ON, Canada) and photographed using an attached Axio MRm camera (Zeiss Ltd.). Images for analysis were scanned at 10x magnification with an exposure time of 1 millisecond. All image analysis was carried out using ImageJ (Research Services Branch, National Institutes of Health, Bethesda, MA, USA, (244)).

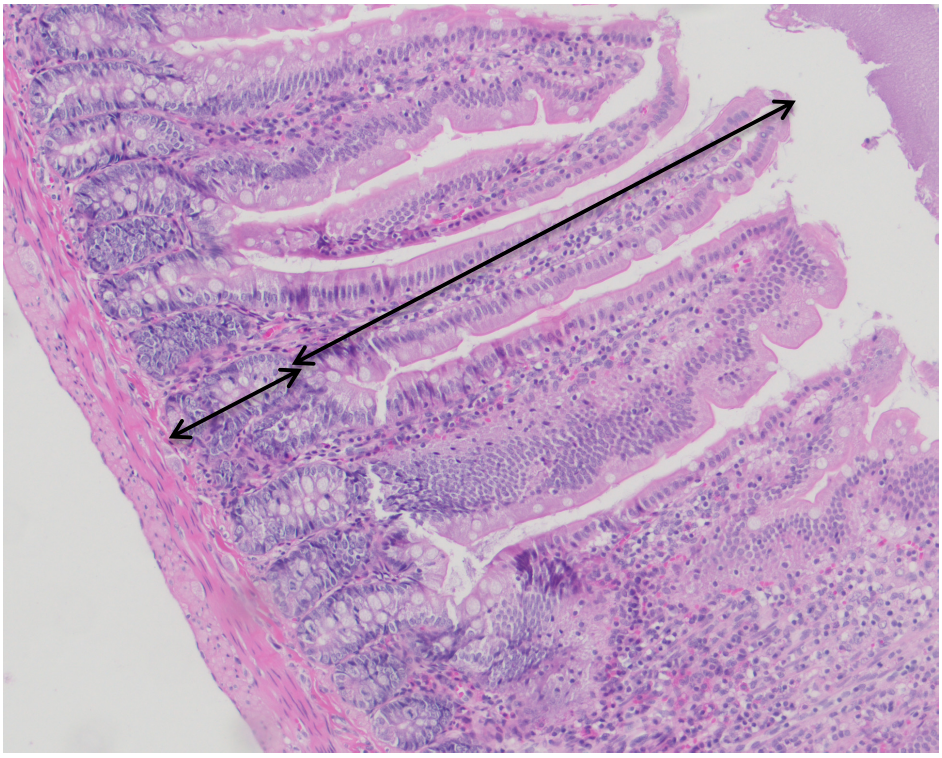


Figure 2-8: Example image of ileum section.

Arrows highlight how the villus height and crypt depth measurements were taken.

5 fields were taken from each slide at 10x magnification, and a scale bar used to calibrate to make villus height measurements in µm. 7-10 villus

height measurements were taken from each field and used to calculate an average villus height for each animal.

2.15.4. Histological processing – University of Nottingham

Samples from the Schothorst study were not fixed for histology at dissection, therefore samples were thawed before being placed in paraffin. Sections of jejunum were cut from frozen tissue and left at -20°C for 12 hours, and 4°C for a further 12 hours. Following thawing samples were placed in formalin and processing carried out as described in sections 2.15.1 and 2.15.2.

Due to the condition of the villi, most likely caused by long-term freezing of the tissue, histological analysis was not carried out following haematoxylin and eosin staining.

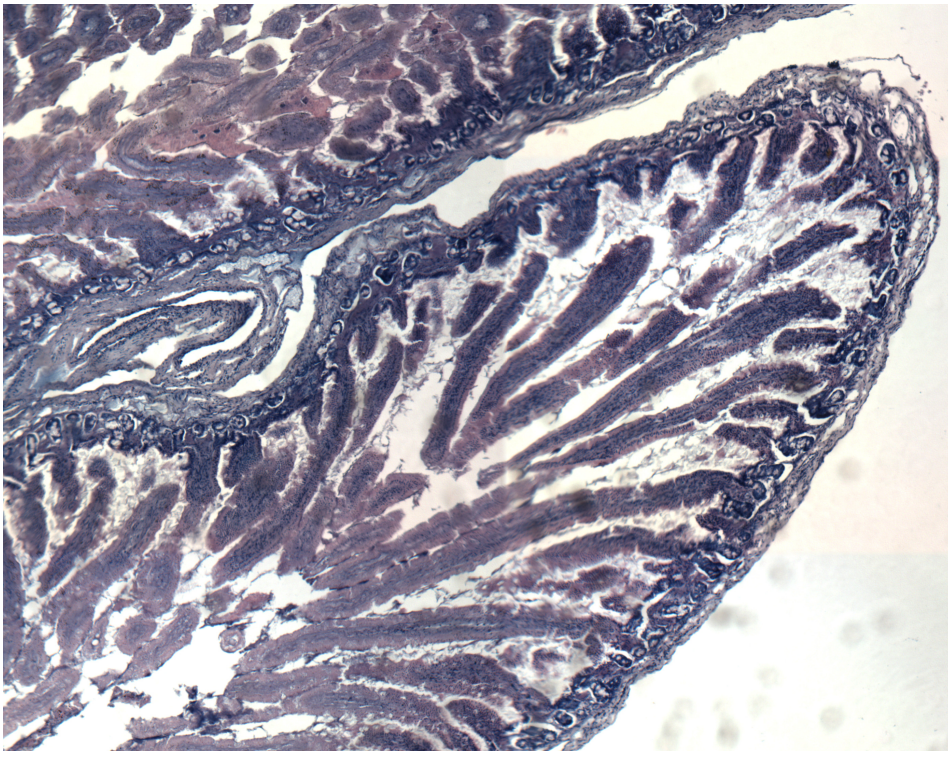


Figure 2-9: Representative section of jejunum stained with haematoxylin and eosin, showing broken and impacted villi.

2.16. Protein expression analysis

Protein immunoblotting or Western blotting is a method used to quantify protein expression in biological cell lysate samples (245). A tissue sample is first homogenised to release the contents of its constituent cells, which can then be measured via bicinchoninic acid (BCA) or Lowry assay. These homogenised samples are usually suspended in a Tris/EDTA buffer along with phenylmethanesulfonylfluoride (PMSF) or other protease inhibitor to prevent sample breakdown. After further dissociation via boiling in β-mercaptoethanol (which will break any disulphide bonds between amino acids and disrupt the tertiary and quaternary structures of proteins) and sodium dodecyl sulphate (SDS), the separated proteins can be applied to a polyacrylamide gel and separated via electrophoresis. The principal here is similar to that outlined in 2.13, as the proteins have been coated in SDS they acquire a negative charge and can be separated purely by size as they move through the acrylamide gel. Samples will generally be dyed at the dissociation step with a dye that moves at a speed equivalent to a known protein size, therefore the movement of samples through the gel can be tracked.

Once the samples have been separated via electrophoresis, they are blotted on to an electrostatic membrane – either nitrocellulose or polyvinylidene fluoride. By "sandwiching" the acrylamide gel and this membrane in a suitable transfer buffer and passing an electric current through the gel, the proteins will migrate from the gel and adhere to the membrane (246). This membrane can then be probed by incubating with an antibody complementary to the protein of interest, followed by a secondary antibody to enable imaging. This secondary antibody can either be conjugated to a compound that will emit light when excited with light of a certain wavelength (such as fluorescein isothyocyanate, FITC) or a chemiluminescent compound such as horseradish peroxidase (HRP), which will fluoresce when incubated with an appropriate substrate. When imaged, the proteins of interest will fluoresce as bands of the appropriate

molecular weight (measured in kilodaltons, kDa), which can be compared against a ladder of known protein sizes. The intensity of bands of the same protein in different samples can then be compared (densitometry) to determine differences in protein expression.

2.16.1. Protein extraction and bicinchoninic acid (BCA) assay procedure

~300mg of each sample was weighed and placed into a Dispomix tube (Miltenyi Biotec), and homogenised in 6ml CellLytic MT extraction buffer (C3228, Sigma-Aldrich). Directly before homogenisation 15µl protease inhibitor cocktail (P8340, Sigma-Aldrich) was added to the mixture. All samples were homogenised at 3000rpm for 30s. The homogenate was then transferred to a sterile 1.5ml Eppendorf tube and centrifuged for 10 minutes at 12000g to remove debris; the supernatant was then transferred to a new 1.5ml tube.

The protein concentration of each sample was then measured using the BCA assay; using a mixture of 100 parts 1% BCA to 2 parts 4% copper sulphate. Standards containing known quantities of bovine serum albumin (BSA) ranging from 0.05 to 1mg/ml of protein were used to prepare a standard curve. 10µl of samples and standards were pipetted on to 96-well plates along with 200µl of the BCA reagent. The plate was then incubated at 37°C whilst shaking for 30 minutes, before being read at 570nm.

2.16.2. Acrylamide gel electrophoresis procedure

Based on the concentrations obtained from the BCA assay, samples were diluted in CellLytic reagent to 4.2mg/ml, and 20µl of this further diluted with 50µl protein dissociation buffer (a mixture of 50mM Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and distilled water at pH 6.8), and 14µl of bromophenol blue dye. This resulted in a “master mix” of

protein at 1mg/ml ready for loading onto polyacrylamide gel. Before loading all samples were heated at 100°C for 10 minutes in a water bath and kept on ice.

12% polyacrylamide resolving gel was made up with the following components per 2 gels:

30% polyacrylamide mix (37:5:1)	3.3ml
Distilled water	4.0ml
1.5M Tris pH 8.8	2.5ml
10% SDS (sodium dodecyl sulphate)	100µl

Immediately prior to pouring:

10% APS (ammonium persulphate)	100µl
TEMED (tetramethylethylenediamine)	4µl

Following addition of APS and TEMED, gels were filled via syringe injection (0.75cm plates, Bio-Rad, Hercules, CA, USA), leaving a 3-4cm gap above for the addition of stacking gel. Water saturated butanol was pipetted on top of the resolving gel whilst setting to eliminate air bubbles. Gels were left to set for 30-40 minutes.

Stacking gel was made up with the following components per 2 gels:

Distilled water	3.4ml
30% polyacrylamide mix (37:5:1)	0.85ml
1.0M Tris p.H 6.8	0.625ml
10% SDS	50µl

Immediately prior to pouring:

10% APS	50µl
TEMED	5.0µl

Once the resolving gel was set, butanol was poured off and blotted using paper towel, and the stacking gel was pipetted on top using a syringe. A 15-well 0.75cm gel comb was placed in between the glass plates and the gel was left to set (15-20 minutes).

Once set, the gel comb was removed and the wells washed with distilled water to ensure even loading of protein samples. Gels were removed from their clamps and placed into a gel tank (Bio-Rad), and the inner chamber was filled with 1x running buffer (5mM Tris, 50mM glycine (Thermo Fisher) and 2% SDS in distilled water).

The denatured samples were then added to wells in duplicate (10µg/well), along with 5µl of molecular weight marker/ladder in the leftmost and rightmost wells (Full Range Rainbow Molecular Weight Marker, GE Healthcare, Buckinghamshire, UK). Any empty wells were filled with 10µl glycerol-bromophenol blue dye to ensure even running of samples. The outer electrophoresis chamber was filled with 1x running buffer and electrophoresis carried out at 150v for ~60minutes, or until the bromophenol blue dye front approached the bottom of the glass plates.

2.16.3. Western blotting procedure

For each gel, two pieces of Whatman filter paper and one nitrocellulose membrane (both GE Healthcare) were prepared. Gels were removed from the electrophoresis tanks and the glass plates separated. Following removal of the stacking gel the resolving gel was carefully placed onto the nitrocellulose membrane. This was then sandwiched between the two sheets of filter paper and two sponges of the same size, all of which were soaked in blotting buffer (48mM Tris base, 39mM glycine, 20% v/v methanol and 0.037% v/v SDS) beforehand. On addition of the filter paper the “sandwich” was carefully pressed down to eliminate air bubbles that may prevent protein transfer and cause artefacts on the membrane. The

gels were then placed into a blotting tank, which was run at 80v for one hour whilst cooled (either via ice packs placed into the tank or constant running cold water).

Following protein transfer, the membrane was submerged in Tris buffered saline with Tween 20 (TTBS), before being submerged in Ponceau S staining solution (a mixture of 0.2g Ponceau S dye, 3g trichloroacetic acid, 3g sulphosalicylic acid (both Fisher Scientific), and distilled water) for 10 minutes. The membrane was then removed from the Ponceau solution and the excess stain washed off in distilled water to confirm protein transfer.

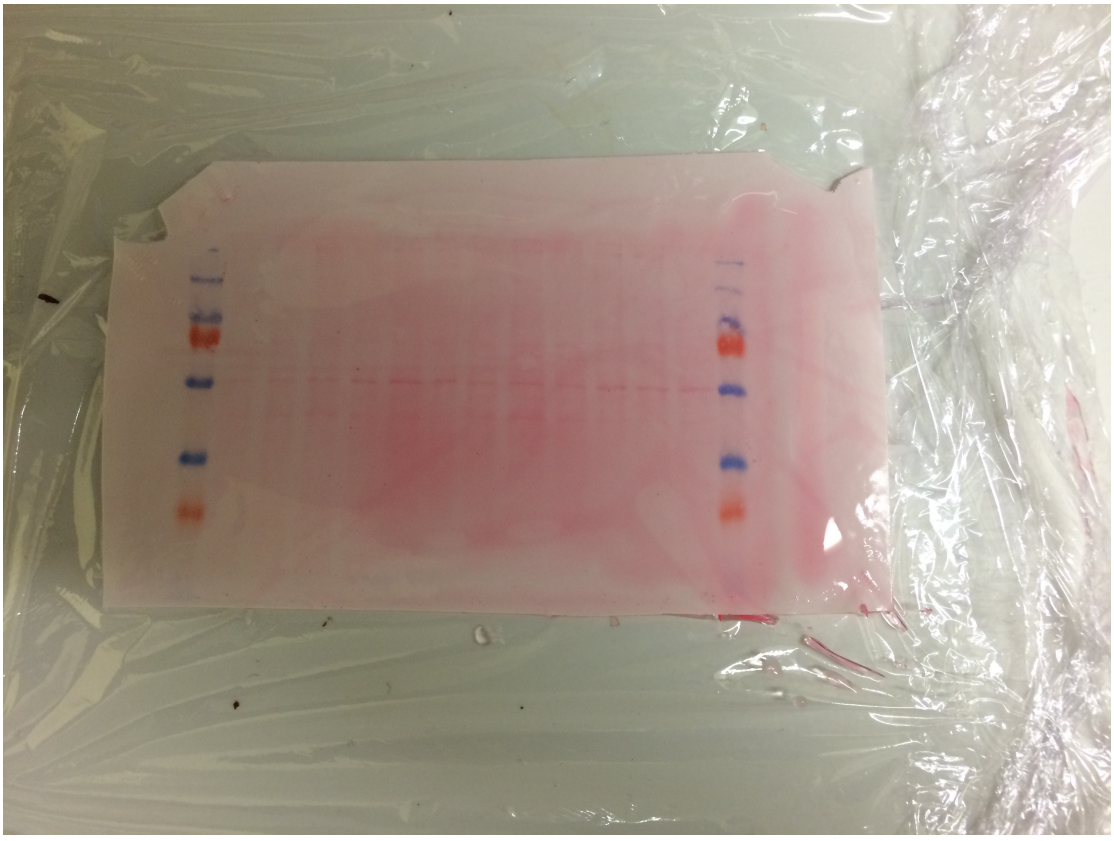


Figure 2-10: Example membrane following Ponceau S staining, showing non-specific protein bands.

Following confirmation of protein bands the Ponceau S was washed off the membrane using distilled water and the membrane was then submerged in 10% milk (Marvel Powder) in TTBS and either blocked for 1 hour at room temperature or overnight at 4°C, both whilst shaking.

After blocking membranes were washed three times in TTBS whilst shaking, with 10 minutes for each wash. Membranes were then incubated with the primary antibody, this was either dissolved in 3% milk and TTBS or 5% bovine serum albumin (BSA) in TTBS, and left for 1 hour at room temperature. The primary antibody was then washed from the membrane using a 3 x minute procedure as before, and the membrane was then submerged in the secondary antibody solution and left for 1 hour at room temperature. Details of antibodies used, dilutions and diluents are given in Table 2-9 and Table 2-10.

Antibody	Dilution used	Tissue
Rabbit Anti-Occludin (ab31721)	Rat: 1:1000 in 5% BSA and TTBS, Pig: 1:1000 in 3% milk and TTBS	Rat, Pig
Rabbit Anti-Claudin 3 (ab15102)	1:1000 in 3% milk and TTBS	Pig
Rabbit Anti-GLUT5 (ab41533)	1:1000 in 5% BSA and TTBS	Rat
Rabbit Anti-GLUT2 (ab104622)	1:1000 in 5% BSA and TTBS	Rat
Goat Anti-JAM1 (ab91501)	1:500 in 3% milk and TTBS	Pig
Rabbit Anti-Beta actin (ab8227)	1:2000 in 5% BSA and TTBS	Rat

Table 2-9: Details of primary antibodies used in Western blotting in both the Schothorst and SM studies.

All antibodies were sourced from Abcam (Cambridge, UK).

Antibody	Dilution used	Tissue
Goat anti-Rabbit IgG (HRP) (ab6721-1)	1:10000 in 3% BSA and TTBS	Rat
Donkey anti-Rabbit IgG FITC (sc2090)	1:300 in 5% milk and TTBS	Pig
Rabbit anti-Goat IgG FITC (sc2777)	1:300 in 5% milk and TTBS	Pig

Table 2-10: Details of secondary antibodies used in Western blotting in both the Schothorst and SM studies.

All antibodies were sourced from Abcam with the exception of sc2090 from Santa Cruz Biotech (Heidelberg, Germany).

The secondary antibody was then washed from the membrane using 2 10 minute washes in TTBS and a single 15 minute wash in TBS. For chemiluminescent detection, a mixture of 3ml of hydrogen peroxide and 3ml luminol (Immobilon Western Chemiluminescent HRP Substrate, Millipore, MA, USA) was applied directly to the membrane using a Pasteur pipette and left shaking for 5 minutes. The HRP substrate was then drained from the membrane, which was then visualised using a cooled CCD camera (ChemiDoc MP, BioRad) firstly under visible light followed by 5-minute exposures under chemiluminescence. 5-minute exposures continued until the software (Image Lab, BioRad) detected overexposed bands on the gel.

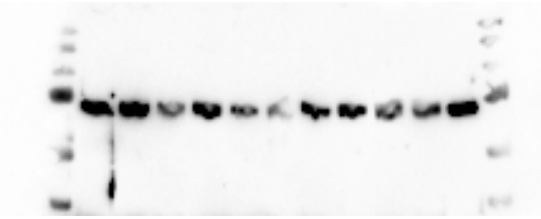


Figure 2-11: Representative blot of claudin-3 and molecular weight marker in rat jejunum, showing bands at ~70kDa.

For fluorescent detection in pig tissues using the fluorescein isothiocyanate (FITC) conjugated secondary antibody, membranes were washed as above before being exposed using a CCD camera (LAS-1000, Fujifilm Europe) under a blue light for 20 minutes. Optimisation of both primary antibodies was unsuccessful in the pig tissue, and due to time constraints these blots were not completed.

Densitometry was carried out using ImageJ (244).

2.17. Colorimetric assays

Samples taken from the Schothorst study were auto-analysed (RX Imola, Randox Laboratories Ltd.) for glucose, NEFA, triglycerides, potassium, chloride and sodium by Dr Kayleigh Almond and the data analysed by myself. Insulin ELISAs were carried out by Dr Kayleigh Almond and Dr Pablo Fainberg and analysed by myself. Samples taken from the SM study were analysed for glucose, triglycerides and insulin by Aleida Song and myself, and analysed by myself.

2.17.1. Glucose analysis

Glucose assays were carried out according to the Trinder method (247). Using this method, the glucose contained in plasma samples is oxidised to gluconic acid and hydrogen peroxide. The addition of 4-aminophenazone, phenol and peroxidase enzyme catalyses the production of a violet coloured quinoneimine dye which can be measured using a spectrophotometer at 500nm. The reaction is described in Figure 2-12.

$$Glucose + O_2 + H_2O \xrightarrow{Glucose\ oxidase} glcuonic\ acid + H_2O_2$$

$$2H_2O_2 + 4\ aminophenazone + phenol \xrightarrow{Peroxidase} quinoneimine + 4H_2O$$

Figure 2-12: Reactions utilised in the Trinder assay (247).

This assay was carried out using the Trinder Assay Kit (Sekisui Diagnostics, Charlottetown, PE, Canada). Samples were run in duplicate along with a standard curve of known concentrations, also run in duplicate. Plates were read using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA) before analysis in Microsoft Excel and SPSS.

2.17.2. Triglyceride analysis

The triglyceride assay works using a similar principle to the Trinder assay, in that a quinoneamine dye is produced which can be measured using a spectrophotometer. Addition of a lipase enzyme to plasma will break down the triglycerides into glycerol and fatty acids. The glycerol can then be treated with glycerol kinase to produce glycerol-3-phosphate, and a further two oxidation steps yield hydrogen peroxide. As before, the addition of 4-aminophenazone and phenol will produce quinoneamine. The enzymatic reactions used are listed in Figure 2-13.

$$Triglycerides + H_2O \xrightarrow{Lipases} glycerol + fatty\ acids$$

$$Glycerol + ATP \xrightarrow{Glycerol\ kinase} glycerol - 3 - phosphate + ADP$$

$$Glycerol - 3 - phosphate + O_2 \xrightarrow{Glycerol\ phosphate\ oxidase} dihydroxyacetone + phosphate + H_2O_2$$

$$2H_2O_2 + 4\ aminophenazone + 4\ chorophenol \xrightarrow{Peroxidase} quinoneimine + 4H_2O + HCl$$

Figure 2-13: Reactions utilised in the triglyceride assay.

For samples taken from the SM study, the Triglyceride-SL assay kit (Sekisui Diagnostics) was used, following the same method as the Trinder assay.

2.18. Insulin enzyme-linked immunosorbent assay (ELISA)

ELISAs enable the detection of a specific antigen within plasma or any other biological sample. Most commonly, commercial kits are used consisting of pre-coated 96 well plates, which will already contain the antibody required. This primary antibody will be conjugated to a substance such as HRP, which will fluoresce on addition of a suitable substrate such as 3,3',5,5'-tetramethylbenzidine (TMB), in a similar process to that described in section 2.16. Incubation of TMB with HRP will produce a blue colour, which will change to yellow on addition of a suitable stop solution such as sulphuric acid. This can then be read at 450nm in a spectrophotometer. Figure 2-14 illustrates the antibody binding procedure.

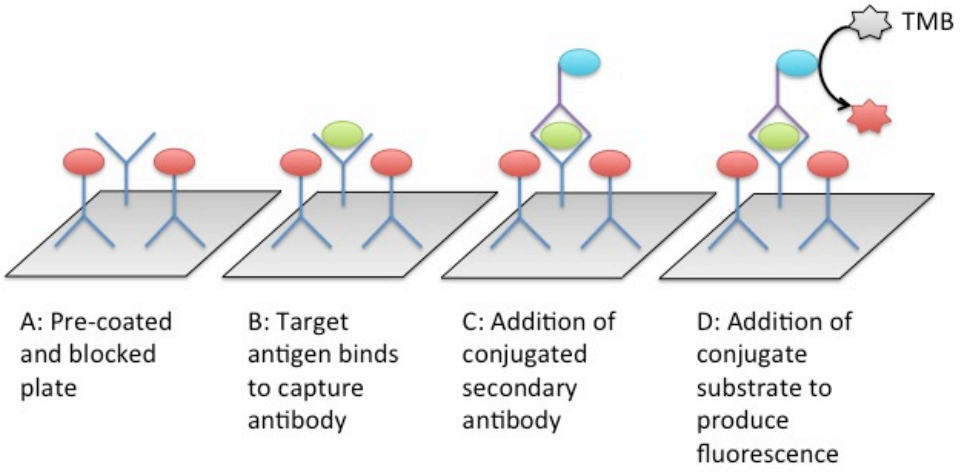


Figure 2-14: Standard "sandwich" ELISA.

A: Plates are pre-coated with the relevant primary antibody, and are either pre-blocked or a blocking agent is applied. B: The sample is added to the plate and incubated; the specific antigens will bind to the primary antibody. C: Conjugated secondary antibody is added and incubated. Addition of an appropriate substrate causes the conjugate to fluoresce.

ELISAs for insulin were carried out in the same way as sections 2.17.1 and 2.17.2, using a standard curve of known insulin concentrations and running all samples in duplicate. Pre-coated insulin plates (Rat

Ultrasensitive Insulin ELISA, ALPCO Diagnostics, Salem, NH, USA) were used for all samples in the SM study. The Porcine Isulin ELISA kit (Mercodia AB) was used for all samples taken from the Schothorst study.

2.19. Glycogen analysis

The assay for tissue glycogen concentration works using the same principle as the assay for glucose concentration, by first treating the homogenised samples with amyloglucosidase, the glycogen present in the tissue is converted in to glucose. The samples can then be read using a spectrophotometer as described in section 2.17.1. Each sample is also run with a control that is not treated with the enzyme, the amount of endogenous glucose in these untreated samples can then be subtracted.

2.19.1. Tissue glycogen extraction

0.5g sections of jejunum were weighed and thawed on ice in 2ml of perchloric acid (PCA, 8% v/v), and homogenised using a Dispomix homogeniser (Miltenyi Biotec). A further 3ml of PCA was added and the samples were spun at 3220g for 1 minute at 4°C. 1ml of the supernatant was transferred to a sterile 1.5ml Eppendorf tube and 100µl sodium bicarbonate solution was added to bring the pH to 7. Sodium acetate (0.2M) was then added to each sample until the pH was reduced to 4.8 to enable the amyloglucosidase to function. Two aliquots of 200µl were then transferred to new 1.5ml tubes – to one of these 5µl of amyloglucosidase enzyme (at 80U/ml) was added and gently pipette mixed, to the other set of control tubes 5µl of distilled water was added. All samples were incubated at 37°C for 30 minutes with gentle agitation to allow the amyloglucosidase to completely reduce the glucose in each sample, followed by being placed in a water bath at 100°C for 5 minutes to stop the reaction. Samples were then centrifuged at 13250g for 10 minutes at 4°C

and the supernatant collected. The method followed above is based on (248).

2.19.2. Glycogen assay procedure

Samples were analysed using the Trinder method covered in 2.17.1, with the exception that known glycogen standards from bovine liver were used to produce a standard curve of 0-200µg/µl. Control samples not containing amyloglucosidase were used to measure the amount of endogenous glucose in each sample, this value was then subtracted from samples treated with amyloglucosidase.

2.20. Faecal bacterial DNA sequencing

2.20.1. Pyrosequencing

Pyrosequencing is a form of DNA sequencing based on the "sequencing by synthesis" principle (249). Single stranded DNA to be sequenced is amplified in the presence of a fluorescent compound, which will produce visible light in proportion to the amount of template DNA. As new nucleotides are incorporated, pyrophosphate (PPi) molecules are released and converted to ATP by ATP sulfurylase, this ATP is then available for the oxidation reaction in which light is produced by luciferase (250). By measuring the amount of light emitted as each base is incorporated into the complementary strand, the DNA sequence can be determined.

$$(NA)_n + nucelotide \xrightarrow{polymerase} (NA)_{n+1} + PPi$$

$$PPi + APS \xrightarrow{ATP\ sulfurylase} ATP + SO_4^{2-}$$

$$ATP + luciferin + O_2 \xrightarrow{luciferase} AMP + PPi + oxyluciferin + CO_2 + light$$

Figure 2-15: Production of light by DNA polymerase and luciferase in the pyrosequencing reaction (250).

In the case of microbiome studies, sequences are generally assigned to operational taxonomic units (OTUs). Due to errors in the sequencing process, such as the generation of chimeras (the binding of two DNA sequences from different subjects caused by termination of the DNA polymerase before the sequence has finished, resulting in a single DNA product produced from two template sequences), OTUs are matched to target sequences with a percentage similarity, usually 97%. Target sequences are acquired from publically available datasets, for this study the GreenGenes database of 16s rRNA sequences (251) was used.

2.20.2. Sample collection and DNA extraction

Faecal samples were collected from rats in the SM study at three time points: pre-mating (PM, 10 weeks), early pregnancy (EP, 12 weeks) and late pregnancy (LP, 14 weeks). Samples were stored in 1.5ml Eppendorf tubes at -20°C until extraction. 180-220g of each sample were homogenised in a bead beater homogeniser (FastPrep 24, MP Biomedicals, Solon, OH, USA) and the recommended protocol followed using the QIAmp DNA Stool Mini Kit (Qiagen). This DNA extraction process works in a similar fashion to that described in section 2.13.1, with additional steps to remove the compounds present in faeces that will degrade DNA. The extracted DNA samples were quantified using a Nanodrop

spectrophotometer (Thermo Fisher), samples with a DNA concentration below 10ng/µl were repeated. The above DNA extraction work was carried out by myself, Jayden MacPherson and Brent Nielsen in the Department of Animal Science, University of Alberta.

Once extracted, 16s ribosomal RNA primers (27F, AGAGTTTGATCMTGGCTCAG and 519R, GWATTACCGCGGCKGCTG, Integrated DNA Technologies) were used to amplify the bacterial DNA in each sample. The primers used in microbial sequencing are degenerate, therefore some positions in the primer can be occupied by different bases (in this case M=A/C, W=A/T and K= T/G). The final primer contains an equal distribution of the different bases. Each sample was amplified using a primer pair with a unique barcode on the forward primer, to enable subsequent identification of the amplified DNA sequences. PCR reactions were carried out under the following conditions:

20µl reaction volume containing:

0.4µl Phusion HotStart II High-Fidelity DNA polymerase (Thermo Scientific)
4µl 5x Phusion HF Buffer (Thermo Scientific)
8pmol forward primer (IDT)
8pmol reverse primer (IDT)
4mM dNTP (Thermo Scientific)
25ng DNA template

The PCR run method was as follows:

Step	Temperature (°C)	Duration
Initial Denaturation	98	1min
Denaturation*	98	10s
Annealing*	59	30s
Extension*	72	30s
Final extension	72	7min
Hold	4	∞

Table 2-11: PCR run method for 16s ribosomal RNA amplification.

***Repeated for 30 cycles.**

PCR products were then run on 1% agarose gels and the bands excised from the gel. The gels and subsequent DNA extraction of the products were carried out following the method described in section 2.13. The concentration of these samples was also verified by Nanodrop, with 20ng/µl being the minimum required for accurate sequencing. Samples were then pooled by time point, giving three tubes – one each for PM, EP and LP, and sent for 454 pyrosequencing at Génome Québec and McGill University Innovation Centre, Montréal, Québec, Canada.

2.21. Statistical analysis

In all cases z-scores were calculated for numerical data, values determined to be ±2SD from the mean were excluded as outliers from that particular analysis. Following exclusion of outliers the distribution of data was determined by Shapiro-Wilk test for data of <50 samples and the Kolmogorov-Smirnov test for data of >50 samples. For parametric data with two groups comparisons were made by Unpaired t-test, data with more than two groups were compared by analysis of variance (ANOVA), using contrast coefficients to make *a priori* comparisons between groups of interest. Where data were nonparametric the Mann-Whitney U test was used to make comparisons between two groups, data with more than two groups were compared first using the Kruskal-Wallace test, followed by

Mann-Whitney U test (with the Bonferroni correction applied based on the number of comparisons made). Statistical analyses were carried out in SPSS v.22 (IBM Corp., Armonk, NY, USA), and presented using Prism v.6.0 (GraphPad Software Inc., La Jolla, CA, USA) or Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

2.21.1. Analysis of DNA sequencing data

Sequencing data were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) workflow (252), using the recommended settings. Samples with fewer than 1,500 sequencing reads were discarded. Remaining samples were then assigned operational taxonomic units (OTUs), through comparison with the Greengenes database (251). Alpha and beta diversity were then calculated using Shannon's diversity index (253) and UniFrac (254) respectively. Microbial community composition was calculated using an analysis of similarity (ANOSIM) model within QIIME. The abundance of each OTU as well as beta diversity was compared between the control and fructose diet and each time point of pregnancy using ANOVA (SAS v9.2, SAS Institute, Cary, NC, USA). Significance was assumed at $p<0.05$. Data were presented using Microsoft Excel, and Venn diagrams generated using the web-based tool Venny (255). The above analyses were carried out by Dr Mi Zhou in the Department of Animal Science, University of Alberta and all data was made available to myself.

Chapter 3: The effect of a fructose supplemented diet during pregnancy on maternal and offspring body composition, gut development and liver gene expression

3.1. Introduction

Chapter 1 has summarised the effects and importance of maternal nutrition in offspring development, and highlighted the potential impact of fructose in causing obesity or diabetes in humans (52,53). I therefore aimed to use a rat model to determine if a maternal diet high in fructose would induce similar adverse effects to other obesogenic diets such as one high in fat (35–37). Studies to date investigating fructose intake in animal models have used an extremely high concentration of fructose, or HFCS (57,256,257) that potentially does not accurately reflect consumption of fructose in humans, since fructose is most commonly being consumed in sugar sweetened beverages (52,258). Therefore, this study utilised a lower concentration of fructose consumed throughout pregnancy in an effort to more accurately mimic frequent consumption of sugar-sweetened beverages in humans.

Research using a lower concentration of fructose throughout pregnancy is limited but several effects have been demonstrated. A 10% fructose solution in rats during pregnancy will lead to impaired glucose tolerance in the mother, and hyperinsulinaemia in the offspring at weaning (259), however analysis of effects on the offspring was limited due to them being euthanised at 21 days. A similar study supplementing drinking water with 10% fructose throughout pregnancy demonstrated increased plasma glucose and triglycerides in dams, but no change in plasma insulin in either dams or non-pregnant controls (260). Metabolic effects on the offspring were not analysed.

Current evidence in rats suggests that a diet high in fructose will significantly restrict placental development, possibly leading to growth restriction in the fetus (56). As there is now a growing body of literature into the effect of fructose on the GI tract (261–264), I have used this model of fructose supplementation in pregnancy to assess how this diet affects intestinal adaptation to pregnancy. This model will also be used to study the effect of the offspring continuing a high fructose diet, and if this adversely affects adaptation to pregnancy in the offspring.

A variety of genes were chosen to act as markers of intestinal permeability, inflammation, and carbohydrate transport. The rationale behind the use of these genes is discussed in chapter 1. Where multiple isoforms of a gene existed (as is the case with the large family of claudin proteins), an isoform was chosen that has been demonstrated to have little variability across the length of the intestine (129). Several markers of liver inflammation have also been chosen, based on the links between gut barrier failure and liver inflammation (40,87).

3.2. Hypothesis

I hypothesise that a maternal diet supplemented with a fructose solution in the drinking water will adversely affect maternal metabolic health during pregnancy, making them more prone to hyperglycaemia, hyperinsulinaemia and hypertriglyceridaemia. Based on previous work directly measuring glucose uptake, and gene expression work in rats in which fructose was directly infused into the small intestine (64,264,265), I hypothesise that the diet used in my study will lead to upregulation of the carbohydrate transporters GLUT2 and 5, and SGLT1.

In accordance with the developmental origins of health and disease hypothesis, I hypothesise that the offspring will display a more exaggerated phenotype than their mothers when placed on the same fructose solution. The already demonstrated effects of fructose on the

placenta (56), suggests that fructose may lead to growth restriction in the offspring, and it is therefore plausible that gut development of the offspring will be compromised, as the GI tract has been shown to be preferentially affected by growth restriction when compared with other organs (99).

An increase in intestinal permeability and failure in gut barrier function may lead to components of the microbiota passing into the portal circulation and leading to liver inflammation (87,266). Therefore to test this hypothesis expression in the liver of a number of inflammatory markers known to respond to lipopolysaccharide (LPS), such as cluster of differentiation 68 (CD68) (267) and toll-like receptor 4 (TLR4) (268) as well as the general inflammatory marker tumour necrosis factor alpha (TNF-α) were measured. I hypothesise that the high-fructose diet will lead to an increase in expression of these inflammatory markers, caused by the adverse effects on intestinal permeability.

Finally, to isolate the effects of diet and pregnancy, a smaller arm of the SM study (described in section 2.4) will use non-pregnant offspring rats which are age-matched and fed the same diets as their pregnant counterparts. The same analyses will be carried out on these rats to determine how much of the effects observed were due to pregnancy adaptations rather than adaptations to the diet.

3.3. Materials and methods

A full description of the methods used for this study is located in chapter 2. Figure 2-2 contains a summary of the animal study. All animal work for this study was carried out between September and December 2012, and April and September 2013 in the Division of Human Nutrition at the University of Alberta. All plasma metabolite work and subsequent analysis was carried out by myself and Aleida Song. Liver gene expression work

was carried out by Brent Nielsen and verified and analysed by myself. Intestinal gene and protein expression work was carried out by myself.

3.4. Results

3.4.1. First generation body composition and organ weights

In first generation dams placental weights were significantly reduced in the fructose group. All fat depots measured were significantly heavier in the fructose group (Table 3-1).

	C1 (n=9)	F1 (n=6)
Number of pups	15.5±1.7	16.0±3.2
Placental weight (g)	3.86±0.7	3.26±0.6*
Weight at dissection (14wk/GD20)	422.2±8.7	434.8±12.3
Liver weight (g)	18.7±0.6	19.6±0.8
Pancreas weight (g)	1.14±0.1	1.15±0.1
Small intestine length (cm)	124.8±3.4	126.5±2.6
Retroperitoneal fat (g)	1.92±0.1	3.56±0.6*
Uterine fat (g)	2.80±0.2	3.97±0.4*
Interscapular fat (g)	0.40±0.1	0.85±0.1*

Table 3-1: First generation body composition.

All organs were weighed directly after removal at dissection (i.e. wet weight). Small intestine was flushed with 10% PBS before length measurement. All values are mean±SEM, *p<0.05, Unpaired t-test.

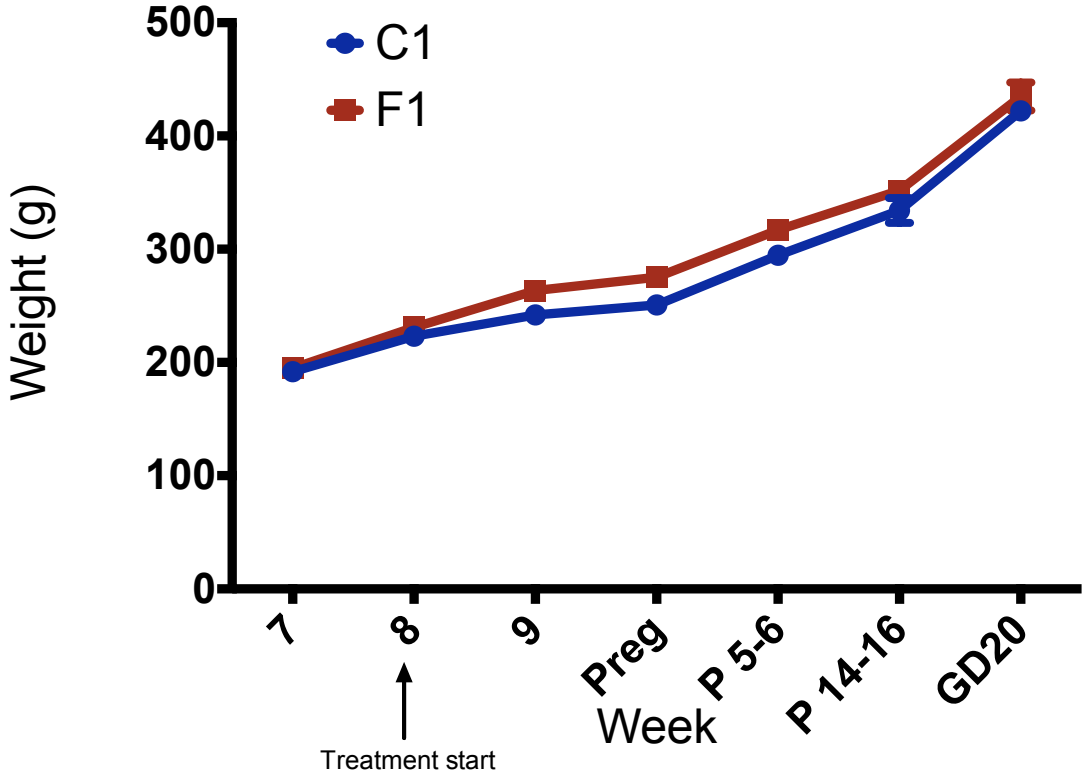


Figure 3-1: Mean body weights of first generation mothers from arrival at the animal facility to euthanasia.

C1 n=9, F1 n=6. Both groups were fed the control diet and regular distilled water until 8 weeks of age at which point those randomly assigned to the F1 group were switched to 10% fructose in their drinking water. No significant differences were observed between groups at any time point.

3.4.2. Offspring body composition

Birth weights were significantly lower in F2 offspring compared to C2. Offspring were mated at approximately 8 weeks of age and their placental and pancreas weights, and small intestinal length were significantly reduced at 20 days of gestation. All fat depots were significantly heavier at GD20 in the fructose group.

	C2 (n=10)	F2 (n=10)
Birth weight (g)	3.86±3.3	3.26±0.6*
Number of pups	16.3±2.3	17.0±3.3
Placental weight (g)	4.07±0.2	3.31±0.7*
Weight at dissection (14wk/GD20)	454.4±19.9	468.5±19.2
Liver weight (g)	21.05±0.5	21.93±1.4
Pancreas weight (g)	1.34±0.1	1.08±0.1*
Small intestine length (cm)	128.6±1.8	123.8±0.4*
Retroperitoneal fat (g)	5.06±0.5	10.29±1.1*
Uterine fat (g)	6.30±0.4	10.81±1.0*
Interscapular fat (g)	0.60±0.1	1.02±0.1*

Table 3-2: Second generation body composition.

C2 dams were born to mothers fed regular chow and distilled water, F2 dams were born to mothers fed regular chow and 10% fructose in distilled water. Both groups were continued on the same diets as their mothers following weaning. All values are mean±SEM, *p<0.05, Unpaired t-test.

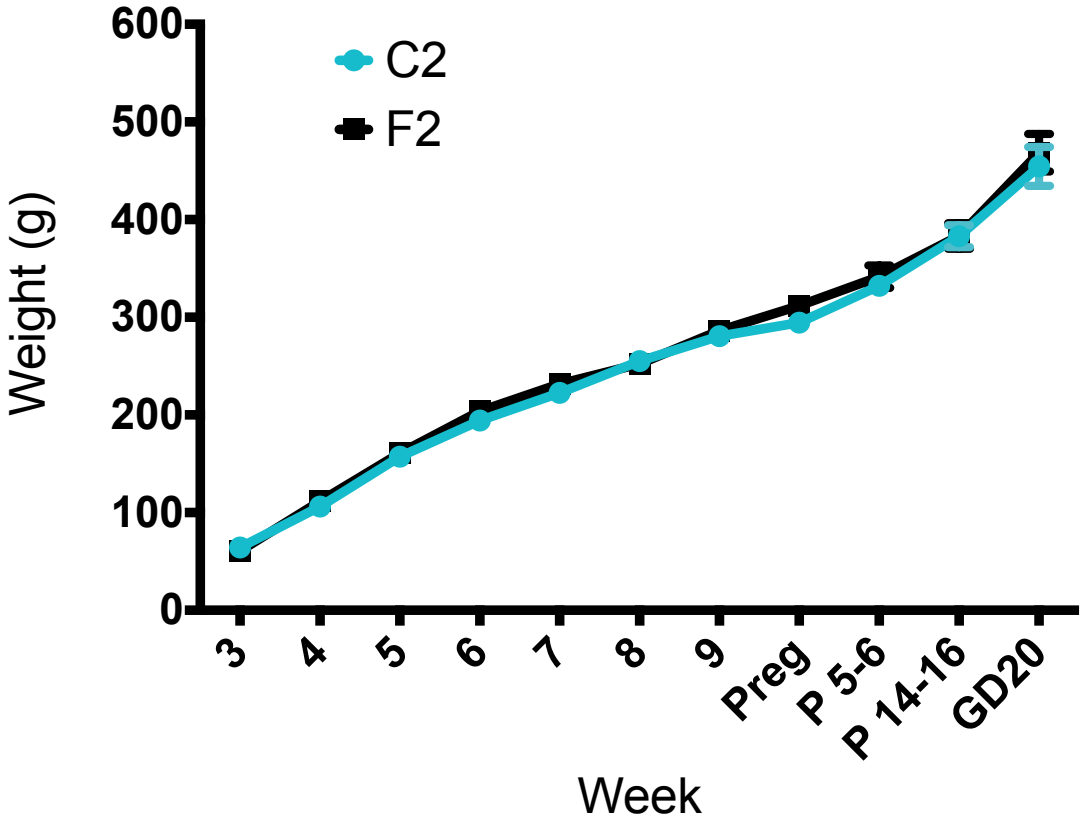


Figure 3-2: Mean weights of second generation (offspring) from weaning to euthanasia at GD20.

C2 n=10, F2 n=10. Both groups were suckled with their mothers and fed the control diet following weaning until 8 weeks of age, when dams born to F1 mothers were switched to 10% fructose solution and C2 remained on distilled water. There are no significant differences between weights at each time point in control or fructose groups. All values are mean±SEM.

No effect of diet on weight at GD20/13 weeks of age was observed in either generation studied.

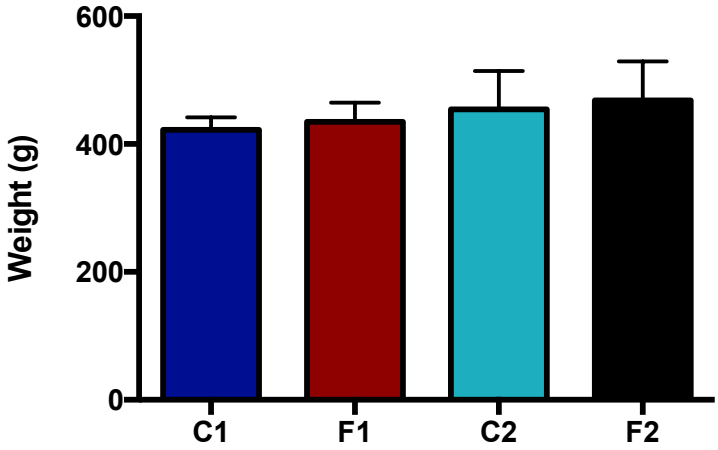


Figure 3-3: Body weights at gestational day 20.

C1 n=9, F1 n=6, C2 n=10, F2 n=10. All dams were weighed immediately prior to euthanasia. C dams consumed regular chow and distilled water throughout pregnancy, F dams consumed regular chow and 10% fructose in distilled water. Diets started at 8 weeks of age in both generations. All values are mean±SEM. Significant differences were not observed comparing C1 vs F1 or C2 vs F2.

Magnetic resonance imaging (MRI) prior to dissection showed a significant increase in percentage fat mass in rats in the F2 group when compared to controls, and a reduction in percentage lean mass.

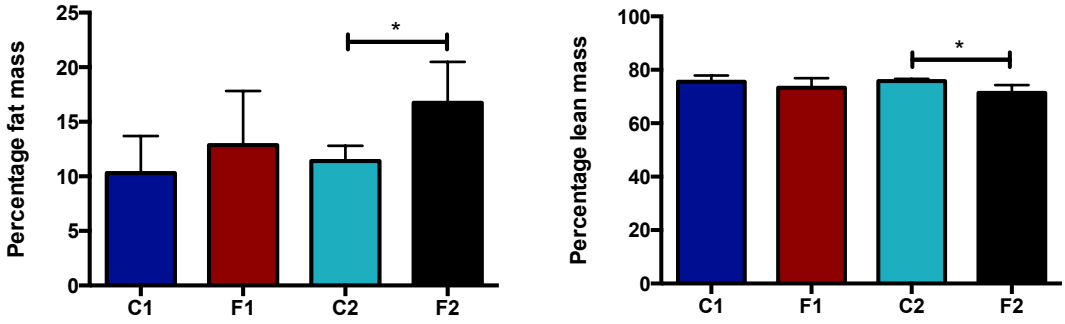


Figure 3-4: Percentage fat (L) and lean (R) mass at gestational day 20.

MRI was taken immediately prior to dissection using EchoMRI (Echo Medical Systems). C1 n=9, F1 n=6, C2 n=10, F2 n=10. Values are mean±SEM, *p<0.05, ANOVA with contrasts.

3.4.3. Nutrient intakes during pregnancy

Fructose fed dams demonstrated a significantly higher water intake in each 24-hour period, and significantly lower chow consumption than the control dams. This resulted in total caloric intake being similar between diet groups. There were no significant differences in food or water intake between first and second generation.

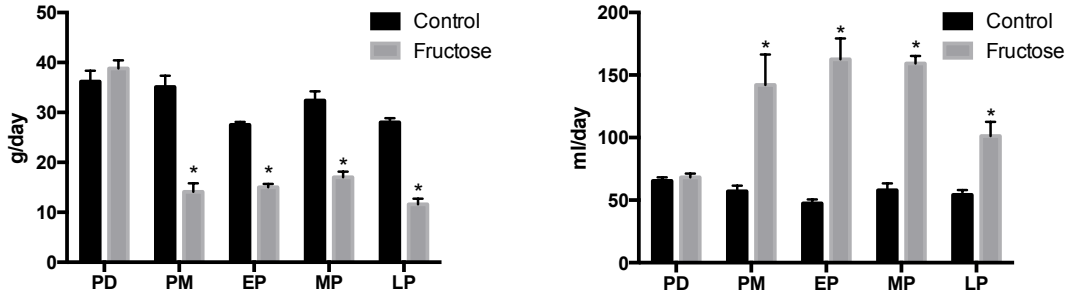


Figure 3-5: 24 hour food (L) and water/fructose solution (R) intake in first generation dams throughout pregnancy.

PD=pre-diet (7 weeks), PM=pre-mating (11 weeks), EP=early pregnancy (12 weeks), MP=mid pregnancy (13 weeks), LP=late pregnancy (14 weeks). All values are mean±SEM, *p<0.05, Unpaired t-test, control vs fructose at each time point.

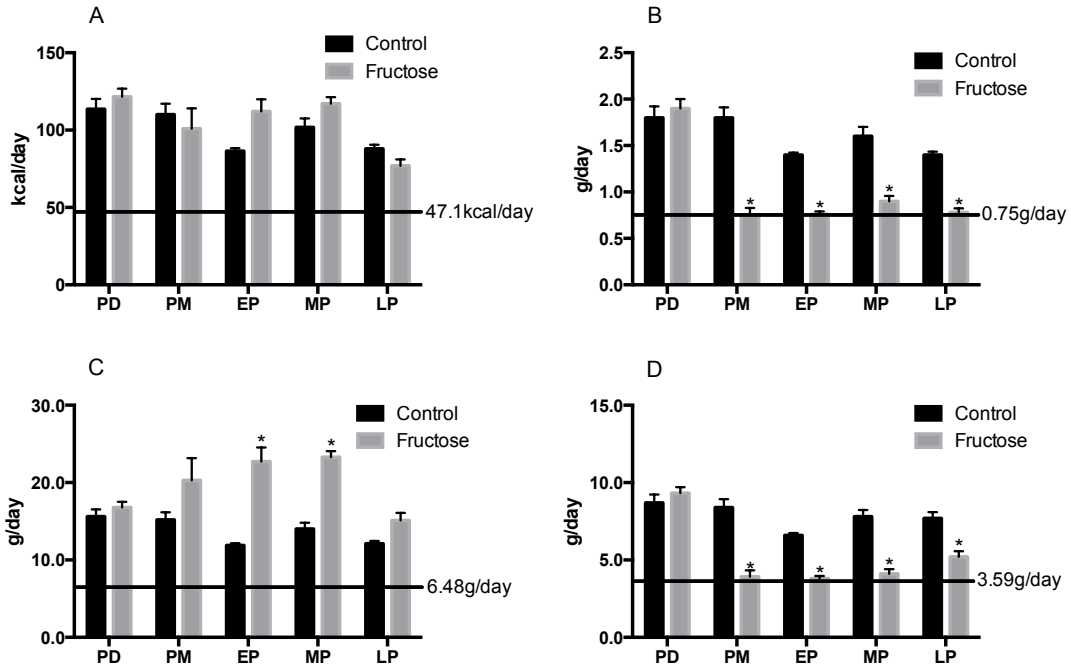


Figure 3-6: 24 hour calories (A), fat (B), carbohydrate (C) and protein (D) intake in first generation dams throughout pregnancy.

PD=pre-diet (7 weeks), PM=pre-mating (11 weeks), EP=early pregnancy (12 weeks), MP=mid pregnancy (13 weeks), LP=late pregnancy (14 weeks). All values are mean±SEM, *p<0.05, Unpaired t-test, control vs fructose at each time point. Recommended daily intakes for pregnancy (269) are highlighted.

3.4.4. Plasma glucose, insulin and triglyceride levels throughout pregnancy

Plasma triglycerides were significantly increased in F1 compared to C1 rats from pre-mating (10 weeks) onwards. Insulin concentrations did not differ between F1 and C1 at any time point, while glucose was significantly higher in F1 compared to C1 in early pregnancy. Plasma glucose, insulin and triglycerides were all significantly higher in F2 dams compared to C2 from pre-mating onwards. By late pregnancy, glucose was no longer significantly elevated in F2 compared to C2 dams (Table 3-3).

	C1 (n=9)	F1 (n=6)	C2 (n=10)	F2 (n=10)
Glucose (mmol/L)				
Pre-diet	7.9±0.6	8.0±0.8	7.3±0.7	7.30±0.5
Pre-mating	8.0±0.7	8.0±1.1	7.0±0.4	8.6±0.9*
Early pregnancy	7.5±0.9	8.3±0.8*	6.8±0.6	7.7±0.5*
Mid pregnancy	6.5±0.7	6.6±0.3	5.6±0.5	6.7±0.8*
Late pregnancy	5.4±0.6	6.3±0.5	5.7±0.3	6.0±0.1
Insulin (ng/ml)				
Pre-diet	0.9±0.5	0.8±0.3	0.9±0.5	1.0±0.5
Pre-mating	1.0±0.7	1.0±0.5	1.4±0.6	2.3±1.2*
Early pregnancy	0.8±0.5	1.1±0.2	1.3±1.0	2.8±1.6*
Mid pregnancy	0.6±0.2	1.5±0.5	1.0±0.5	2.8±1.7*
Late pregnancy	0.9±0.7	1.7±0.4	0.9±0.2	1.7±0.5*
Triglyceride (mmol/L)				
Pre-diet	3.6±1.2	3.3±0.9	3.9±1.5	3.7±1.3
Pre-mating	4.4±1.8	7.1±2.2*	4.7±1.0	17.6±5.1*
Early pregnancy	5.2±1.1	9.5±3.2*	4.5±1.3	14.1±4.2*
Mid pregnancy	11.0±4.7	18.4±1.6*	10.4±2.7	21.3±6.7*
Late pregnancy	12.1±1.0	20.3±1.5*	11.4±3.2	23.4±3.1*

Table 3-3: Plasma glucose, insulin and triglyceride levels through pregnancy.

Pre-diet=7wks, pre-mating=10wks, early pregnancy=12wks/GD4-7d, mid pregnancy=13wks/GD14=17, late pregnancy=14wks/GD19-20. All values are mean±SEM, *p<0.05 compared to the age-matched control group, Unpaired t-test.

3.4.5. Oral glucose tolerance tests (OGTT)

Plasma glucose was not significantly different between C1 and F1 dams at the peak of the OGTT. In F2 dams, plasma glucose was significantly higher than C2 dams at the peak of the OGTT but not significantly different at any other time point (Figure 3-7, L). Area under the curve (AUC) for glucose was not significantly different between groups in either generation (Figure 3-8, L).

Plasma insulin was also significantly increased in both F1 and F2 compared with controls at 15 minutes following glucose administration, and not significantly different following this peak (Figure 3-7, R). AUC for insulin was significantly greater in both F1 and F2 dams compared with their respective controls (Figure 3-8, R).

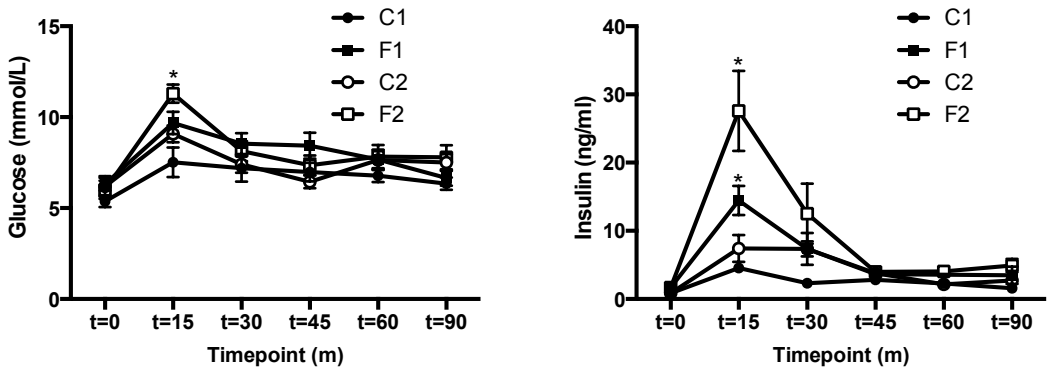


Figure 3-7: OGTT for plasma glucose (L) and insulin (R) during OGTT.

C1 n=9, F1 n=6, C2 n=10, F2 n=10. At t=0 3g glucose/kg body weight was administered by gavage and blood samples taken from the tail vein at each timepoint. Values are mean±SEM, *p<0.05, C1 vs F1, C2 vs F2 and F1 vs F2, ANOVA with contrasts.

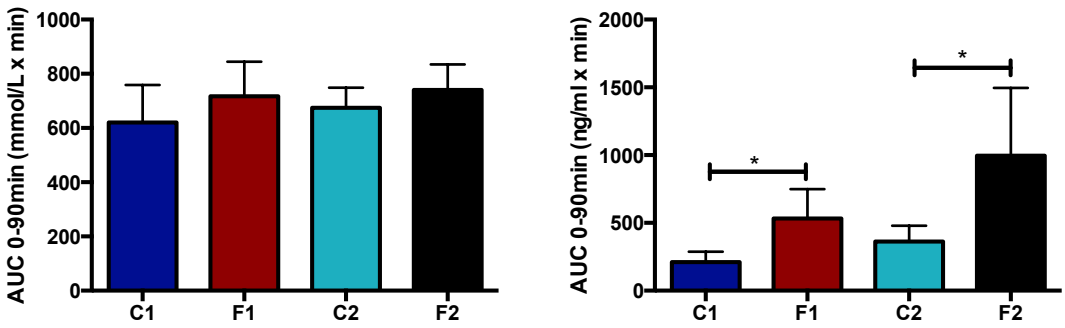


Figure 3-8: Plasma glucose (L) and insulin (R) area under the curve (AUC) for OGTT (Figure 3-7).

C1 n=9, F1 n=6, C2 n=10, F2 n=10. Values are mean±SEM. (L) *p<0.05 C1 vs F1 and C2 vs F2, ANOVA with contrasts.

3.4.6. The effect of a high fructose diet on intestinal morphology in both generations of dams

Villus heights were significantly increased in F1 dams compared to C1 in the jejunum but not the ileum. Villus heights were not significantly different between offspring in the control and fructose group, in either the jejunum or ileum (Figure 3-9).

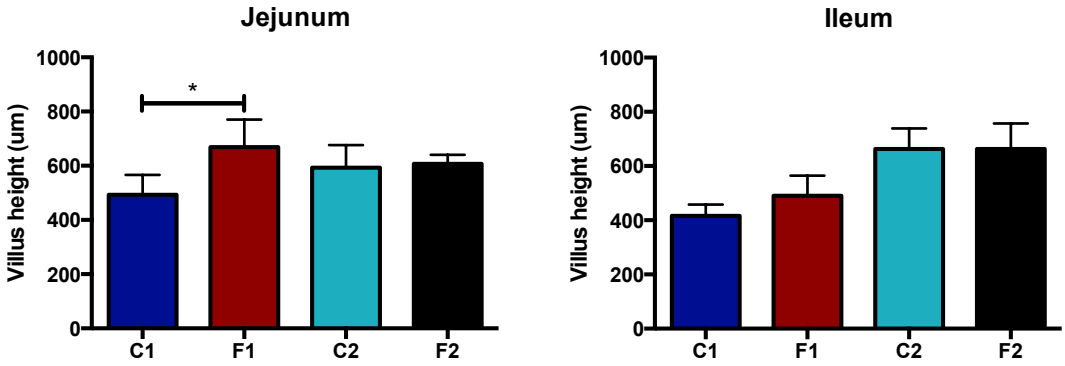


Figure 3-9: Villus height measurements in the jejunum (L) and ileum (R) of C1 (n=9), F1 (n=6), C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM. *p<0.05, ANOVA with contrasts.

Crypt depths in the jejunum and ileum were not significantly changed in control or fructose groups in either generation (Figure 3-10).

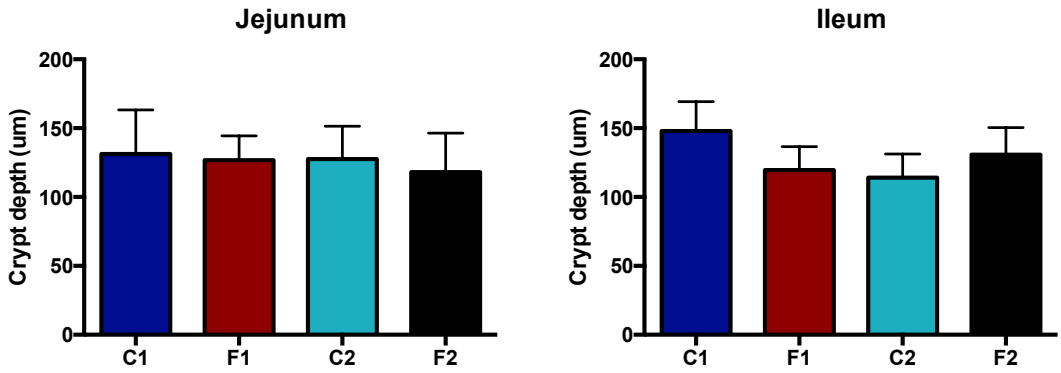


Figure 3-10: Crypt depth measurements in the jejunum (L) and ileum (R) of C1 (n=9), F1 (n=6), C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM. No significant differences were observed comparing C1 vs F1 and C2 vs F2.

3.4.7. The effect of consumption of 10% fructose solution on intestinal permeability markers during pregnancy

Of the four intestinal permeability markers tested, none were significantly different between diet groups in first generation dams (Table 3-4).

	Jejunum C1	Jejunum F1	Ileum C1	Ileum F1
Junctional adhesion molecule A (JAMA)	1.00±0.1	0.90±0.3	1.00±0.1	1.69±0.2
Occludin (OCLN)	1.00±0.1	1.04±0.1	1.00±0.1	1.16±0.1
Zonulin-1 (ZO-1)	1.00±0.1	1.39±0.2	1.00±0.1	1.12±0.1
Claudin-3 (CLDN-3)	1.00±0.2	1.80±0.5	1.00±0.1	1.28±0.3

Table 3-4: Gene expression of markers of intestinal permeability in C1 (n=9) and F1 (n=6) dams.

All values are mean±SEM relative expression vs the control group, all expression values are adjusted to the reference genes GAPDH and RPLP0.

3.4.8. The effect of consumption of a 10% fructose solution over two generations on gene expression of intestinal permeability markers in the offspring

mRNA analysis of junctional adhesion molecule (JAMA) showed a significant downregulation in jejunal expression in rats in the fructose group, shown in Figure 3-11, although expression in the ileum was not significantly different between diet groups.

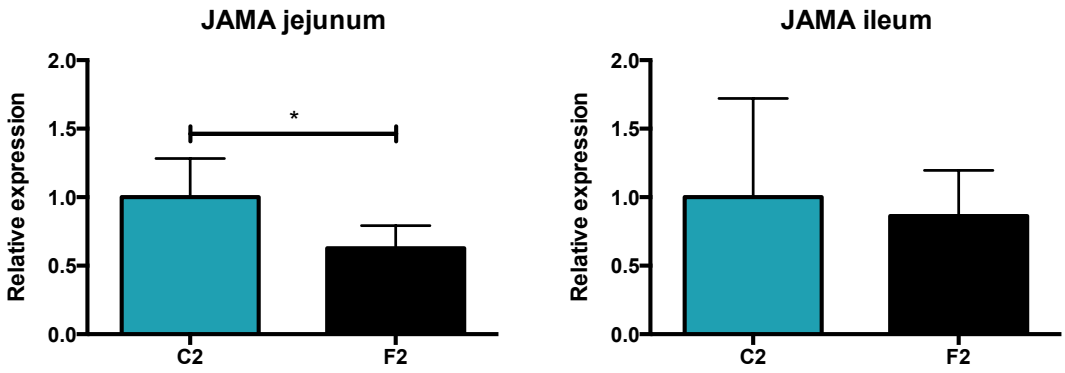


Figure 3-11: Junctional adhesion molecule (JAMA) expression in the jejunum (L) and ileum (R) of C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

Analysis of occludin (OCLN) expression showed a similar downregulation in the jejunum of fructose fed rats relative to controls, but no change in the ileum (Figure 3-12).

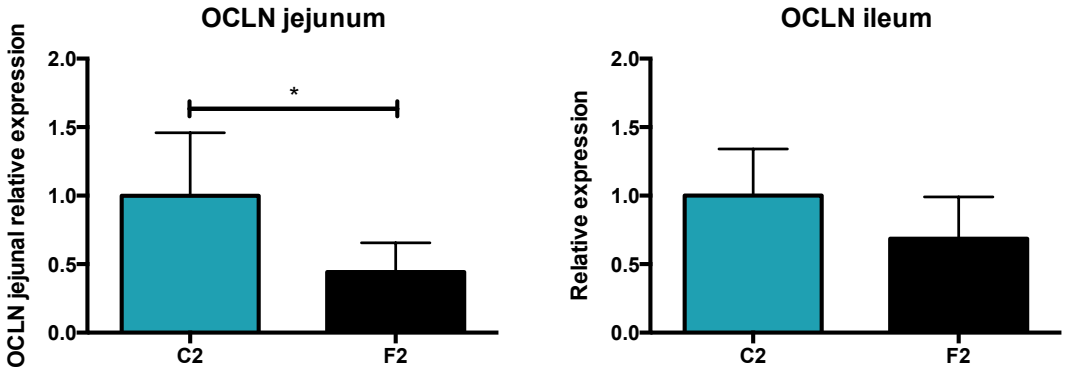


Figure 3-12: Occludin (OCLN) expression in the jejunum (L) and ileum (R) of control (n=10) and fructose (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

Expression of zonulin-1 (ZO-1) also followed this pattern of downregulation in the jejunum of dams in the fructose group relative to controls and no change in the ileum (Figure 3-13).

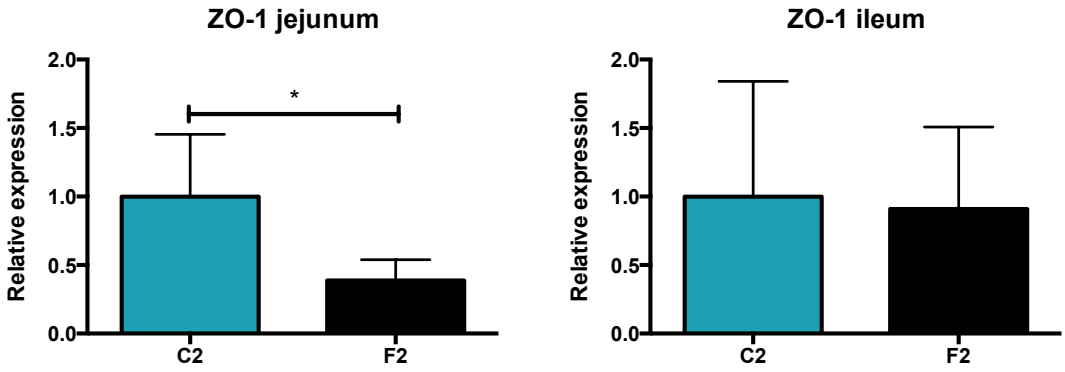


Figure 3-13: Zonulin-1 (ZO-1) expression in the jejunum (L) and ileum (R) of C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

Finally, claudin-3 (CLDN3) expression did not fit the pattern demonstrated in Figure 3-11 to Figure 3-13, with no significant changes in gene expression in either of the small intestinal sections tested (Figure 3-14).

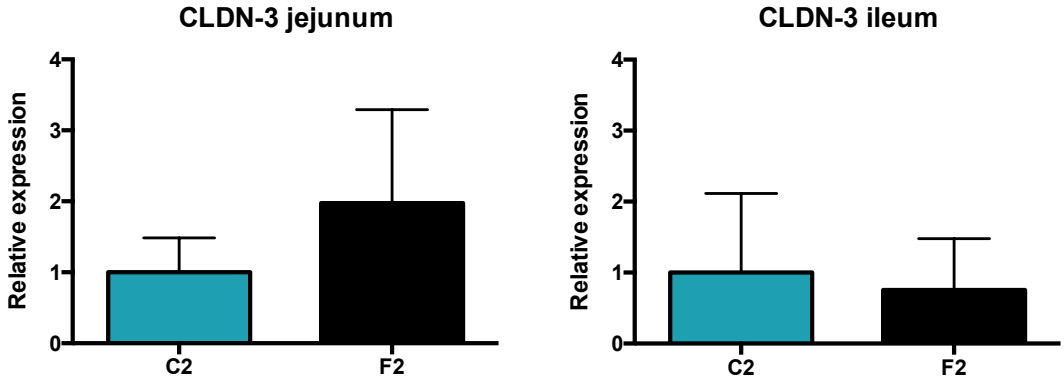


Figure 3-14: Claudin-3 (CLDN3) expression in the jejunum (L) and ileum (R) of C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0.

3.4.9. The effect of consumption of a 10% fructose solution over two generations on protein expression of intestinal permeability markers in the offspring

Two of the intestinal permeability genes were successfully analysed via Western blot. Protein expression of CLDN3 was consistent with mRNA expression, with no difference in expression between control and fructose groups in either section of small intestine (Figure 3-15). Due to time and cost constraints, C1 and F1 samples with no significant gene expression changes were not analysed.

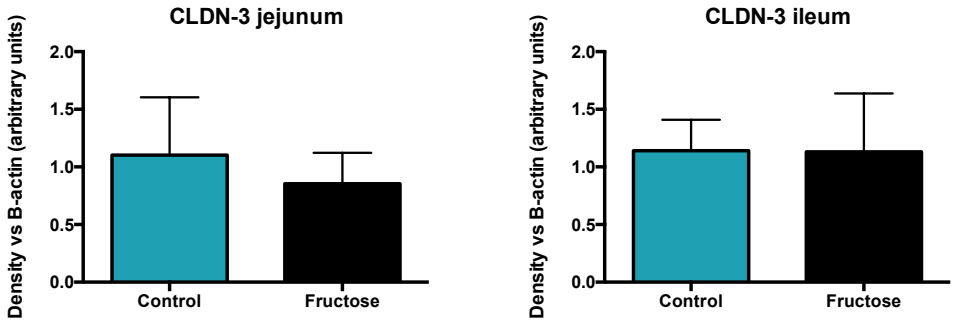


Figure 3-15: Protein expression of claudin-3 (CLDN3) in the jejunum (L) and ileum (R) of randomly chosen samples of C2 (n=5) and F2 (n=6) dams.

All values are density of CLDN-3 in each sample relative to β-actin in the same sample run on the same gel, jejunum and ileum are run on separate gels. Values are mean±SEM, *p<0.05, Unpaired t-test.

Protein expression of OCLN in the jejunum showed the same trend as mRNA expression, with a significant reduction in fructose dams compared to controls. This same reduction was also observed in the ileum, which did not correlate with mRNA abundance (Figure 3-16).

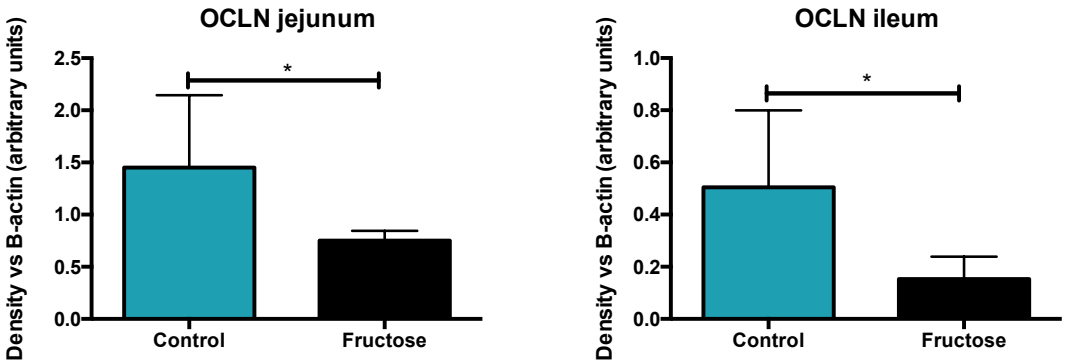


Figure 3-16: Protein expression of occludin (OCLN) in the jejunum (L) and ileum (R) of randomly chosen samples of C2 (n=5) and F2 (n=5) dams.

All values are density of OCLN in each sample relative to β-actin in the same sample run on the same gel, jejunum and ileum are run on separate gels. Values are mean±SEM, *p<0.05, Unpaired t-test.

3.4.10. The effect of consumption of a 10% fructose solution on expression of intestinal carbohydrate transporters in pregnancy

Glucose transporter 2 (GLUT2) expression was significantly increased in both the ileum and jejunum of F1 dams (Figure 3-17).

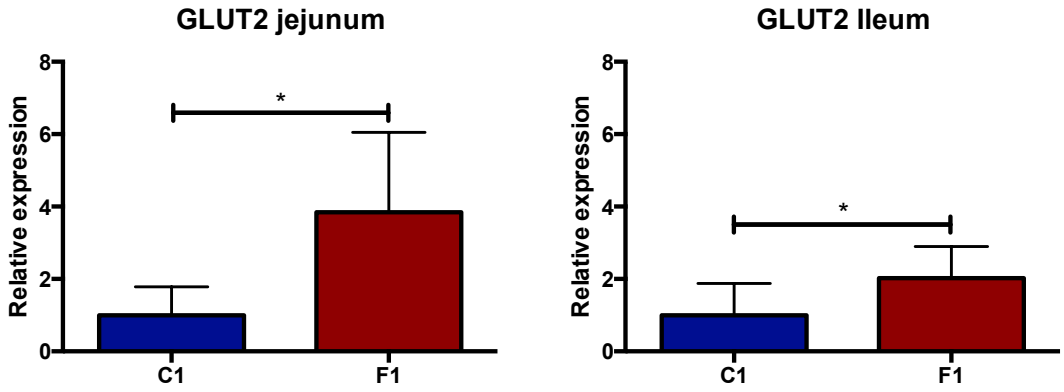


Figure 3-17: Glucose transporter 2 (GLUT2) expression in the jejunum (L) and ileum (R) of C1 (n=9) and F1 (n=6) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

Glucose transporter 5 (GLUT5) expression was markedly increased in the jejunum of F1 dams relative to controls, and not significantly different in the ileum Figure 3-18.

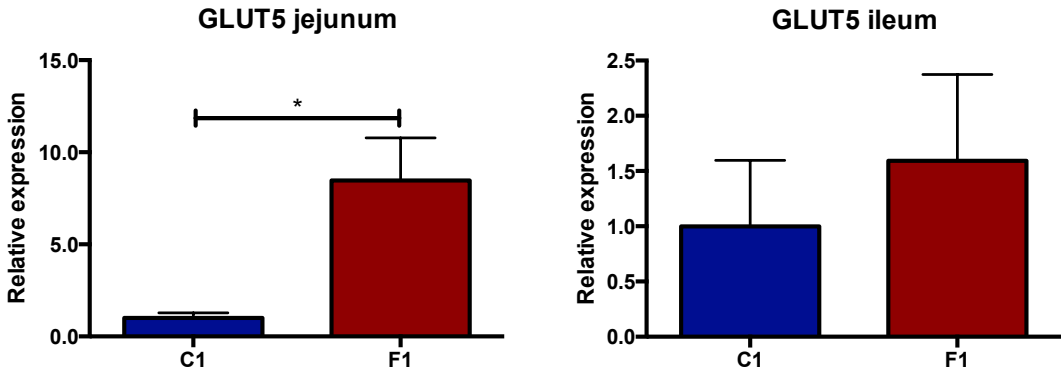


Figure 3-18: Glucose transporter 5 (GLUT5) expression in the jejunum (L) and ileum (R) of C1 (n=9) and F1 (n=6) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

Expression of sodium-glucose linked transporter 1 (SGLT1) was significantly increased in both the ileum and jejunum of F1 dams compared to controls (Figure 3-19).

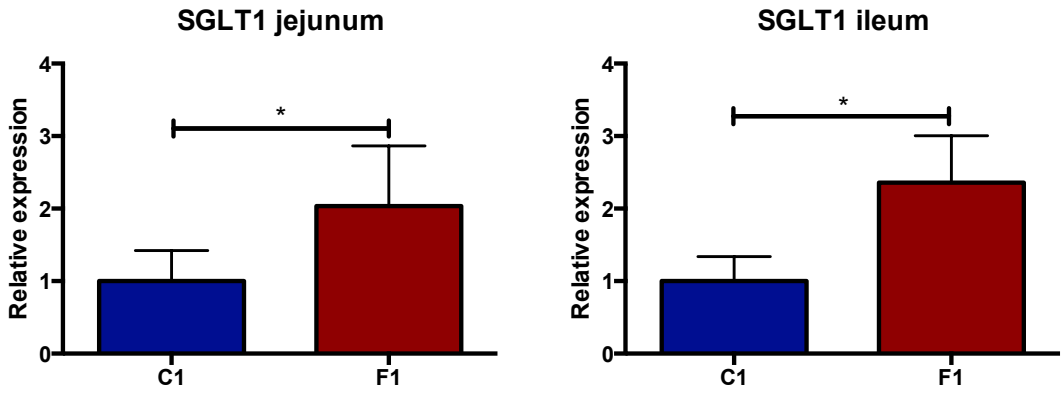


Figure 3-19: Sodim-glucose linked transporter 1 (SGLT1) expression in the jejunum (L) and ileum (R) of C1 (n=9) and F1 (n=6) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

3.4.11. The effect of consumption of a 10% fructose solution over two generations on expression of intestinal carbohydrate transporters in the offspring

GLUT2 expression was significantly increased in the jejunum of dams in the offspring F2 group compared to controls (C2), no change was observed in the ileum (Figure 3-20).

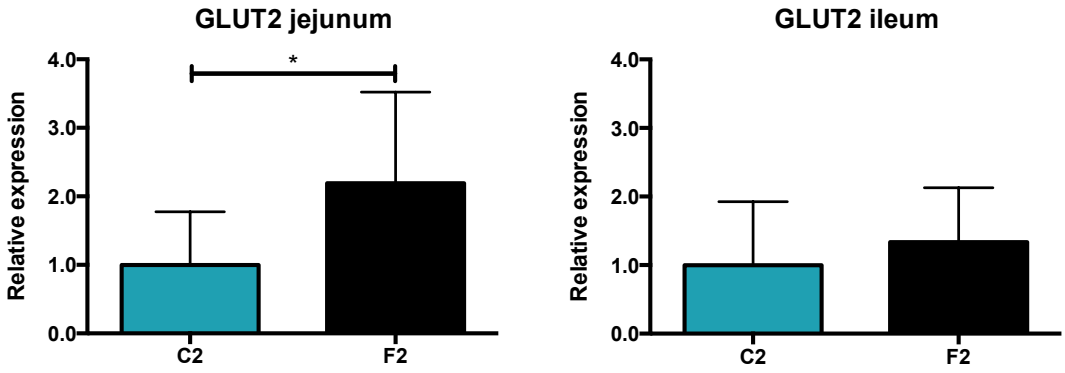


Figure 3-20: Glucose transporter 2 (GLUT2) expression in the jejunum (L) and ileum (R) of C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

GLUT5 expression was also significantly increased in the jejunum of dams in the fructose group compared with controls, and unlike GLUT2 was also significantly increased in the ileum of dams in the fructose group (Figure 3-21).

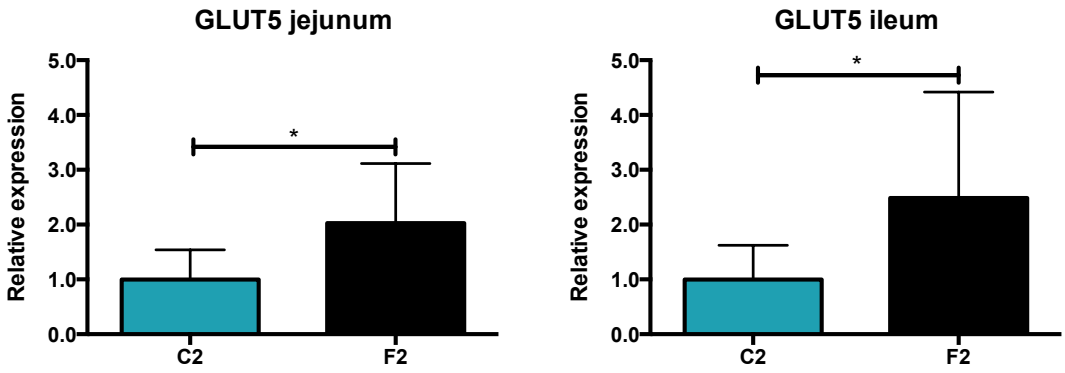


Figure 3-21: Glucose transporter 5 (GLUT5) expression in the jejunum (L) and ileum (R) of C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

Finally, expression of sodium-glucose linked transporter 1 (SGLT1) was also significantly increased in both the ileum and jejunum of dams in the fructose group compared to controls (Figure 3-22).

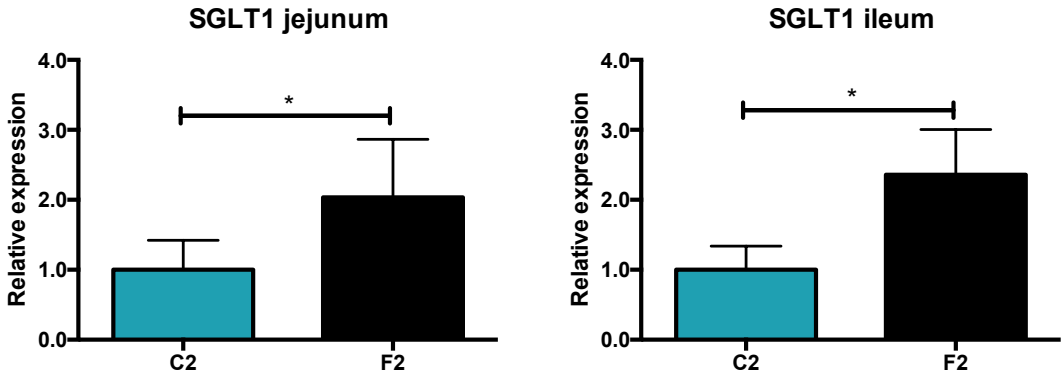


Figure 3-22: Sodium-glucose linked transporter 1 (SGLT1) expression in the jejunum (L) and ileum (R) of C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM relative expression, adjusted to the reference genes GAPDH and RPLP0, *p<0.05, Unpaired t-test.

3.4.12. The effect of a consumption of a 10% fructose solution on expression of inflammatory genes in the liver during pregnancy in two generations

In both generations, none of the inflammatory markers were significantly changed in the liver when comparing control and fructose fed dams (Table 3-5).

Gene	C1	F1	C2	F2
Toll-like receptor 4 (TLR4)	1.00±0.3	0.90±0.2	1.00±0.1	0.93±0.2
Cluster of differentiation 68 (CD68)	1.00±0.1	0.71±0.1	1.00±0.2	1.18±0.6
Tumour necrosis factor α	1.00±0.1	0.94±0.5	1.00±0.2	0.91±0.2
C-jun N-terminal kinase (JNK1)	1.00±0.2	0.83±0.6	1.00±0.1	0.75±0.1

Table 3-5: Expression of inflammatory genes in the liver of C1 (n=9), F1 (n=6), C2 (n=10) and F2 (n=10) dams.

All values are mean±SEM and presented as fold change relative to controls, all expression is adjusted to the reference genes GAPDH and RPLP0.

3.4.13. Non-pregnant body composition data

	C1 (n=4)	F1 (n=4)	C2 (n=5)	F2 (n=5)
Birth weight (g)	-	-	3.91±0.20	3.25±0.2*
Weight at dissection (14wk)	302.3±7.4	336.3±10.6	335.2±13.2	341.8±14.1
Liver weight (g)	13.2±0.3	15.9±0.5	15.5±0.6	18.6±0.5
Pancreas weight (g)	1.27±0.2	0.91±0.2	1.61±0.1	1.34±1.1
Small intestine length (cm)	120.3±5.1	117.0±2.9	124.6±3.1	119.5±2.8
Retroperitoneal fat (g)	1.25±0.1	3.32±1.1	3.91±1.4	3.26±3.1
Uterine fat (g)	1.83±0.3	3.84±1.1	2.47±0.9	3.04±0.7
Interscapular fat (g)	0.43±0.1	0.67±0.1	0.51±0.4	0.78±0.9

Table 3-6: Body composition data in non-pregnant rats.

C1 n=4, F1 n=4, C2 n=4, F2 n=4. *p<0.05 C1 vs F1 and C2 vs F2, ANOVA with contrasts.

3.4.14. Non pregnant blood results

Plasma glucose was not significantly affected by diet in the first generation. Plasma insulin was significantly increased in fructose fed first generation rats by 14 weeks, and plasma triglycerides were significantly elevated from 12 weeks onwards. Plasma triglycerides in second generation rats were significantly elevated in the fructose group at 14 weeks. Plasma insulin and glucose were not significantly affected at any time point in second generation rats (Table 3-7).

	C1 (n=4)	F1 (n=4)	C2 (n=5)	F2 (n=5)
Glucose (mmol/L)				
7 weeks	7.8±0.2	8.2±0.2	13.8±0.6	15.1±0.4
11 weeks	7.7±0.2	6.0±2.1	14.4±0.1	14.1±0.1
12 weeks	8.1±0.4	8.4±0.3	14.6±0.5	14.7±0.3
13 weeks	7.8±0.3	8.2±0.1	13.2±0.4	10.1±0.5
14 weeks	7.9±0.2	8.7±0.3	7.9±0.1	9.0±1.1
Insulin (ng/ml)				
7 weeks	0.9±0.1	1.4±0.2	1.0±0.2	1.3±0.1
11 weeks	1.3±0.2	1.3±0.32	1.0±0.2	1.1±0.1
12 weeks	1.1±0.2	2.1±0.72	1.6±0.1	1.6±0.1
13 weeks	0.9±0.3	2.2±0.64	0.9±0.4	2.2±0.6
14 weeks	1.6±0.1	3.4±0.5*	1.9±0.1	2.0±0.2
Triglyceride (mmol/L)				
7 weeks	2.9±0.4	4.5±0.7	4.0±0.6	3.8±0.9
11 weeks	3.1±0.6	2.9±1.0	7.0±0.4	5.8±1.8
12 weeks	4.4±1.34	7.4±1.0*	5.1±0.7	5.8±0.8
13 weeks	5.4±0.8	9.2±0.6*	6.1±0.3	6.1±1.2
14 weeks	6.5±0.3	26.0±5.2*	3.9±0.7	10.2±1.4*

Table 3-7: Monitoring blood results in non-pregnant female rats.

C1 n=6, F1 n=6, C2 n=4, F2 n=4. *p<0.05 C1 vs F1 and C2 vs F2, ANOVA with contrasts.

3.4.15. Non-pregnant OGTT

Plasma glucose was not significantly affected by diet at any time point in the OGTT. Plasma insulin was significantly increased in non-pregnant F2 rats compared to their control counterparts at 15 minutes (Figure 3-23). AUC for insulin was significantly increased in both F1 and F2 rats compared to controls (Figure 3-24).

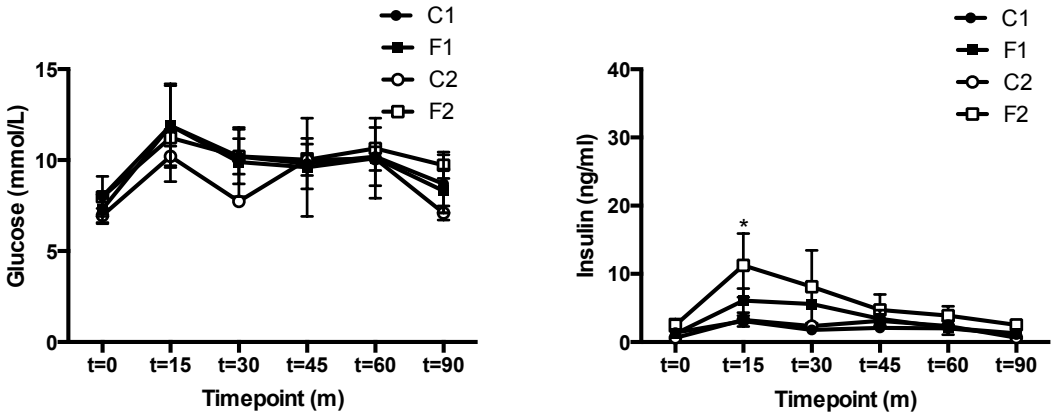


Figure 3-23: Plasma glucose (L) and insulin (R) results from OGTT in non-pregnant female rats.

C1 n=4, F1 n=4, C2 n=5, F2 n=5. At t=0, 3g glucose/kg body weight was administered by gavage, blood samples were taken from the tail vein at each timepoint. Values are mean±SEM, *p<0.05, C1 vs F1 and C2 vs F2, ANOVA with contrasts.

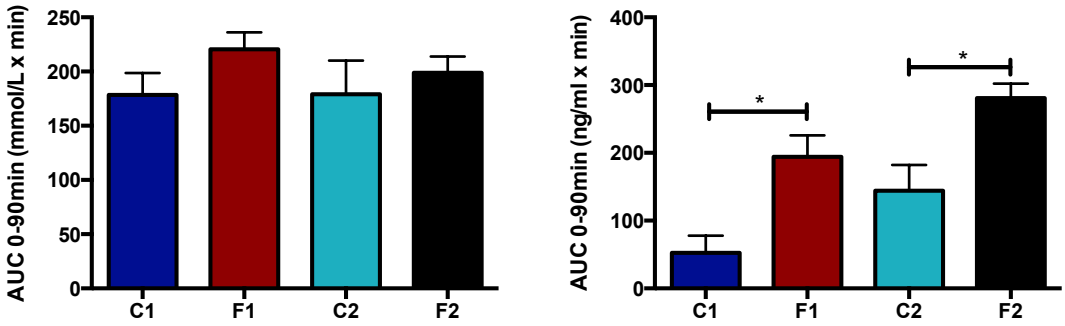


Figure 3-24: Plasma glucose (L) and insulin (R) area under the curve from OGTT in non-pregnant female rats (Figure 3-23).

C1 n=4, F1 n=4, C2 n=5, F2 n=5. All values are mean±SEM, *p<0.05, ANOVA with contrasts.

3.4.16. Gene expression results in first generation non-pregnant rats

JAMA expression was significantly reduced in the jejunum of fructose fed non-pregnant female rats relative to F1 non-pregnant rats, while expression of the other genes tested was not significantly different between diets (Table 3-8).

Gene	**Jejunum C1**	**Jejunum F1**	**Ileum C1**	**Ileum F1**
Claudin-3 (CLDN-3)	1.00±0.4	1.48±0.2	1.00±0.2	0.45±0.9
Junctional adhesion molecule (JAMA)	1.00±0.2	0.51±0.6*	1.00±0.2	0.95±0.4
Occludin (OCLN)	1.00±0.1	1.2±0.5	1.00±0.1	1.5±0.3
Zonulin-1 (ZO-1)	1.00±0.1	1.0±0.7	1.00±0.2	1.3±1.0

Table 3-8: Gene expression results in intestinal permeability markers in age matched non-pregnant female rats.

Non-pregnant rats followed the same diet as their pregnant counterparts. C1 n=4, F1 n=4. *p<0.05 fold change vs control. All values are mean ±SEM relative expression adjusted to the reference genes GAPDH and RPLP0.

GLUT2 expression was significantly increased in the ileum and jejunum of fructose fed non-pregnant rats. GLUT5 expression was increased in the jejunum only of fructose fed rats. SGLT1 expression was increased in the ileum of fructose fed rats (Table 3-9).

Gene	Jejunum C1	Jejunum F1	Ileum C1	Ileum F1
Glucose transporter 2 (GLUT2)	1.00±0.1	4.17±1.2*	1.00±0.3	10.01±0.3*
Glucose transporter 5 (GLUT5)	1.00±0.5	9.80±0.8*	1.00±0.2	1.23±0.1
Sodium-linked glucose transporter (SGLT1)	1.00±0.1	0.78±0.1	1.00±0.4	4.90±0.3*

Table 3-9: Gene expression results in carbohydrate transport proteins in age matched non-pregnant female rats.

C1 n=4, F1 n=4. *p<0.05 fold change C1 vs F1, unpaired t-test. All values are mean ±SEM relative expression adjusted to the reference genes GAPDH and RPLP0.

3.4.17. Gene expression results in second generation non-pregnant rats

In age matched non-pregnant offspring rats following the same diet as their pregnant counterparts there were no significant changes in any of the tight-junction genes tested (Table 3-10).

Gene	Jejunum C2	Jejunum F2	Ileum C2	Ileum F2
Claudin-3 (CLDN-3)	1.00±0.4	1.00±0.2	1.00±1.0	3.00±0.9
Junctional adhesion molecule (JAMA)	1.00±0.1	1.07±0.2	1.00±0.4	0.62±0.2
Occludin (OCLN)	1.00±0.5	1.36±0.7	1.00±0.4	0.81±0.1
Zonulin-1 (ZO-1)	1.00±0.1	1.49±0.2	1.00±0.1	0.96±0.3

Table 3-10: Gene expression results in intestinal permeability markers in second generation age matched non-pregnant female rats

C2 n=5, F2 n=5. All values are mean±SEM relative expression adjusted to the reference genes GAPDH and RPLP0.

GLUT2 and SGLT1 were both significantly upregulated in the ileum and jejunum of second generation non-pregnant rats. GLUT5 upregulation was observed in the jejunum only (Table 3-11).

Gene	**Jejunum C2**	**Jejunum F2**	**Ileum C2**	**Ileum F2**
Glucose transporter 2 (GLUT2)	1.00±0.2	1.99±0.3*	1.00±0.4	3.43±1.9*
Glucose transporter 5 (GLUT5)	1.00±0.1	2.22±1.2*	1.00±0.1	1.20±0.2
Sodium-linked glucose transporter (SGLT1)	1.00±0.3	1.80±0.1*	1.00±0.3	2.94±0.3*

Table 3-11: Gene expression results in carbohydrate transport proteins in second generation age matched non-pregnant female rats.

C2 n=5, F2 n=5. All values are mean±SEM relative expression adjusted to the reference genes GAPDH and RPLP0. *p<0.05 fold change vs control, unpaired t-test.

3.5. Discussion

3.5.1. The effects of a maternal high-fructose diet on body composition and metabolism in the mother and the offspring

In both generations of dams, the 10% fructose solution did not affect overall body weight by 13 weeks of age. In the second generation, offspring born to mothers consuming the 10% fructose solution were significantly growth restricted, yet displayed catch-up growth to reach an equivalent weight to controls by 13 weeks. The role of fructose in fetal growth restriction appears to be consistent with the limited research already carried out on this topic (56). There are some proposed mechanisms for high fructose intake having this effect on offspring birthweight: hyperinsulinaemia has been shown to reduce blood flow to visceral tissues (270), and increase blood pressure in animal models and human studies (271), these changes may be reducing placental blood flow and causing the fetal growth restriction observed in the F2 rats.

Insulin resistance may also be having independent effects on fetal growth, however specific mechanisms by which insulin resistance can affect placental development are not well understood. Human studies suggest that poor glucose tolerance will lead to increased placental growth, as substrate delivery is thought to be its main mediator, with a rise in maternal substrates leading to an increase in expression of insulin-like growth factors resulting in an increase in fetal growth (272,273). However fructose supplementation appears to have the opposite effect. The reduced protein intake in fructose fed dams could explain this difference, but as in the similar study by Vickers *et al.* (56), the reduced protein intakes in the fructose fed dams were not below AIN93 requirements for rodents (216,269).

Increases in fat mass were observed in both generations, with a greater effect in the F2 dams. Single generation rodent studies have produced

similar findings. In male mice fed 15% fructose *ad libitum* for 60 days a similar increase in total body fat measured by MRI is observed, however the fructose fed group are also significantly heavier than their control counterparts (274). Male rats fed *ad libitum* HFCS (8% solution in water, containing 55% fructose) for 8 weeks showed an increase in fat mass and body weight compared to controls (275). In contrast to these studies, I have shown a high fructose diet fed for two generations will lead to the increase in total fat mass consistent with observations described above, but also a redistribution of lean and fat mass. This is potentially an effect of pregnancy, maternal adipose tissue increases in pregnancy (276) and it would appear that an increased intake of fructose exacerbates this. However, as this study was focussed solely on females, further research using the same model to analyse fat depots and total fat mass would be beneficial based on the acknowledged differences in adipose tissue deposition between the sexes (277).

The role of fructose in nutritional programming has been previously demonstrated to a limited extent (278), with studies in rats showing that high fructose intake in gestation will lead to an increase in both fat mass and adipocyte size in retroperitoneal adipose tissue of 60 day old offspring (279). A high concentration of fructose (40%) in maternal drinking water throughout pregnancy will also lead to offspring which are hyperglycaemic at birth (256). I have shown that chronic consumption of a moderate concentration of fructose will induce adipogenesis in the offspring, with retroperitoneal, interscapular and uterine adipose tissue all being markedly increased in the offspring, and to a greater degree than in the first generation. This is consistent with the acknowledged role of fructose in adipocyte differentiation (280) with the treatment of cultured murine preadipocytes with fructose leading to an increase in the differentiation markers peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer binding protein alpha (C/EBPα) (281).

In addition to a fructose supplemented diet, growth restriction has also been shown to lead to increased adiposity in later life (282). Growth restriction in pigs will lead to increased fat depth at 12 months of age compared to normal birth weight offspring, with a more significant increase in females (283). Similar effects have been demonstrated in sheep, IUGR induced by placental restriction will lead to a significant increase in central adiposity and reduction in lean mass in 43 day old lambs (284). Finally, uterine artery ligation in pregnant rats will lead to growth restricted offspring with significantly increased retroperitoneal fat mass by 21 days of age (285).

In first generation dams, plasma glucose levels were not significantly different between control and fructose groups at any time point. Several studies using high concentrations of fructose ranging from 34% of total calories to 60% dissolved in drinking water that allowed *ad libitum* consumption of fructose have shown that fructose can increase plasma glucose concentrations (46,286). Research focusing on the liver in fructose fed rats has shown that fructose will have a direct effect on insulin receptor phosphorylation, whilst the expression of insulin receptors remains constant (45). This is consistent with other models of insulin resistance (287,288), and has also been shown to occur during pregnancy (289). My data suggests that dietary supplementation with a smaller amount of fructose may not lead to hyperglycaemia, even during pregnancy. Potentially this is due to the age of the rats and the lower concentration of fructose used, with a study in non-pregnant rats using 15% fructose dissolved in drinking water showed significant increases in fasting glucose after 7 months of feeding (290).

Both insulin levels throughout pregnancy and during OGTT in F1 rats are consistent with what has been previously shown in in both humans (291) and rats (292,293), however a progressive worsening of insulin resistance over two generations of fructose supplementation has not been previously demonstrated. Our results fit with the idea that insulin resistance can be

"programmed" in the offspring via suboptimal maternal nutrition, but the development of symptoms of the metabolic syndrome such as insulin resistance will become apparent with a second dietary and metabolic challenge in the offspring (19,294). The results in the non-pregnant rats indicate that in this model a challenge such as pregnancy in conjunction with the 10% fructose solution is sufficient to induce a more severe insulin resistance in the offspring than diet alone.

The fact that the F1 rats in this study are only showing signs of developing pre-diabetes, yet have F2 offspring which are clearly insulin resistant suggests that fructose *in utero* may have important metabolic effects on the offspring which warrant further exploration. Epigenetic mechanisms have been proposed to explain these changes, particularly with regard to the effect of uteroplacental insufficiency. IUGR rat models have shown increases in binding site specific histone H3 acetylation of a number of genes associated with IUGR (295), including those potentially linked to insulin resistance (296). Research in a primate model has shown similar results induced by a maternal high-fat diet (297). Previous research in the same model (293) does not show these effects in non-pregnant offspring, suggesting that insulin resistance induced during pregnancy (298–300) may be a contributing factor. Notably, I have shown that consumption of 10% fructose will have more deleterious effects at an earlier age in the offspring of mothers who have consumed the same diet.

Finally, serum triglycerides were significantly increased in the fructose groups of both generations from pre-mating onwards (10 weeks of age). Based on the evidence covered in chapter 1 on fructose being diverted to lipogenesis this is an expected finding, and has been demonstrated in a number of human and rat studies (301–303). However, I have again shown that exposure to fructose during gestation will increase the effects of the same diet given postnatally.

3.5.2. The effects of a maternal high-fructose diet on GI adaptation in the mother and offspring

Of the intestinal permeability markers studied, none were found to be significantly altered in first generation dams, suggesting that the fructose diet is not inducing any changes in intestinal permeability that have been previously associated with high fructose intake and subsequent inflammation (40,174,304). Whether these results are due to the milder dietary challenge or the pregnancy, or an interaction between these factors remains to be determined. Therefore further research should focus on whether the changes observed in the second generation dams are due to growth restriction (and reduction of small intestinal length) caused by the high fructose diet in the mothers, or a direct effect of the fructose itself. Research into the effect of fructose on the maternal microbiota will be discussed in chapter 4.

My results show a significant decrease in expression of markers of intestinal permeability in the offspring of rats born to mothers consuming a high fructose diet. These F2 rats also displayed significantly lower birth weights than their C2 counterparts. The effect of growth restriction on GI development has been previously studied in rats (85,86) and pigs (305), and is of interest to humans due to the prevalence of GI disorders in low birth weight (LBW) and premature infants (306). Of particular interest is the fact that F2 rats had relatively shorter small intestines, suggesting that the GI tract (as well as the pancreas) is not displaying the same catch-up growth as other organs and is in accord with studies of IUGR in piglets (83).

The significant reduction observed in three of the four epithelial tight junction markers studied in the second generation suggest that fructose plays a role in small intestinal development and adaption of the small intestine to pregnancy. There is evidence to support both the role of fructose directly on the intestine, and the effect of growth restriction. IUGR

in a pig model has been shown to adversely affect gut development from birth onwards, with regard to inflammation and establishing intestinal permeability (307). Chronic consumption of fructose (but not glucose) has been shown to induce endotoxaemia in mice (308). Expression of another claudin isoform, claudin-4 has been shown to be reduced in cultured intestinal epithelial (Caco-2) cells on treatment with fructose (309). To my knowledge this is the first study showing such an effect on the intestine in an *in vivo* model.

I have also shown significant effects of fructose feeding on the three carbohydrate transporters tested: GLUT2, GLUT5 and SGLT1. This upregulation is more marked in the first generation, with fold changes reaching 10 times that of controls in the fructose group. A high intake of fructose has been shown to regulate the expression of both GLUT2 and GLUT5 (265), using direct perfusion of fructose into the jejunum of anaesthetised rats, or a diet containing 65% fructose (263). GLUT5 expression is thought to be extremely low in early life, with an increase in activity in rats occurring regardless of diet around 28 days (310), but expression can be stimulated in neonates by dietary fructose (311). GLUT5 expression is also thought in part to be regulated by diet and age induced histone acetylation of the GLUT5 promoter (264). In contrast, GLUT2 and SGLT1 expression is thought to remain consistently high throughout development, with diet playing less of a role in transporter expression (93). My study has demonstrated similar results to (265) in a more physiologically relevant animal model, with fructose included in the diet rather than direct intestinal perfusion.

SGLT1 expression is also significantly upregulated in the fructose groups of both generations. Fructose infused directly into the small intestine of rats has been shown to upregulate SGLT1 expression to the same degree as D-glucose (312), despite SGLT1 having an extremely low affinity for fructose (313). Again, to my knowledge this is the first example of a comparatively low-level dietary supplementation of fructose showing

similar effects on the intestine. Taken together, these findings suggest that fructose may potentially be upregulating the transport of glucose as well as fructose. SGLT1 has also been shown to act as both a transport protein and nutrient sensor, mediating the secretion of gastric inhibitory peptide (GIP) and GLP1 and thereby promoting insulin secretion (314). This upreguation of SGLT1 would therefore provide another mechanism by which fructose can promote the onset of insulin resistance.

In all cases of significant changes in the intestinal permeability markers, and in the case of GLUT5 in the first generation and GLUT2 in the second generation, the significant changes observed were largely confined to the jejunum. This is consistent with research examining the effects of diets high in fructose or glucose on intestinal uptake of monosaccharides. Other investigators have demonstrated in rats that a diet high in fructose will increase uptake in the jejunum to a far greater degree than the ileum (315). The fact that fewer changes are observed in the ileum suggests that the majority of the fructose ingested is being taken up in the upper small intestine. In cases of malabsorption, fructose will pass further into the ileum and large intestine (316); the lack of significant changes in the ileum in this study would indicate that a 10% fructose supplemented diet is not enough to induce fructose malabsorption.

3.5.3. Non-pregnant rats

In order to test the extent to which pregnancy is involved in the effect of the high fructose diet on gut development, several of the genes tested in the main study were analysed in a smaller cohort of non-pregnant animals. In the four intestinal permeability markers tested there were no significant differences in expression, indicating that the adverse effects seen in the second generation pregnant model are confined to pregnancy. This is consistent with the limited literature that is available, suggesting that the intestine is more susceptible to changes in permeability during

pregnancy (317). There are potential links here with research into the microbiome during pregnancy which will be discussed in chapter 5.

All three carbohydrate transporters analysed showed the same changes in expression as the pregnant model, this is in keeping with previous literature showing that carbohydrate transporters are regulated by diet (64,318). Limited research using mRNA abundance and microarray suggests that both SGLT1 and GLUT5 are upregulated in pregnancy regardless of diet (319).

3.5.4. Liver gene expression

In both generations analysed, no inflammatory markers were significantly changed in the liver. This indicates that although the fructose diet will upregulate markers of intestinal permeability, this is not enough to induce inflammatory effects. Diets using a higher concentration of fructose have induced both inflammatory changes in the liver (320), and endotoxaemia (180), and these changes have been shown to be a hallmark of diet induced NAFLD (40). It is possible that the fructose diet in my study will lead to a noticeable systemic inflammation if consumed over a longer period of time; therefore further research may be warranted. The liver was chosen as a target organ to potentially study the effect of LPS passing through the gut barrier and into the portal vein (87). However LPS has also been shown to lead to inflammation of adipose tissue (321), therefore adipose tissue depots would be a target for further analysis given the significant increases in fat mass observed in the F2 dams. Plasma endotoxin could also potentially be studied, however there is an acknowledged wide variation (317) in the tests used to measure this. Finally, pregnancy has been shown to be a state in which inflammation is supressed, both in humans (322), and rodents (323), therefore this may explain to some extent the lack of inflammation observed in this model.

3.6. Summary

In summary, I have shown that a high fructose diet during pregnancy induced transient hyperglycemia during pregnancy in the first generation, but will significantly increase serum triglycerides and adversely affect glucose tolerance at the end of pregnancy. Offspring born to fructose fed dams are growth restricted compared to offspring of control dams, and continuing on the same diet leads to dramatically reduced glucose tolerance and increased insulin resistance, along with significant increases in fat mass when these offspring are pregnant.

Markers of intestinal permeability are not significantly affected by the fructose diet in the first generation, but a continuation of this diet by the offspring causes a significant reduction in several of the epithelial tight junction proteins studied. This suggests that intestinal permeability may be adversely affected by a long term exposure to a diet supplemented with fructose. Despite these metabolic changes and effects on epithelial tight junctions, no significant indications of liver inflammation were observed.

Based on the studies in intestinal carbohydrate transporters, in both generations of rats I have shown that a relatively low level supplementation of fructose has the potential to significantly increase transport of both glucose and fructose in the intestine. Taken together, these findings have implications for the amount of fructose included in the average "Western diet", and suggest that frequent low-level consumption of fructose in the diet can have equally deleterious effects to those already shown in studies using high concentrations of fructose or HFCS.

Chapter 4: The effect of pregnancy and fructose supplementation on the rat intestinal microbiome

4.1. Introduction

As discussed in chapter 1, the intestinal microbiota is now recognised as an important mediator of human health, particularly with regard to obesity and the metabolic syndrome (183), and it has now been shown that it is likely that components of the diet, rather than host genetics, play a dominant role in shaping the microbiota (324,325). The maternal microbiome undergoes significant changes during pregnancy which are similar in some respects to those observed in obesity (199). Although there is a rapidly growing body of evidence demonstrating how the diet modulates the human microbiome (326–328), there has been little work covering specific effects of fructose, and none studying the effects of fructose during pregnancy.

In humans, the microbiome in pregnancy has been shown to gradually shift from one similar to the non-pregnant state in the first trimester (T1), to one that is more similar to a disease state such as obesity by T3 (199). By T3 there is a high microbial beta diversity, indicating a wide variation between individuals, whilst the diversity of microbes within an individual is significantly reduced compared to T1 and non-pregnant controls, both of these changes are also observed in obese individuals (185,329). These findings are also consistent with a smaller study in pregnant women (330), both studies also highlight the increased abundance of *Proteobacteria* in T3, generally characteristic of intestinal inflammation (203). However looking at changes in individual species the T3 pregnant microbiome is not entirely similar to an obese one, with no changes in the relative abundance of *Bacteroidetes* or *Firmicutes*, which are regarded as a characteristic of the obese microbiome (as discussed in chapter 1).

4.2. Hypothesis

Based on the literature discussed above and in chapter 1, I hypothesise that consumption of a fructose supplemented diet will significantly affect the composition of the microbiome, chiefly by increasing the population of fructose digesting species. These changes may disrupt the shifts in alpha and beta diversity than have been previously demonstrated to occur in pregnancy (199). As the changes in microbial diversity during the third trimester have been suggested to be beneficial by providing a mechanism for the mother to pass on certain microbes on to her offspring, this study may provide further insight into how fructose supplementation adversely affects intestinal adaptation in pregnancy.

4.3. Materials and methods

A full description of the animal study is given in section 2.4 and methods are described in 2.20 and 2.21.1. Faecal samples were taken from dams in the first generation of the SM study (C1 and F1) at three pregnancy time points: Pre-mating (PM, 11 weeks), early pregnancy (EP, 12 weeks/GD 4-7) and late pregnancy (LP, 14 weeks GD 19-20). Sample numbers were as follows: PM n=27, EP n=29, LP n=28.

DNA extraction was carried out by myself, Brent Nielsen and Jayden MacPherson under the supervision of Dr Ben Willing in the Division of Animal Science, University of Alberta. All PCR work was carried out by myself, and samples sent for sequencing at the McGill University and Génome Québec Innovation Centre, Montréal, QC, Canada. Analysis of the sequencing data was carried out by Dr Mi Zhou and all data and results made available to myself for analysis.

4.4. Results

4.4.1. Effect of fructose and pregnancy on alpha and beta diversity

Alpha diversity significantly differed between the control and fructose diets at PM and LP time points, and was unchanged at EP. Similarly, beta diversity was significantly increased in fructose-fed dams at PM and LP time points and unchanged at EP.

Control dams displayed a significant reduction in alpha diversity between PM and LP, whereas fructose fed dams displayed a significant increase between PM and LP (Figure 4-1). Fructose-fed dams did not display significant changes in beta diversity between any pregnancy time point, control dams demonstrated a peak in beta diversity at EP (Figure 4-2).

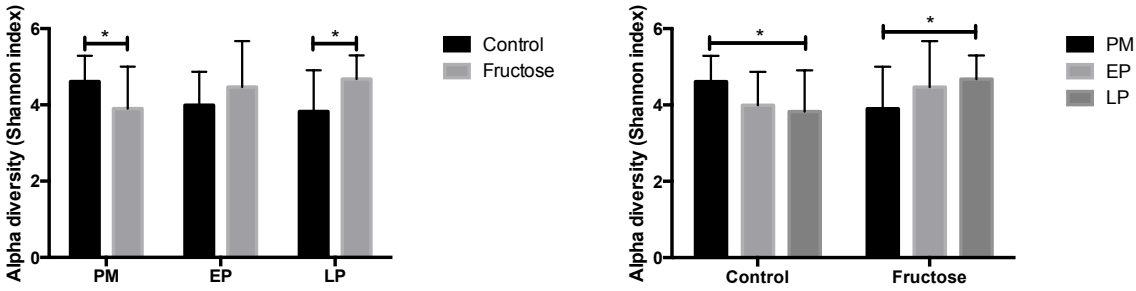


Figure 4-1: Alpha diversity stratified by time point (L) and diet (R).

All values are mean±SEM, arbitrary units. *p<0.05, ANOVA.

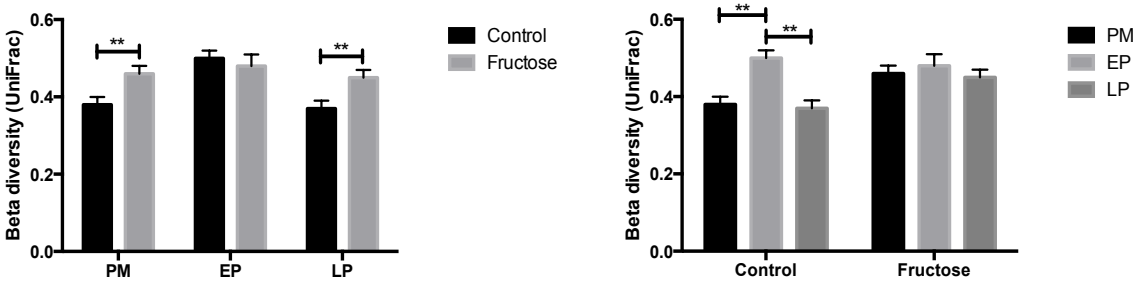


Figure 4-2: Beta diversity stratified by time point (L) and diet (R).

All values are mean ±SEM UniFrac distance (arbitrary units). **p<0.01, ANOVA.

4.4.2. Distribution of phyla among samples at each stage of pregnancy

The sequences (as OTUs) were assigned to 10 phyla in total. At each time point, *Bacteroidetes* and *Firmicutes* were the most abundant phyla (Figure 4-3-Figure 4-5).

At PM, the overall bacterial composition in the faecal samples significantly differed between control and fructose diets (ANOSIM $R=0.1145$, $p<0.05$). No significant differences were observed at EP between diets. Bacterial composition again became significantly different between control and fructose diets at LP (ANOSIM $R=0.1728$, $p<0.05$).

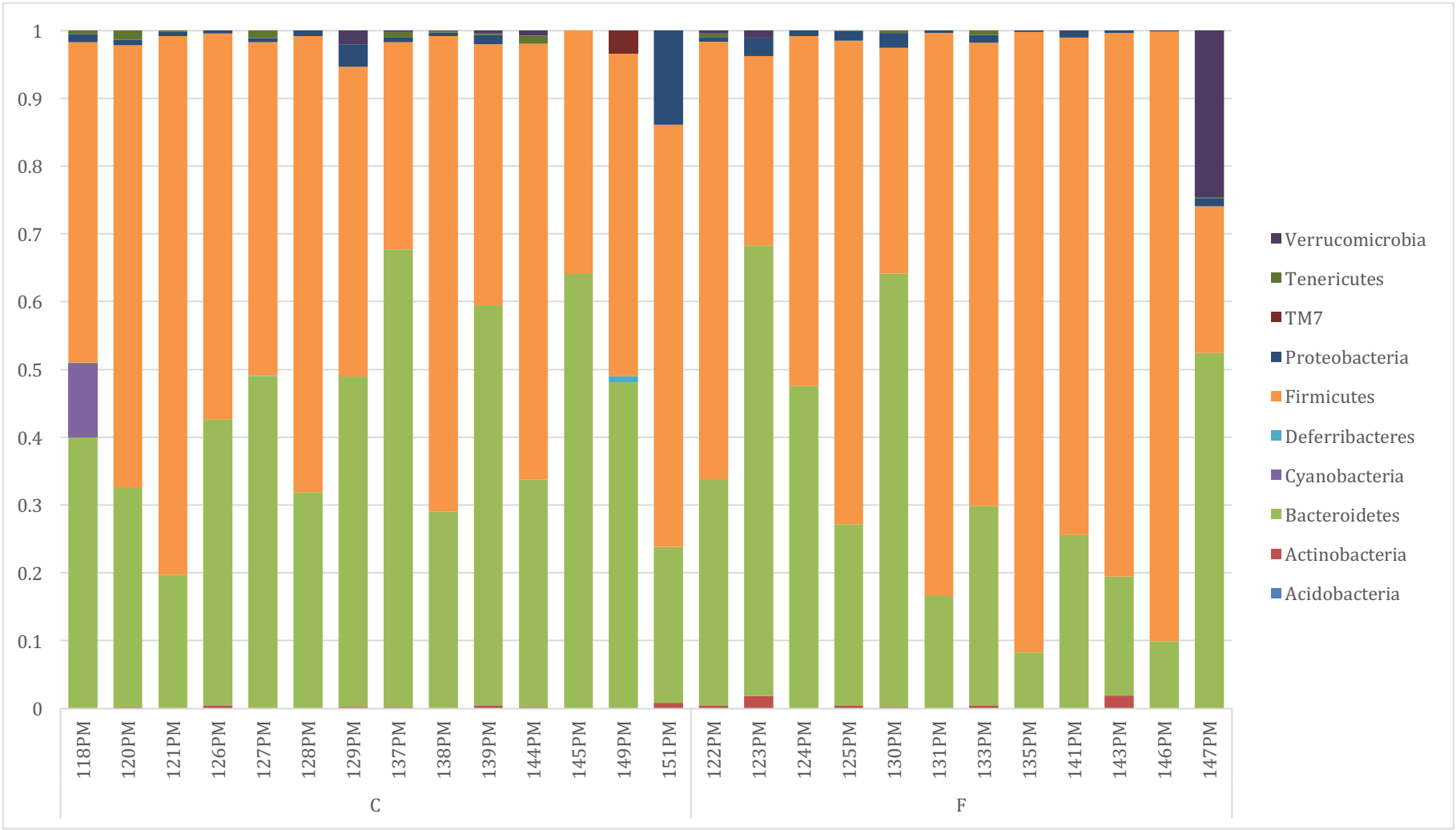


Figure 4-3: Distribution of phyla among PM samples, stratified by diet.

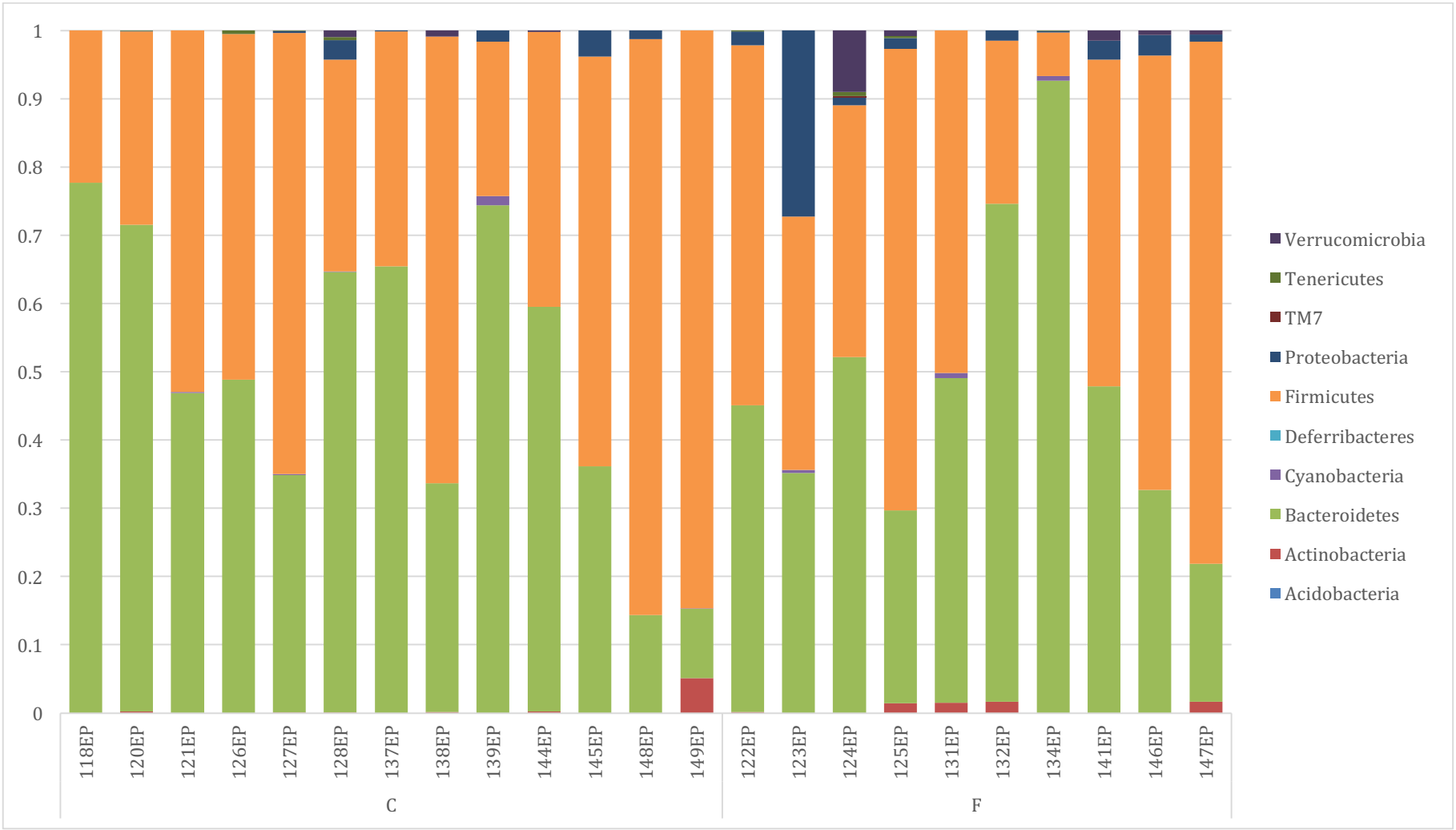


Figure 4-4: Distribution of phyla among EP samples, stratified by diet.

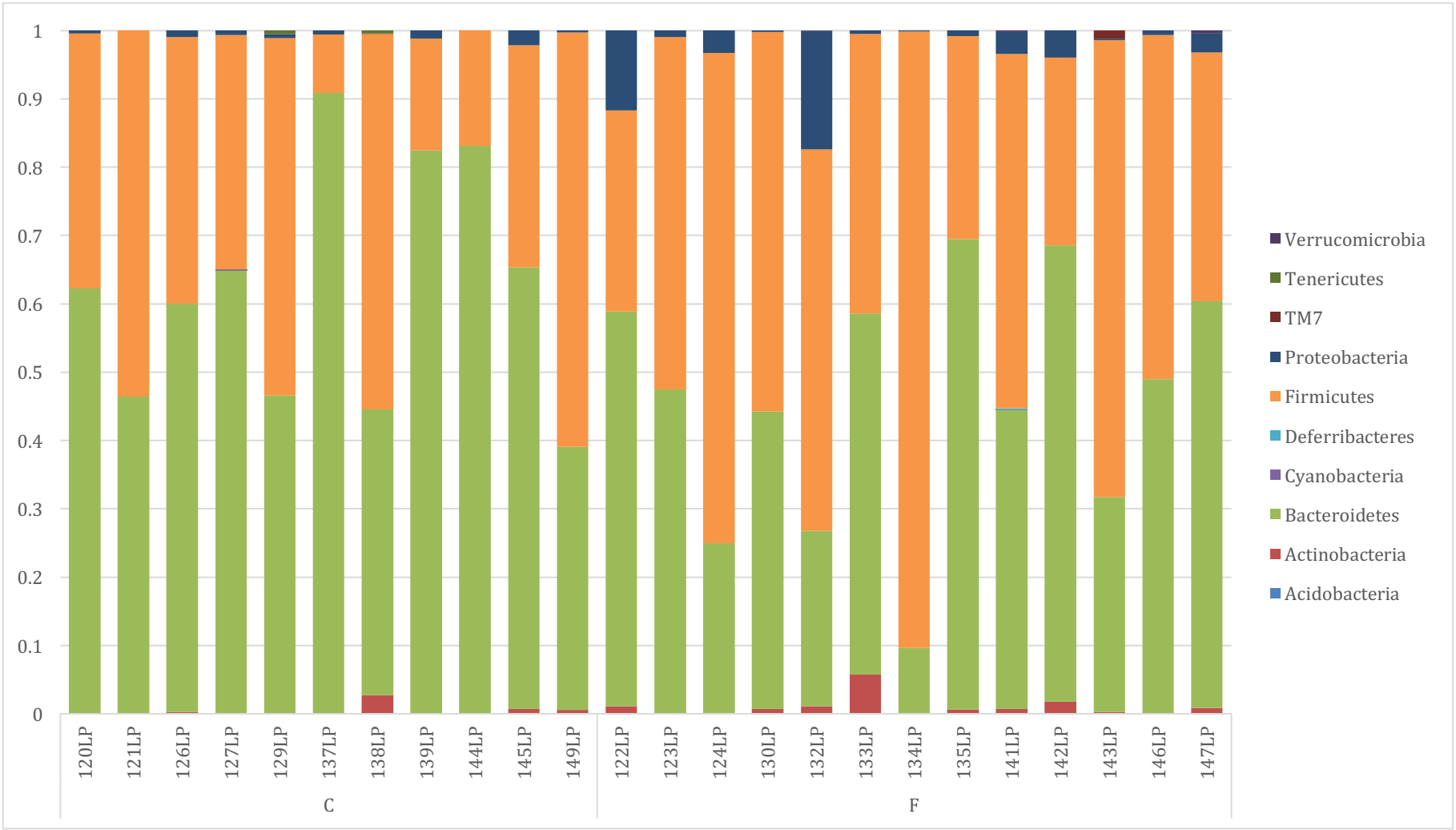


Figure 4-5: Distribution of phyla among LP samples, stratified by diet.

4.4.3. Distribution of operational taxonomic units at each phylogenetic level

Control dams displayed an increased bacterial richness (measured by number of unique OTUs at each phylogenetic level) compared to fructose-fed dams (Figure 4-6-Figure 4-7).

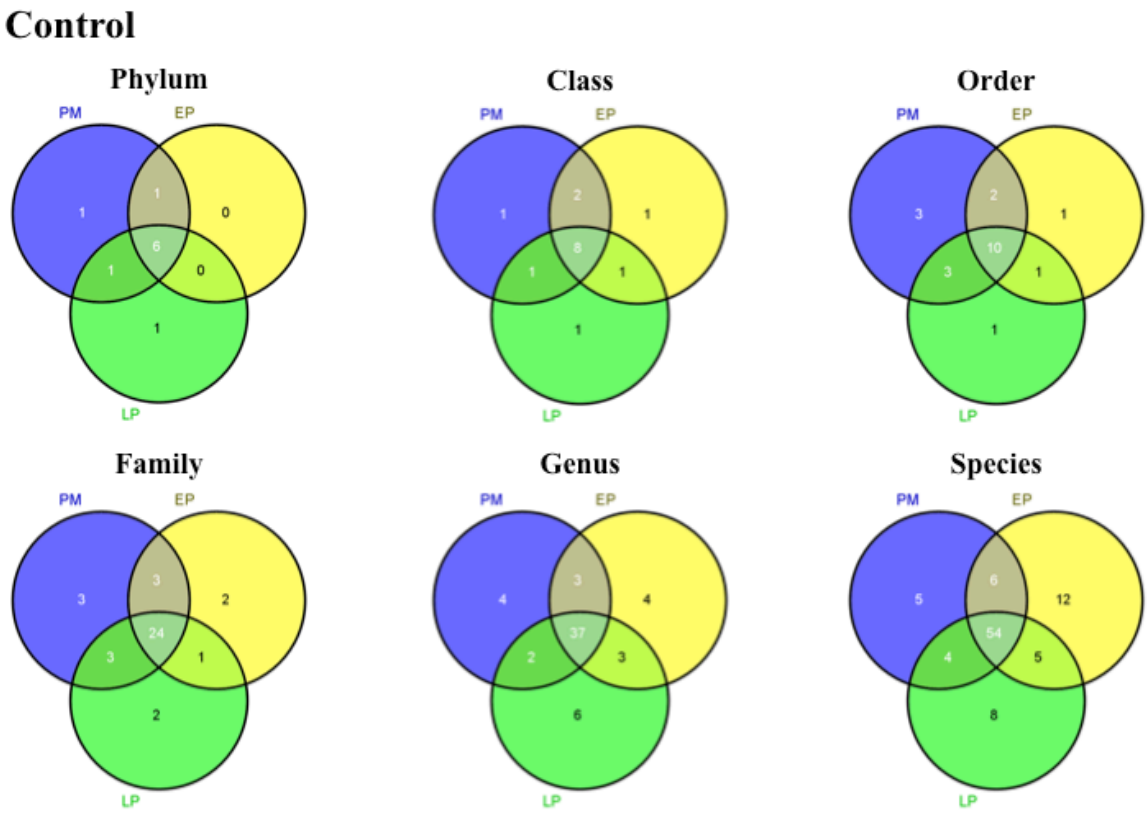


Figure 4-6: Distribution of operational taxonomic units (OTUs) at each phylogenetic level among all control samples.

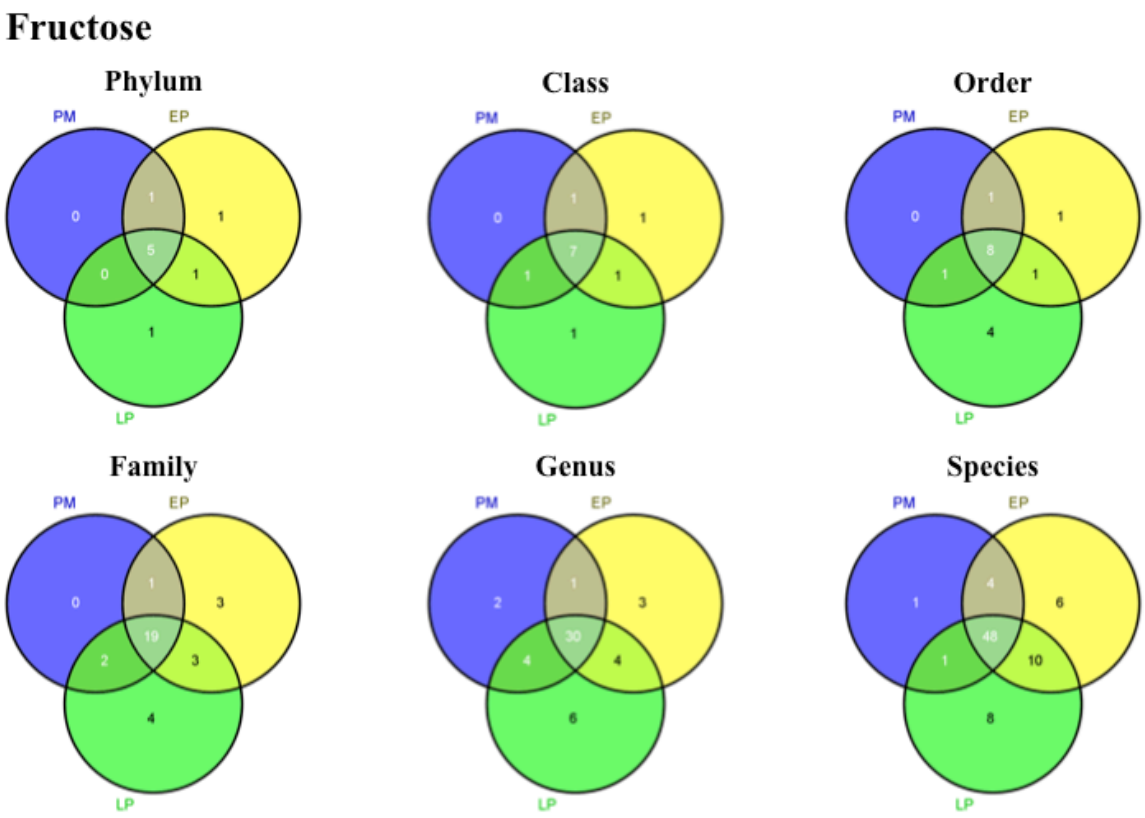


Figure 4-7: Distribution of OTUs at each phylogenetic level among all fructose samples.

4.4.4. Summary of bacterial communities in each sample

In total, obtained sequences from each sample covered 85.9% to 98.3% of the total bacterial population (estimated by Good's coverage).

Sample ID	*Good's coverage*[a]	*Observed species*[b]	*Shannon*[c]
118PM	0.931	142	5.46
120PM	0.930	145	5.44
121PM	0.961	85	3.82
122PM	0.932	120	4.98
123PM	0.960	97	4.41
124PM	0.937	74	4.45
125PM	0.964	84	3.91
126PM	0.923	62	4.21
127PM	0.958	107	4.84
128PM	0.936	78	4.47
129PM	0.921	100	5.43
130PM	0.917	97	5.32
131PM	0.973	51	2.65
133PM	0.919	124	5.36
135PM	0.982	41	2.28
137PM	0.956	100	4.31
138PM	0.967	69	3.71
139PM	0.903	84	5.27
141PM	0.960	85	3.86
143PM	0.969	64	3.59
144PM	0.926	99	5.21
145PM	0.913	38	4.03
146PM	0.983	36	2.15
147PM	0.954	66	3.87
149PM	0.875	50	4.73
151PM	0.967	67	3.59
118EP	0.975	63	3.72

120EP	0.961	91	4.55
121EP	0.955	44	2.89
122EP	0.910	168	5.14
123EP	0.934	103	5.23
124EP	0.93	98	4.81
125EP	0.963	93	4.09
126EP	0.859	78	5.02
127EP	0.935	119	3.92
128EP	0.950	108	4.74
131EP	0.874	54	4.83
132EP	0.960	92	4.28
134EP	0.982	37	1.26
137EP	0.952	89	3.56
138EP	0.939	69	4.39
139EP	0.944	110	3.75
141EP	0.862	85	5.39
144EP	0.896	85	5.01
145EP	0.943	80	4.88
146EP	0.907	116	5.05
147EP	0.925	79	4.63
148EP	0.976	55	2.14
149EP	0.977	58	3.30
120LP	0.941	65	3.59
121LP	0.956	57	4.45
122LP	0.919	54	5.01
123LP	0.901	120	5.26
124LP	0.947	73	4.42
126LP	0.880	87	4.97
127LP	0.948	83	4.01
129LP	0.933	90	4.77
130LP	0.928	51	3.86
132LP	0.959	70	4.57
133LP	0.953	49	4.25

134LP	0.961	50	3.48
135LP	0.935	73	4.64
137LP	0.973	43	1.85
138LP	0.943	92	5.05
139LP	0.960	57	3.66
141LP	0.878	102	5.72
142LP	0.88	60	4.53
143LP	0.898	84	5.33
144LP	0.958	43	2.01
145LP	0.942	53	3.52
146LP	0.866	57	4.58
147LP	0.935	92	5.23
149LP	0.953	67	4.24

Table 4-1: Summary of bacterial communities in all samples across the three timepoints studied.

[a]Good's coverage for OTU definition, [b]Observed species: unique OTU numbers, [c]Shannon diversity index.

4.5. Discussion

There are a range of studies showing the effects of both fructose and HFCS on metabolic health in human and animal models (275,278,293). There is also a limited amount of research demonstrating that fructose will have adverse effects on the mother during pregnancy (293) and that this may lead to adverse effects in the offspring (56,260). Fructose has been shown to modulate the microbiota independently of pregnancy (331,332), and this work is now expanding to include the microbiome (333). The aim of this chapter was therefore to use pyrosequencing to assess the effect of fructose supplementation and pregnancy on the microbiome.

4.5.1. The effect of 10% fructose supplementation on the microbiome

The significantly increased alpha diversity in fructose-fed rats throughout pregnancy indicates that a more diverse microbiome is required for fructose fermentation. Direct comparisons between diets at LP also support this, with a significant increase in diversity in the fructose group. Looking at specific phyla, *Lactobacillus* and *Bacteroides* are both significantly enriched in dams on the fructose diet, which suggests that the fructose diet is increasing the abundance of fructose-digesting species (334,335). *Bacteroides* abundance has also been shown to be increased in obese pregnant women, and are associated with excessive weight gain in pregnancy (198). In this study *Bacteroides* were shown to be increased in the fructose group without an excessive caloric load, or increased weight gain through pregnancy, this suggests that *Bacteroides* may have an affinity for fructose, although the metabolism of sugars by *Bacteroides* is not fully understood (336).

Dams fed the fructose diet demonstrated no significant changes in beta diversity throughout pregnancy, those fed the control diet demonstrated a peak in beta diversity at EP, which was then reduced by LP. This is inconsistent with work done in human pregnancy (199), in which

pregnancy was associated with an increased beta diversity. The dams in this study were all exposed to an identical environment and diet which may explain the lack of diversity when comparing individuals.

4.5.2. Microbial adaptation to pregnancy

In control dams, alpha diversity was shown to be significantly reduced by LP, which is consistent with work in humans (199). Therefore both my study and Koren *et al.* (199) suggest that pregnancy is associated with a decrease in microbial diversity within an individual, likely caused by an increase in the abundance of certain species at the expense of others. This increase in microbial abundance in late pregnancy has been suggested to facilitate transfer of certain species, such as *Lactobacilli* to the offspring at birth (337). However, although the colonisation of the intestinal tract by microbes is thought to be broadly similar between mammals (338), the mechanisms by which microbes are inherited are not fully understood and there may be significant variations between rodents and humans.

A significant increase in the abundance of *Prevotella* species was observed at LP in control dams. Relationships between this genus and pregnancy are not understood, however the abundance of *Prevotella nigrescens* has been shown to be increased in pregnancy in the oral microbiota of pregnant women (339). Therefore, abundance of *Prevotella* is potentially affected by host hormone changes. Due to limitations of the reference database used in my study, it was not possible to match any OTUs to known species of *Prevotella*, making any more detailed analysis of metabolic characteristics impossible.

Levels of *Actinobacteria*, which have been previously shown to be elevated in pregnancy (199,330), were unchanged in my study. *Actinobacteria* have been demonstrated to be elevated in obesity (184), and an increase in abundance is hypothesised to be beneficial in pregnancy (199). The universal bacterial primers used in my study are not capable of amplifying

all *Actinobacteria* genes within a sample (340), leading to a loss of definition in the sequencing data, this may explain the differences between my study and previous work.

4.5.3. Summary

In summary I have shown that a high fructose diet during pregnancy will significantly affect microbial diversity in the mother, with a shift towards a microbiome that is both more diverse, and enriched for species such as *Lactobacillus* and *Bacteroides* which will lead to increased energy availability from this diet. Within the control animals, the results from my study are broadly similar to those in human pregnancy, with a decrease in alpha diversity observed in late pregnancy. The fructose diet appears to induce the opposite effect on alpha diversity, suggesting that the increase in abundance of fructose-fermenting bacteria is preventing pregnancy-specific changes in diversity from taking place. As these changes are thought to facilitate the inheritance of beneficial species by the offspring (337), these findings may suggest another mechanism by which a suboptimal maternal diet will lead to adverse effects in the offspring.

Further studies into how this maternal microbiome will influence colonisation in the offspring, either those continuing on a fructose supplemented diet or switching to a control diet, would be worthwhile. To my knowledge this is the first account of these microbial changes being demonstrated with a low level supplementation of fructose compared to previous work, and this may have implications for the effects of fructose consumption as part of the Western diet.

Chapter 5: The effect of fat supplementation and growth restriction during gestation on gastrointestinal development in the offspring

5.1. Introduction

Chapter 1 has outlined the effects of both fat supplementation and growth restriction during gestation on metabolic health in the offspring. A maternal high fat diet during gestation has been shown to increase blood pressure and plasma insulin in the offspring of rats (341,342), with similar changes occurring if the diet is purely confined to lactation (37). Similar findings have also been demonstrated in a small cohort of primates (343).

The pig has been shown to be a useful model for studying the effect of growth restriction and nutritional programming in the intestine (106). Relative to other large animal models, pigs display severe IUGR, with the low birth weight or "runt" piglets being around 65% lighter than median weight littermates (344). These differences in birth weight have been shown to be established very early in development at around 35 days of gestation (345,346).

The effect of growth restriction on gastrointestinal development has been studied; IUGR has been shown to preferentially affect the small intestine relative to other organs (83), and low birth weight and growth restricted infants display a higher prevalence of GI disorders (306). In pigs, IUGR will significantly delay intestinal development, with lactase and aminopeptidase activity significantly lower in growth restricted pigs at birth (347), suggesting a reduced digestive capacity (348). A decline in intestinal permeability to macromolecules which occurs at the beginning of suckling does not occur with IUGR (305). Taken together this research suggests that the immaturity of the small intestine demonstrated in low

birth weight piglets could lead to increased intestinal permeability and reduced response to inflammation.

The effect of a maternal high-fat diet has been shown to have considerable effects on growth trajectories in the offspring, however this response is disputed in the literature. Wistar rats fed a high fat diet (replacing starch with palm oil) during pregnancy have been shown to have significantly smaller offspring, which do not display any catch-up growth and remain smaller than controls at 28 days of age (349). However pregnant mice fed a lard supplemented diet during pregnancy produced heavier offspring (350,351), that then grew faster up to 14 weeks of age (350,352).

Based on the effects outlined above a maternal high-fat diet may therefore have profound effects on gastrointestinal development, similar to those seen with IUGR. Currently this research is lacking, although a high-fat diet has been shown to induce an increase in intestinal permeability, a decrease in intestinal alkaline phosphatase (vital for ameliorating the inflammatory effects of LPS (353)), and a significant decrease in microbial diversity (145). As covered in section 1.11, there are clear effects of maternal nutrition on GI development (96,98–100), but there is a need to examine more targeted maternal dietary interventions such as a high-fat diet, especially given the effects on other organs such as the liver (343,351,354) and pancreas (355,356).

Based on the literature covered in chapter 1, there are significant differences in intestinal development between low and normal birth weight infants. Growth restriction appears to adversely affect intestinal growth and repair, particularly in early life before and during catch up growth (89,357). This has been demonstrated through differential expression of several genes, with EGF/EGFr being particularly important for intestinal development. However, ubiquitous inflammatory markers such as TNFα and IL-1β and the TLR family, and intestinal specific markers such as pBD can also be used to give an indication of

inflammation in the intestine, and can be used to make a comparison between low and normal birth weight offspring.

Growth restriction may also have an effect on intestinal permeability (117,118). Epithelial tight junctions are of interest in this context due to them providing a means by which bacteria (and LPS) can cross the intestinal epithelium. Dysregulation of the genes ZO-1, CLDN and OCLN can provide an indication of changes in intestinal permeability and therefore be used to make an assessment of the effect of IUGR.

5.2. Hypothesis

Therefore, I hypothesise that growth restriction will induce adverse effects on the maturing GI tract, creating a proinflammatory state and reduction in epithelial tight junction function. Based on links between disrupted intestinal development and glycogen accumulation within enterocytes (100), I expect to observe an increased amount of tissue glycogen in the small intestines taken from piglets born to fat-supplemented mothers compared to controls.

As discussed, the role of individual nutrients (as opposed to the total energy content of the diet) in gut development has not been well researched, particularly with regard to effects on intestinal permeability (106). However, based on the adverse effects of a high fat diet that have been demonstrated on the small intestine (321,358), I hypothesise that a suboptimal maternal diet will induce similar changes in gut development to those observed in low birth weight infants (106,144,145). The role of the intestine in ion transport has been previously researched, particularly with regard to increased intestinal permeability leading to the development of postweaning diarrhoea in the pig (359,360). Therefore this study will enable correlations between markers of tight junction function and plasma ions to be studied.

Four groups from the already completed Schothorst study will be used to test this hypothesis – offspring of both low and median birth weights within their litter, born to sows fed a control or fat supplemented diet. Offspring were sampled at two time points: 7 days and 6 months, therefore longer-term effects on the intestine can be studied.

5.3. Materials and methods

Tissue from the already completed Schothorst study, described in section 2.3. Was used to test the above hypothesis. All animal work was carried out prior to the start of this project (213,214). Tissue samples and data were made available for laboratory work and further analysis respectively. The animal work was carried out at Schothorst Feed Research in the Netherlands, and all laboratory procedures were carried out in the Division of Child Health, Obstetrics and Gynaecology, and the School of Veterinary Medicine and Science, University of Nottingham as described in chapter 2.

5.4. Results

5.4.1. Maternal growth data

Maternal body weights were unaffected by the fat-supplemented diet throughout gestation (Table 5-1).

	Control (n=8)	Fat supplemented (n=8)
Gestational day -4		
Body weight (kg)	215.5±13.42	209.9±9.25
Back fat (mm)	13.9±0.86	13.8±0.59
Gestational day 40		
Body weight (kg)	236.6±11.76	232.6±9.61
Back fat (mm)	15.6±1.01	15.5±0.63
Gestational day 70		
Body weight (kg)	257.8±10.82	254.6±8.60
Back fat (mm)	16.4±1.26	16.9±0.56
Gestational day 108		
Body weight (kg)	287.8±10.47	281.9±8.70
Back fat (mm)	17.3±1.27	16.7±0.46

Table 5-1: Maternal weights and back fat measurements throughout gestation.

All values are mean±SEM.

5.4.2. Maternal fasted plasma glucose, insulin and triglycerides

Gestational diet did not have a significant effect on maternal glucose or triglycerides through gestation.

Gestational day	Gestational diet	Glucose (mM)	Triglyceride (mM)
-4	C (n=5)	3.9±0.57	1.5±0.30
	F (n=8)	3.6±0.51	1.6±0.20
40	C (n=8)	3.9±0.12	1.3±0.09
	F (n=8)	4.1±0.08	1.5±0.32
70	C (n=8)	3.9±0.10	1.2±0.05
	F (n=8)	4.0±0.10	1.4±0.13
108	C (n=8)	3.9±0.10	1.4±0.13
	F (n=8)	3.7±0.17	1.4±0.15

Table 5-2: Maternal fasted plasma glucose and triglycerides (TAG) throughout gestation.

All values are mean±SEM. Number of C sows at gestational day -4 is reduced due to blocked catheters or problems inserting catheters into the ear during sampling.

Maternal fasted plasma insulin was measured at 108 days gestation, no significant effect was observed as a result of maternal diet: C=0.46±0.01; F=0.48±0.01 µg/ml.

5.4.3. Maternal glucose tolerance tests (GTT)

Area under the curve (AUC) for maternal plasma glucose following GTT at 108 days gestation was significantly increased in those fed the fat supplemented diet, indicating reduced glucose tolerance compared to controls (Figure 5-1).

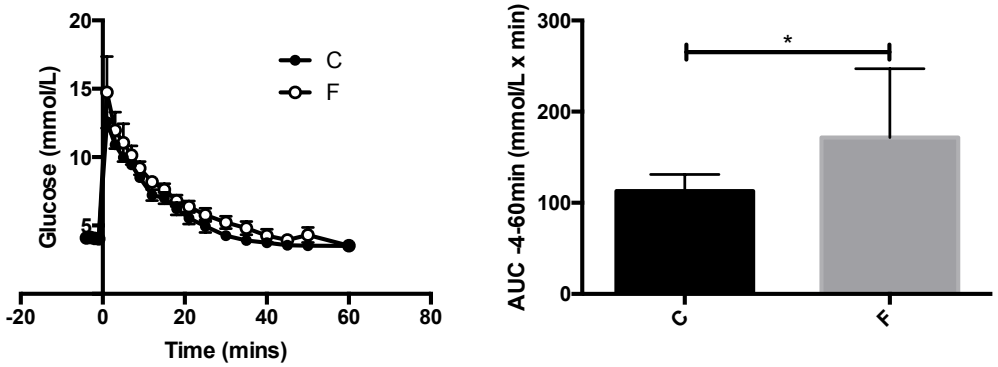


Figure 5-1: Maternal GTT (L) and area under the curve (AUC, R) for glucose in C (n=8) and F (n=8) sows at day 108 of gestation.

All values are mean± SEM, *p<0.05, Unpaired t-test.

5.4.4. Maternal milk composition at 2 days lactation

At 2 days lactation percentage fat, lactose and protein in milk was not significantly affected by maternal diet.

Composition (%)	C (n=8)	F (n=8)
Fat	7.80±0.56	7.87±0.56
Lactose	5.35±0.17	4.85±0.33
Protein	5.55±0.24	6.32±0.51

Table 5-3: Sow milk composition at 2 days lactation.

All values are mean±SEM.

5.4.5. Offspring body composition and growth rates

Both gestational length (C: 117.4±0.32, F: 116.1±0.67 days) and average litter size were not significantly affected by the fat supplementation. Fat supplementation had no significant effect on offspring birth weight, or the distribution of birth weights within a litter. The number of males vs females within a litter was significantly higher in the F group (Table 5-4).

	Control (C, n=8)	Fat supplemented (F, n=8)
Mean litter size (n)	15±0.78	16±0.75
Males	7.0±0.59	9.0±0.65#
Females	7.9±0.85	7.4±0.68
Mean birth weight (kg)	1.22±0.04	1.25±0.07
Males	1.26±0.07	1.30±0.08
Females	1.16±0.03*	1.20±0.06

Table 5-4: Offspring characteristics at birth grouped by maternal diet.

Values are mean±SEM, or median±range where stated. *p<0.05 male vs female, #p<0.05, C vs FS, Unpaired t-test.

As expected given the study design, piglets allocated to the low birth weight (LBW) groups at birth were significantly lighter than their median counterparts, these differences were still apparent at 7 days of age. By 9 weeks of age LBW piglets had displayed catch-up growth and were no longer significantly lighter than controls. At 7 days, fat supplemented LBW piglets were significantly heavier than their control diet LBW counterparts (Table 5-5).

Maternal diet	Age at dissection	Birth weight Group	Birth weight (kg)	Weight at 9 weeks (kg)	Weight at euthanasia (kg)
Control	**7 days**	Low (n=8)	0.72±0.07	-	1.12±0.13
		Median (n=8)	1.25±0.06*	-	2.13±0.13*
	6 months	Low (n=7)	0.99±0.07	22.44±0.93	115.21±5.92
		Median (n=7)	1.37±0.05*	24.77±1.46	120.14±3.27
Fat supplemented	**7 days**	Low (n=8)	0.90±0.07	-	1.65±0.20#
		Median (n=8)	1.27±0.12*	-	2.61±0.25*
	6 months	Low (n=7)	1.05±0.10	23.85±2.23	115.9±5.06
		Median (n=7)	1.38±0.05*	27.33±2.20	125.7±4.81

Table 5-5: Offspring weights at birth, 9 weeks of age and euthanasia.

***p<0.05 low vs median weight, Unpaired t-test, #p<0.05 C vs F, Unpaired t-test. All values are mean±SEM.**

Fractional growth rates (FGR) were not significantly different between groups at 6 months of age, but there was a significant correlation between FGR and birth weight (R^2=0.84, p<0.01, Figure 5-2).

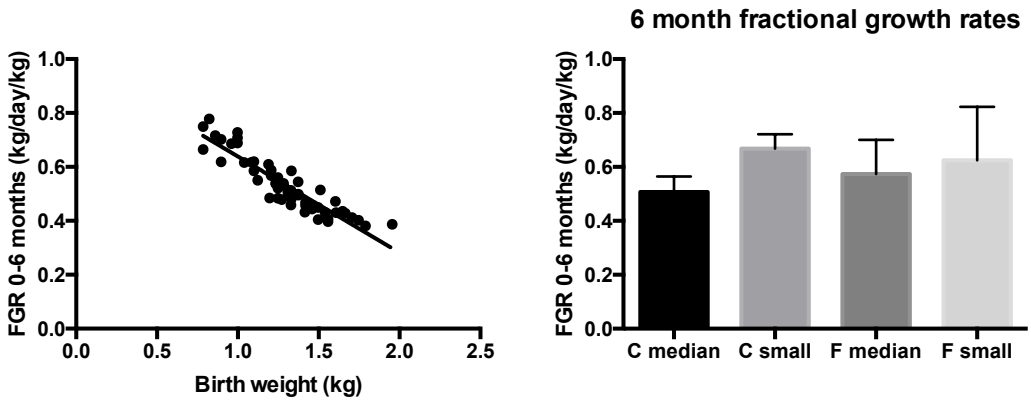


Figure 5-2: Correlation between fractional growth rate (0-6 months) and birth weight (L), R2=0.84, p<0.01) and 6 month fractional growth rates divided by group (R).

All values are mean±SEM.

Relative to total body weight, the offspring small intestinal weight was not significantly affected by maternal diet or size at birth (Figure 5-3).

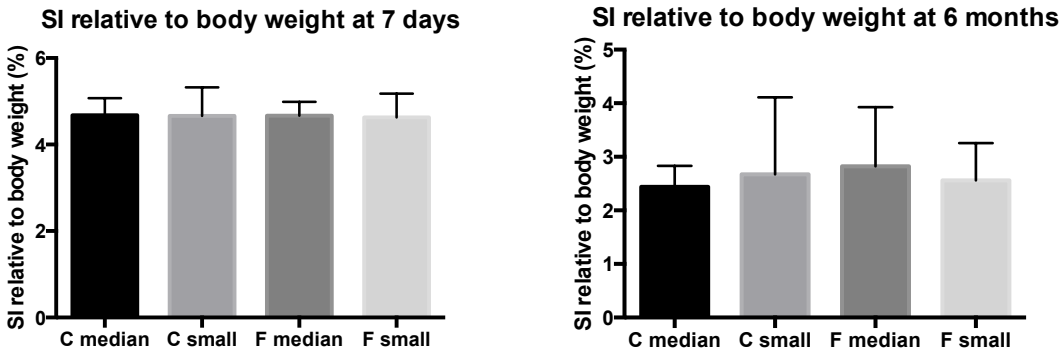


Figure 5-3: Small intestinal weights as a proportion of body weight in piglets euthanised at 7 days (L) and 6 months (R).

All values are mean±SEM. 7 day n=8/group, 6 months n=7/group.

5.4.6. Offspring back fat measurements at 6 months

Back fat measurements were taken from median birthweight piglets at 6 months of age not selected for euthanasia. No significant differences were observed between piglets born to sows consuming the control and fat supplemented diets.

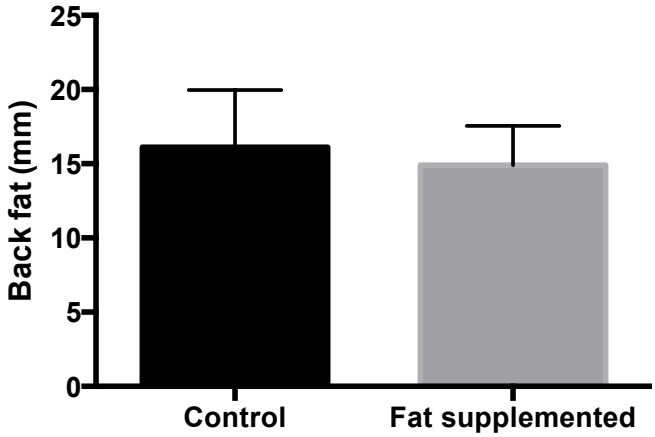


Figure 5-4: Back fat measurements from piglets at 6 months.

All values are mean ±SEM, n=8/group. Back fat was measured 5cm from the midline over the last rib by LeanMeater (Renco Corp.).

5.4.7. Offspring plasma glucose, insulin, triglycerides and non-esterified fatty acids

Offspring plasma glucose, insulin, triglycerides (TAG) and non-esterified fatty acids (NEFA) were not significantly different between groups at either time point measured (Table 5-6).

Maternal diet	Age at dissection	Group	Glucose (mM)	TAG (mM)	NEFA (mM)
Control	**7 days**	Low (n=8)	7.52±0.40	3.64±0.71	1.18±0.15
		Median (n=8)	7.97±0.23	3.16±0.58	0.99±0.11
	6 month	Low (n=7)	4.22±0.47	1.14±0.22	0.96±0.14
		Median (n=7)	4.71±0.41	1.26±0.09	0.94±0.15
Fat supplemented	**7 days**	Low (n=8)	7.37±0.46	4.12±1.27	1.14±0.24
		Median (n=8)	8.14±0.45	3.78±0.96	1.17±0.32
	6 month	Low (n=7)	4.23±0.31	1.51±0.12	0.79±0.17
		Median (n=7)	4.28±0.24	1.10±0.22	0.98±0.18

Table 5-6: Offspring fasted plasma glucose, TAG and NEFA at 7 days and 6 months of age, grouped by maternal diet and birth weight.

All values are mean±SEM.

Fasted plasma insulin was measured in offspring at 6 months, with no effect observed between the two maternal diet groups (Low: C: 0.16±0.03, F: 0.15±0.03, Median: C: 0.22±0.08, F: 0.22±0.08). As birth weight groups were compared using separate 96-well insulin plates, and the coefficient of variation (CV) of a sample included on both plates was considered unacceptably high (16%), it is inappropriate to compare these groups.

5.4.8. Gene expression of intestinal permeability markers in 7 day and 6 month offspring

Expression of OCLN and CLDN-3, but not ZO-1 was significantly reduced in F median piglets relative to C median controls at 7 days. No changes were observed in 6 month old piglets (Figure 5-5 - Figure 5-7).

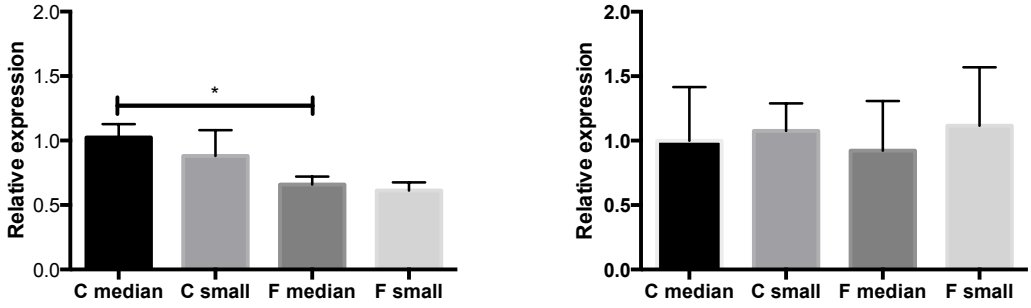


Figure 5-5: Occludin (OCLN) expression in the jejunum of 7 day (L) and 6 month (R) piglets born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

n=8/group. All values are mean±SEM, relative expression adjusted to the reference genes 18s and RPLP0. *p<0.05, ANOVA with contrasts (C median vs C small, C median vs F median, C small vs F small).

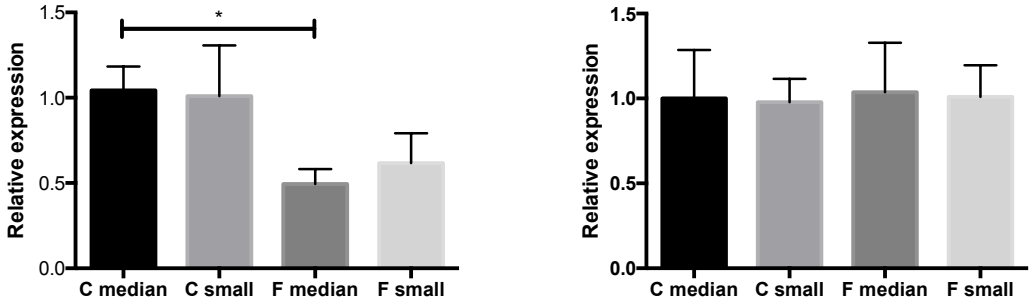


Figure 5-6: Claudin-3 (CLDN3) expression in the jejunum of 7 day (L) and 6 month (R) piglets born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

n=8/group. All values are mean±SEM relative expression adjusted to the referene genes 18s and RPLP0. *p<0.05, ANOVA with contrasts (C median vs C small, C median vs F median, C small vs F small).

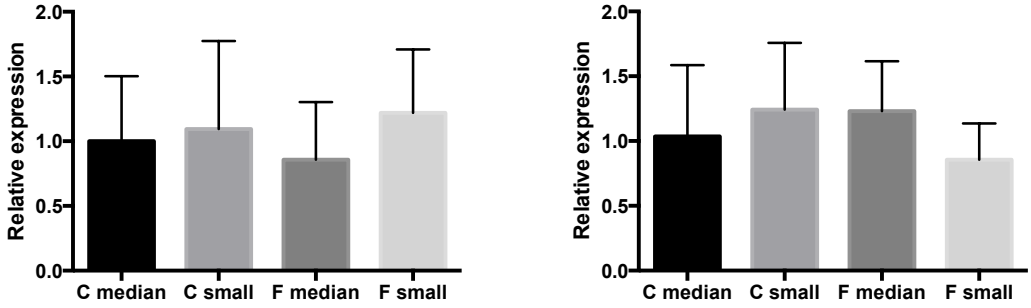


Figure 5-7: Zonulin-1 (ZO-1) expression in the jejunum of 7 day (L) and 6 month (R) piglets born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

n=8/group. All values are mean±SEM relative expression adjusted to the referene genes 18s and RPLP0. *p<0.05, ANOVA with contrasts (C median vs C small, C median vs F median, C small vs F small).

5.4.9. Expression of inflammatory genes in the small intestine of 7 day offspring

Expression of EGFr was significantly reduced in both C small and F median offspring relative to C median at 7 days (Figure 5-8). pBD2 and TLR4 were not affected by maternal diet or birth weight (Figure 5-9 - Figure 5-10).

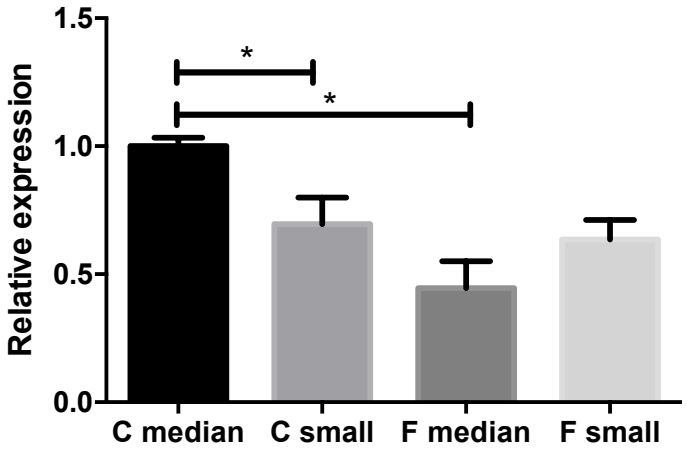


Figure 5-8: Epidermal growth factor receptor (EGFr) expression in the jejunum of 7 day old piglets born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

n=8/group. All values are mean±SEM relative expression adjusted to the referene genes 18s and RPLP0. *p<0.05, ANOVA with contrasts (C median vs C small, C median vs F median, C small vs F small).

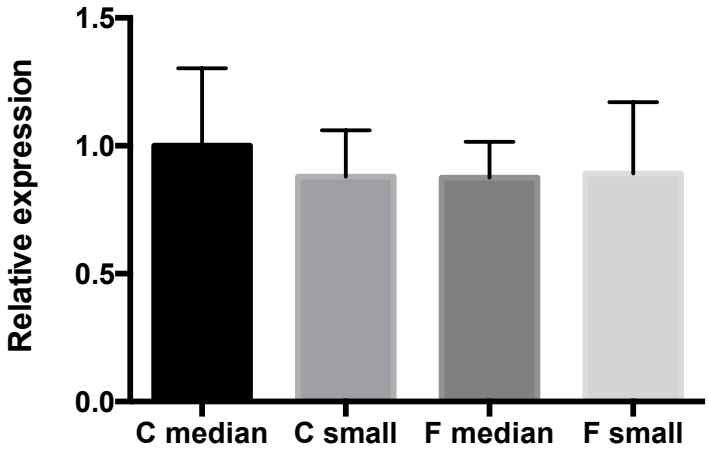


Figure 5-9: Porcine beta defensin 2 (pBD2) expression in the jejunum of 7 day old piglets born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

n=8/group. All values are mean±SEM relative expression adjusted to the referene genes 18s and RPLP0. *p<0.05, ANOVA with contrasts (C median vs C small, C median vs F median, C small vs F small).

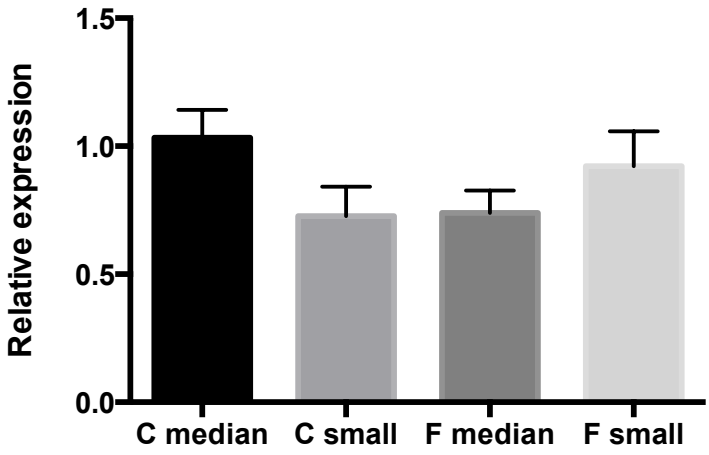


Figure 5-10: Toll-like receptor 4 (TLR4) expression in the jejunum of 7 day old piglets born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

n=8/group. All values are mean±SEM relative expression adjusted to the referene genes 18s and RPLP0. *p<0.05, ANOVA with contrasts (C median vs C small, C median vs F median, C small vs F small).

5.4.10. Intestinal glycogen accumulation in 7 day offspring

Intestinal tissue glycogen was not significantly different when comparing maternal diet or birth weight (Figure 5-11).

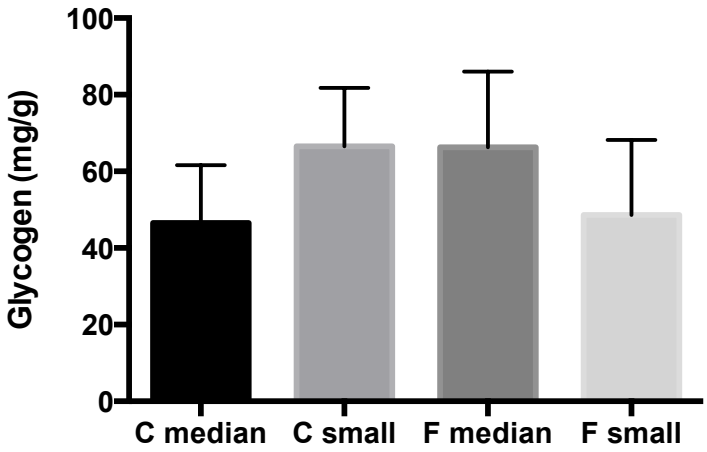


Figure 5-11: Intestinal tissue glycogen in 7 day offspring born to sows fed control (C) or fat supplemented (F) diets and grouped by birth weight.

All values are mean±SEM mg glycogen per gram of small intestinal (jejunum) tissue.

5.4.11. Plasma chloride, potassium and sodium at 7 days

At 7 days, plasma chloride and sodium were significantly higher in C small and F small piglets relative to their median weight counterparts (Table 5-7). Expression of EFGr, CLDN-3, OCLN and ZO-1 were not significantly correlated with plasma chloride, potassium or sodium (Figure 5-12).

	C median (n=8)	C small (n=8)	F median (n=8)	F small (n=8)
Chloride (mmol/L)	88.7±4.4	107.4±3.0*	95.1±8.8	109.1±3.4
Potassium (mmol/L)	2.5±0.2	2.9±0.2	2.6±0.2	2.9±0.2
Sodium (mmol/L)	96.0±10.2	145.9±4.1*	104.3±11.4	147±5.4*

Table 5-7: Plasma ions in piglets at 7 days of age.

All values are mean±SEM. *p<0.05, ANOVA with contrasts, C median vs C small and F median vs F small.

Across all four groups, significant correlations between EGFr and the three intestinal permeability markers studied were observed: EGFr and CLDN-3 (R=0.45, p=0.021), ZO-1 (R=0.48, p=0.014), and OCLN (R=0.58, p=0.003). Expression of CLDN-3 and ZO-1 was also significantly correlated (R=0.41, p=0.033).

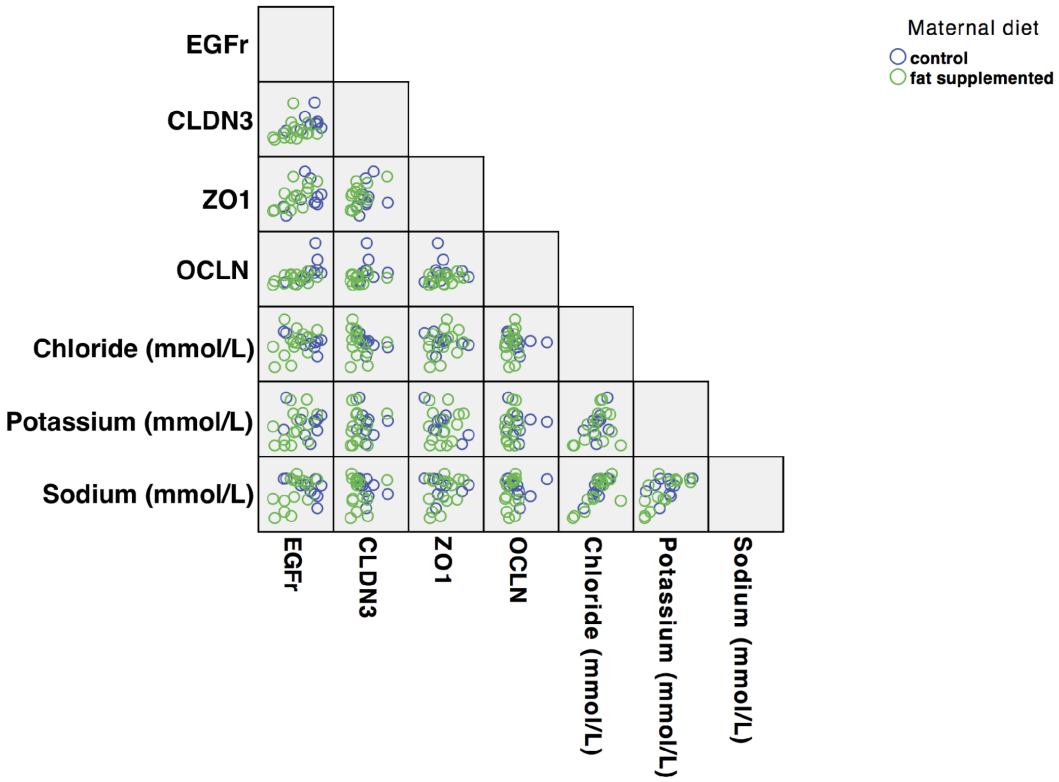


Figure 5-12: Correlations between plasma ions (sodium, chloride and potassium) and expression of EGFr, CLDN-3, ZO-1 and OCLN in the jejunum of piglets at 7 days grouped by maternal diet.

5.4.12. Plasma chloride, potassium and sodium at 6 months

At 6 months, plasma chloride, potassium and sodium were not significantly affected by birth weight or maternal diet (Table 1-8).

	C median (n=8)	C small (n=8)	F median (n=8)	F small (n=8)
Chloride (mmol/L)	100.9±1.7	98.7±2.5	91.6±3.7	103.7±2.4
Potassium (mmol/L)	4.7±0.2	4.5±0.2	4.0±0.3	4.8±0.3
Sodium (mmol/L)	135.9±2.3	135.7±4.5	117.9±8.2	137.0±4.0

Table 5-8: Plasma ions in piglets at 6 months of age. All values are mean±SEM.

At 6 months, expression of OCLN was significantly correlated with plasma potassium (Pearson's R 0.449, p=0.047). There were no significant correlations in gene expression between any of the genes tested (Figure 5-13).

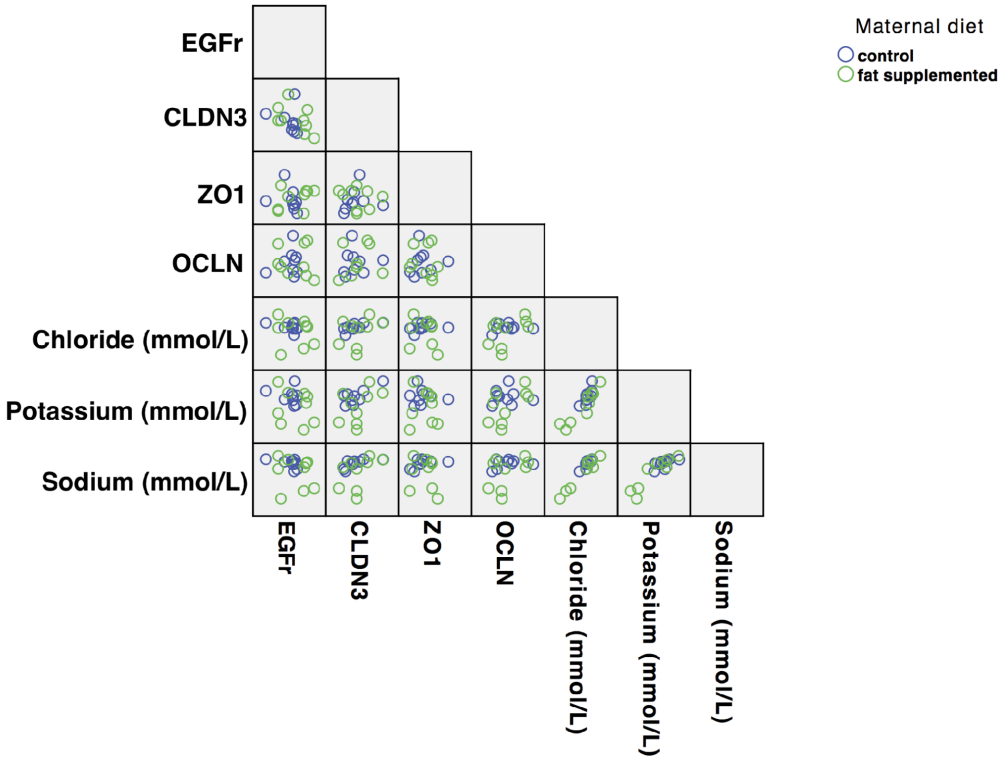


Figure 5-13: Correlations between plasma ions (sodium, chloride and potassium) and expression of EGFr, CLDN-3, ZO-1 and OCLN in the jejunum of piglets at 6 months grouped by maternal diet.

5.5. Discussion

5.5.1. The effect of fat supplementation on maternal glucose tolerance and metabolism

Insulin resistance and a reduced glucose tolerance has previously been demonstrated in response to pregnancy in humans (298–300), rats (33) and pigs (34,361). This is thought to be a response to the gradually increasing energy demands of the fetus, and results in glucose tolerance being at its lowest at the end of gestation. Studies using high fat diets in rats (356,362) and pigs (363) have shown that fat supplementation exaggerates these effects and further reduces glucose tolerance. The maternal glucose tolerance test values observed in my study are similar to others conducted in pigs, suggesting that palm oil supplementation negatively affects glucose tolerance. Van der Peet-Schweering *et al.* (363) supplemented sows with 9.6% total calories from fat (vs 5.2% in controls) from day 85 of gestation and saw similar GTT results to my study at 108 days gestation. Despite both this study and my own showing a reduction in glucose tolerance, the AUC and peak glucose measured in my study is markedly higher than that observed by van der Peet-Schweering *et al.* This is likely due to the milder glucose challenge used relative to my study, or the shorter dietary intervention, with diets starting at 85 days gestation (363).

In contrast to a study supplementing rat dams with a high fat diet (20% w/w lard added to the control diet, with additional protein, vitamins and minerals to account for the dilution), which showed significant increases in fasted plasma insulin (364), no significant changes were observed in my study at 108 days gestation. Due to the constraints of conducting the study within a commercial setting it was not possible to measure insulin during the GTT or throughout gestation.

5.5.2. The effect of maternal fat supplementation on offspring metabolism

The role of maternal fat supplementation on metabolism in the offspring has not been well studied. Clinical studies in mothers with gestational diabetes have shown an increased risk in the offspring of developing diabetes and/or obesity (341,342). However in these cases the offspring are usually born macrosomic, thought to be caused by maternal hyperglycaemia (365,366), or increased lipid supply to the fetus (367). Studies using maternal high fat diets in rats demonstrate development of insulin resistance and hypertension in the offspring with no change in birth weights (35,37,356). In contrast, my study demonstrated no changes in offspring metabolic profile, with plasma glucose, insulin, triglyceride and NEFA unchanged between diet groups. Rat studies generally measure the weight of all adipose depots, and have demonstrated a significant increase in abdominal fat relative to other depots (36,37), whereas in this study only back fat was measured, which may explain the failure to detect any significant differences between groups.

Differences in diet composition also explains in part the contrasting results between my study and those carried out in rats. High-fat diets used in rat studies are generally the control diet supplemented with lard (37,356), resulting in a diet high in saturated fatty acids. Supplementation of saturated fats has been shown in humans (368–370), rats (362,364), and pigs (371) to decrease insulin sensitivity and increase the risk of developing obesity. In porcine studies dietary supplementation of fat is usually achieved by addition of soy bean or corn oil (372–375), which contain a higher ratio of polyunsaturated (PUFA) to saturated fatty acids. In humans, studies of diets containing high amounts of the PUFAs linoleic and linolenic acid relative to saturated fatty acids have been shown to reduce the risk of developing cardiovascular disease in humans (370). Therefore this may also explain the difference in the results of this study, with palm oil containing an equal ratio of unsaturated and saturated fatty acids.

Finally, the length of feeding and overall macronutrient composition of the fat supplemented diet may be important. The above rat studies (35,37,364) dramatically increase the energy content of the diet, with reduced intake in the dams making the diets effectively isocaloric but also resulting in a reduced protein intake (as discussed in section 1.5). In my study, protein intake between control and fat supplemented groups was identical, potentially explaining the lack of adverse metabolic effects observed in the offspring. The effect of high-fat feeding in lactation alone has been demonstrated (37), with rats born to dams fed a control diet and cross-fostered to high-fat fed dams during suckling becoming hyperinsulinaemic and displaying reduced endothelial function. As all sows in this study were switched to the control diet after birth, this may reduce the effect of the high-fat diet on offspring development.

5.5.3. The effect of maternal fat supplementation on early small intestinal development

Despite the lack of changes in growth rate and effects on the weight of the small intestine, a significant reduction in EGFr expression was observed in both median weight offspring born to sows fed the high fat diet and low birth weight offspring born to sows fed the control diet. Reduced EGFr has been hypothesised to be a contributing factor to the development of intestinal inflammation in growth restricted and preterm newborns (376,377). Enteral administration of EGF in neonatal rats also reduces the incidence of NEC (155). The same study also showed a significant increase in EGFr mRNA abundance within damaged sections of ileum studied, relative to healthy tissue. This suggests that a reduction in EGFr expression will inhibit the intestinal response to inflammation.

Whereas the effect of growth restriction on EGFr expression has been previously demonstrated (89), I observed that a maternal high-fat diet

had an effect independent of birth weight. Research into the effect of fat and individual fatty acids on EGFr expression in the small intestine is limited. Cell culture models suggest however that incorporation of unsaturated fatty acids into lipid rafts on cell membranes reduces gene expression of EGFr (378,379). If these findings can be extrapolated to the small intestine they may provide a mechanism for dietary fat supplementation having a similar response. Although no significant results were found in the milk analysis with regard to total fat content (Table 5-3), more detailed milk analysis may be worthwhile to determine the effect of specific fatty acids on EGFr expression.

Two of the three genes coding for epithelial tight junction proteins (OCLN and CLDN-3) were reduced in the F median piglets at 7 days compared to controls. Deletion of a CLDN isoform in mice (380), and enhancing ZO-1 expression in pigs (125) increase or reduce intestinal permeability respectively. Feeding of a high fat diet increases intestinal permeability in mice, leading to endotoxaemia (321). The authors hypothesised that this was due to the reduction in epithelial tight junction proteins. Antibiotic treatment reversed these changes and reduced expression of inflammatory markers TNF-α and interleukin-1, and the macrophage marker monocyte chemotactic protein 1 (MCP1). These findings suggest that the microbiota plays a role in the mediation of intestinal permeability (321). Feeding of a high-fat diet has been shown to reduce the abundance of *Bifidobacteria* (321,358), which have been shown to decrease intestinal LPS (381,382) and intestinal permeability (383). Therefore the findings in my study are in keeping with the above, and show that exposure to a high-fat diet during gestation has similar effects on intestinal permeability. However further analysis of the microbial population in the mother and offspring would be required to confirm this.

A high-fat diet in the mother has been shown to affect the microbiome in offspring switched to a control diet at weaning (384), providing a potential route for maternal diet to modulate offspring intestinal development

independently of breast milk. Studies using cultured kidney cells treated with EGF raise their permeability (measured by transepithelial resistance) through increasing the expression of various claudin isoforms (385). Therefore it is still unclear whether diet, the microbial population, a reduction in expression of EGFr, or a combination of all three, mediates the changes in tight junction gene expression observed in my study.

Aside from reduced EGFr expression, low birth weight piglets showed lower expression of tight junction genes but this was not statistically significant. The role of growth restriction in adversely affecting small intestinal development has been previously studied (84,85). Low birth weight piglets also displayed accelerated growth (Figure 5-2), suggesting that neither birth weight or growth rate have a significant effect on the chosen markers of intestinal permeability.

Correlation of expression of tight junction genes has not been previously demonstrated, but is expected given that regulation of these genes has been previously shown to be closely coupled with zonulin-1 providing a link between occludin and the cytoskeleton (119).

5.5.4. The effect of maternal fat supplementation on the small intestine in 6 month old offspring

Weaning has been shown to induce a cluster of maturational changes in the GI tract, although the extent of these changes is debated. Species born with a relatively immature GI tract such as rats and mice display much more pronounced changes at weaning relative to humans and other large mammals (89). In pigs, weaning may cause persistent changes in intestinal permeability and is a significant cause of growth stasis in commercial settings (386). This could be one factor contributing to increased mortality caused by post-weaning diarrhoea (387). These adverse effects are thought to be caused by the increase in expression of inflammatory cytokines (including IL-1β, IL-6 and TNF-α) in the small intestine at

weaning (388). No mechanism has been proposed for this, however in pigs it is likely a part of the stress response to the abrupt change in diet, as well as separation from the sow, a change in environment and mixing with non-littermates. 2D-gel electrophoresis followed by mass spectrophotometry in protein extracts taken from the jejunum of IUGR piglets at weaning showed a significant increase in expression of proteins related to apoptosis and oxidative stress, and a decrease in those related to absorption and metabolism of carbohydrates, lipids and amino acids (389). This suggests that growth restriction will reduce the ability of the small intestine to respond to the stresses associated with weaning.

In my study I have shown that both maternal diet and offspring birth weight have no effect on the markers of intestinal permeability tested. This is consistent with similar research in pigs showing weaning upregulated several mucosal enzymes and glucose absorption, without changing intestinal permeability (390). Currently there is a lack of research that follows up potential weaning-related changes in the small intestine, with all of the above studies taking tissue samples no later than 28 days. Taken together, the findings at 7 days and 6 months in my study suggest that the significant effects on intestinal permeability markers observed at 7 days are likely to be transient, but further time points would be necessary to determine if the normalisation in tight junction gene expression at 6 months occurs as a result of weaning.

5.5.5. Correlation of tight junction gene expression and plasma ions

At both ages the expression of EGFr and the tight junction genes measured was not significantly correlated with plasma sodium, potassium or chloride ions. Based on the existing literature, a decrease in the function of tight junctions would lead to increased permeability through "leak pathways" (391,392), leading to an increase in various plasma metabolites, including sodium and potassium. Transfection of human claudin-4 into cultured kidney (MDCK) cells lead to a decrease in the

passage of sodium, but not chloride ions across the epithelial membrane (393).

There are a number of potential explanations for the lack of observed correlations in my study. Firstly the change in tight junction permeability in the small intestine may not be drastic enough to alter the transfer of ions into plasma. Although changes in plasma ion concentration have been reported in cases of postweaning diarrhoea (394), it is likely that any changes in intestinal ion transport are being corrected by the kidneys. Therefore analysis of the ion concentration of luminal contents would be beneficial but was not possible in my study. Secondly, although expression of tight junction genes has been correlated with direct measurement of permeability via Ussing chamber (167,395), this work is confined to cell culture studies. Therefore protein expression of the genes studied may be required to more accurately determine the effect of a reduction in tight junction gene expression. Tight junctions have been shown in some cases to be regulated by post-translational modifications such as phosphorylation (396,397), making study of protein expression of further interest.

5.5.6. Summary

In conclusion, this study has shown that supplementation of the maternal diet with fat will significantly reduce maternal glucose tolerance and affect the offspring intestine in early life. The pig is regarded as an applicable model for the human intestine, particularly with regard to growth restriction (106), therefore these findings are potentially of relevance to humans. The reduction in expression of EGFr in low birth weight pigs may be of relevance to growth restricted or preterm human infants, with a reduction in EGFr resulting in a lack of response to inflammation at a time when the small intestine is most vulnerable (376,398). However these findings currently lack a mechanism, the maternal high-fat diet did not appear to induce significant accelerated growth, which has previously

been shown to lead to small intestinal dysfunction (89,357), and no significant fat increase was observed in the sow's milk during lactation. The changes observed at 7 days do not appear to persist at 6 months of age, suggesting that the changes that occur in the intestine at weaning are drastic enough to compensate for the adverse effects seen at 7 days (386,390). Studies linking the microbiome, a high-fat diet and intestinal barrier failure (321) make following-up this work with microbial analysis of either faecal samples or the mucosal layer of the small and large intestine of interest. Further study into whether exposure to LPS or other components of the microbiota can program the immune system, thereby increasing the risk of autoimmune disease is of interest, as such interactions have been demonstrated in the development of conditions such as type II diabetes (399,400).

Chapter 6: Conclusions

6.1. Aims

The overall aim of this work was to study the effect of macronutrient supplementation during pregnancy, and to study their effects on gastrointestinal development in the offspring. Two studies were used: a rat model of fructose supplementation, and a porcine model of fat supplementation. In both studies the small intestine was the focus, and primarily the use of gene and protein expression analysis was utilised to study the function of epithelial tight junctions, inflammatory response and nutrient transport. In the rat study tissue was taken from both the mothers and offspring, to enable the effect of a high-fructose diet to be studied in two generations. In both models the metabolic profile of both mothers and offspring was studied to observe how the respective diets may lead to the development of the metabolic syndrome. This chapter will summarise the conclusions from both studies carried out and highlight similarities and inconsistencies with the existing literature, as well as limitations of the work.

6.2. Summary of findings

6.2.1. The effect of fat supplementation in pregnancy on metabolism in the mother and offspring

Despite the significant reduction in sow glucose tolerance when subjected to the fat-supplemented diet, which are consistent with the literature (363), no significant changes were observed in offspring at either 7 days or 6 months of age. In rats, fat supplementation has been shown to induce hypertension, obesity and impaired glucose tolerance (35,37,356), and in humans metabolic dysregulation has been demonstrated independently of a high-fat diet, in offspring born to mothers with impaired glucose tolerance (341,342). Potentially this is due to the lower level of fat

supplementation in my study relative to the above work in rats, with the above studies using diets containing ~25% fat as opposed to the 9% used in my study.

6.2.2. The effect of growth restriction on offspring gastrointestinal development

IUGR and preterm infants are at increased risk of developing gastrointestinal infection and inflammation (401,402), with evidence suggesting the GI tract is preferentially affected by growth restriction relative to other major organs (99). With pigs providing a model of naturally occurring growth restriction (344–346), an objective of my study was to use two of the offspring groups classed as low birth weight within their respective litters to observe the effect of growth restriction on the small intestine.

A significant drop in EGFr expression was observed in LBW piglets born to sows consuming the control diet at 7 days of age, and a non-significant reduction was observed in LBW piglets born to sows consuming the fat-supplemented diet. To my knowledge this has not been previously demonstrated in this model, but is consistent with previous work in which administration of EGF, (which leads to upregulation of the receptor (403)), will ameliorate intestinal inflammation in IUGR and preterm pigs (156) and rats (155). The reduction in EGFr observed in my study therefore provides a potential mechanism by which growth restriction leads to increased risk of small intestinal inflammation.

Over 6 months, growth restricted piglets displayed increased growth rates, however no significant changes in gene expression were observed in these animals. This suggests that although the changes shown above may increase susceptibility to inflammation in early life, they do not persist beyond weaning.

6.2.3. The effect of fat supplementation in pregnancy on gastrointestinal development

At 7 days, median birth weight piglets born to sows fed the high fat diet showed similar changes in EGFr expression as LBW piglets discussed above. These piglets also displayed a significant reduction in expression of several tight junction genes, indicating an increase in intestinal permeability. In rats, a high fat diet (achieved by reducing carbohydrate) has been shown to increase intestinal permeability, thought to be a result of a reduction in the abundance of *Bifidobacteria* (321). Although identical effects have been demonstrated in my study, this mechanism is impossible to prove with this model, as fecal samples, or samples of the intestinal mucosa were not taken. Additionally, analysis of the milk taken from sows in the study suggests no change in total fat composition, however more detailed analysis of the fatty acid composition may be worthwhile.

As described in the previous section, no changes were observed at 6 months. This suggests that the changes in the intestine at weaning are likely "re-setting" tight junction function. My initial hypothesis based on work suggesting increased intestinal permeability could predispose to the development of obesity (87), has therefore not been proven, and in this model it is likely that the increased permeability is not drastic enough to induce any metabolic dysfunction, and is no longer present at 6 months of age.

6.2.4. The effect of fructose supplementation in pregnancy

The aim of the work conducted as part of the SM study (chapters 3 and 4), was to study the effect of fructose supplementation first on small intestinal adaptation to pregnancy, followed by intestinal adaptation in a subsequent offspring pregnancy. Previous studies have shown that supplementation of 10% fructose during pregnancy can lead to impaired

glucose tolerance in the offspring (259,260), however there has been no work investigating offspring over a longer term into their own pregnancies, and none focusing on the intestine. This is potentially of interest as high fructose intake has been shown to adversely affect the intestinal barrier (174), and alter the metabolic capacity of the microbiome in a way that may contribute to the development of obesity (328).

My study demonstrated a small reduction in glucose tolerance in first generation dams subject to the fructose supplementation, with offspring continuing on the fructose diet clearly displaying insulin resistance. Rats born to fructose-fed mothers displayed growth restriction, and similar to the growth restricted pigs displayed catch-up growth, reaching an equivalent weight to control counterparts by 14 weeks of age. Second generation fructose-fed rats displayed a significant reduction in several markers of intestinal permeability, potentially indicating a reduction in gut barrier function. Further study of expression of inflammatory markers showed no change in TLR4 or CD68 expression, which have previously been associated with an increase in intestinal permeability leading to liver and adipose tissue inflammation (87,267). This suggests that either expression of intestinal tight junction genes does not correlate with actual permeability, or that the fructose intervention is required over a longer period of time before these changes are noticeable.

Fructose has been shown to regulate the carbohydrate transporters GLUT2 and GLUT5 (264,404,405), suggesting that consumption of a diet high in fructose will lead to an increase in absorptive capacity of carbohydrates. My study has demonstrated similar results, with an upregulation in the three carbohydrate transporters studied, to my knowledge this has not been shown previously with a relatively low-level supplementation of fructose in the diet, as opposed to direct perfusion of the small intestine with fructose (265) or a much higher concentration of fructose in the diet or drinking water (263). However, the speed of the

response of carbohydrate transporters to changes of carbohydrate in the diet is currently debated, with studies showing a upregulation in uptake in response to glucose within hours (63) or 1-2 days (406), and the effect of diurnal rhythm (407,408) therefore the study of additional time-points would be beneficial.

In addition to the above physiological and metabolic changes, the results from the microbiome sequencing presented in chapter 5 demonstrate that a fructose diet will lead to profound shifts in microbial diversity. This results in a more diverse microbiome, likely due to the increase in abundance of fructose digesting species. These results complement the changes in abundance of carbohydrate transporters above, and suggest that the small intestine adapts to an increased intake of fructose. This may lead to a gradual worsening of the metabolic effects of fructose as its availability for intestinal absorption increases.

6.3. Limitations and future work

6.3.1. SM study

In comparison with the Schothorst study in which it was possible to analyse tissue from two time-points, the design of the SM study only allowed for tissue collection at a single time-point. As some of the work conducted, particularly that on nutrient transporters, has been previously demonstrated to be subject to diurnal variation (404,407), additional sampling time-points would be beneficial to either study or rule out the effect of this variation. Further sampling would also have been beneficial with regard to studying the microbiome; research in mice has shown diet induced changes in the microbiome can be transient (409), therefore further time-points, such as those after birth would enable the degree and speed of this transience to be measured. The design and cost constraints of pyrosequencing prevented these further time-points from being studied.

In order to study developmental effects of fructose in a single generation, as opposed to offspring continuing on an identical diet, a crossover design could have been employed. By switching offspring born to fructose-fed mothers on to a control diet and studying their metabolism and GI adaptation, further insight could be gained into the effects of fructose in pregnancy.

In order to more fully study the effects of an increase in intestinal permeability leading to microbe-induced inflammation, other tissues such as adipose depots could be studied for inflammatory response. The study of plasma LPS would also be beneficial in confirming this hypothesis, however this was not attempted due to the difficulties in accurately measuring LPS in plasma (317). Direct measurements of intestinal permeability, such as those obtained using an Ussing chamber, would also help to confirm the hypothesis that a high fructose diet is inducing increased permeability.

The viscosity of the 10% fructose solution used in the SM study resulted in frequent leaking of the water bottles, and contributed to inaccurate measurements of water intake during the 24-hour measurement periods. Therefore whilst the measurements of fructose intake in chapter 2 appear to show a clear increase in water consumption in rats on the fructose diet, these measurements should be interpreted with caution. Further studies using metabolic cages would provide a more accurate measure of energy intake and expenditure. Although rats fed fructose showed a marked decrease in chow consumption, nutritional requirements (216) were still met, and no signs of protein deficiency were observed.

6.3.2. Rats as a gastrointestinal model

Despite their widespread use as an animal model (discussed in section 1.20.1) rats have a markedly short gestation (~3 weeks), and are born with a GI tract that is much less well developed compared to a human or pig. Rats display the same critical windows of gut development occurring around birth and at weaning but maturation occurs at a much faster rate, leading to a GI tract that is fully mature at around 5 weeks of age (89). Therefore studying the small intestine at multiple time-points (for example pre-weaning at around 20 days of age) would have been worthwhile to compare the rapid intestinal development in the rat with the slower maturation in the pig. Specific differences between rat, human and porcine GI development and function have not been well studied, however changes in absorption rates of various compounds have been documented between rats and humans (410).

6.3.3. Schothorst study

Samples taken from the Schothorst study were not fixed for histological preparation, and attempts to thaw frozen samples for analysis were unsuccessful (section 2.15.4), this meant that measurement of villus height, an important indicator of intestinal health, was not possible. Additionally, work studying the localisation of tight junction proteins using immunohistochemistry would be of value in this model, as the location of various tight junction proteins within the junction itself has been demonstrated to change in disease states (411,412).

As discussed in section 5.4.5, the sex ratio in the Schothorst study is unequal, with fat supplemented sows having significantly more male offspring. Within the LBW groups there was also a significantly higher number of females, due to the higher postnatal mortality displayed by LBW males in the first days of life. Therefore the possible role of sex in the changes observed in the study cannot be discounted. Specific mechanistic

reasons for this postnatal mortality have not been covered in the literature but similar cases been previously demonstrated (413). Male piglets generally have higher birthweights compared to females (413), therefore the fact that lower birthweight male piglets display a higher rate of mortality suggests that they are less able to adapt to postnatal life compared to their lower birthweight female counterparts. Studying the role of sex in this model would potentially be of interest, but would require a significantly higher number of animals than that used to achieve the balance of male and female offspring, the amount of wastage involved in such a study would be difficult to justify ethically.

6.4. Final remarks

My thesis has demonstrated significant developmental effects of maternal diets supplemented with both fat and fructose. Supplementation with fructose in a rat model over two generations will lead to significant microbial adaptation in the mother, and potentially alterations in carbohydrate transport. Offspring displayed significant reductions in markers of intestinal permeability. Fat supplementation in a pig model lead to a similar reduction in markers of intestinal permeability in 7 day old offspring which was no longer present at 6 months. These findings may have implications for humans consuming a high amount of fat and fructose as part of the Western diet, and may aid in understanding programming effects of the maternal diet on disease risk in the offspring.

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Appendices

Appendix A: Paper abstracts

Astbury S, Mostyn A, Symonds ME, Bell RC (2015) Nutrient availability, the microbiome and intestinal transport during pregnancy. *Applied Physiology, Nutrition, and Metabolism* (in press, doi: 10.1139/apnm-2015-0117).

Adequate adaptation of the gastrointestinal (GI) tract is important during pregnancy to ensure that the increased metabolic demands by the developing fetus are met. These include changes in surface area mediated by villus hypertrophy and enhanced functional capacity of individual nutrient receptors including those transporting glucose, fructose, leucine, and calcium. These processes are regulated either by the enhanced nutrient demand or are facilitated by changes in the secretion of pregnancy hormones. Our review also covers recent research into the microbiome, and how pregnancy could lead to microbial adaptations, which are beneficial to the mother, yet are also similar to those seen in the metabolic syndrome. The potential role of diet in modulating the microbiome during pregnancy, as well as the potential for the intestinal microbiota to induce pregnancy complications are examined.

Gaps in the current literature are highlighted including those where only historical evidence is available, and we suggest areas that should be a priority for further research. In summary, although a significant degree of adaptation has been described, there are both well-established processes, and more recent discoveries such as changes within the maternal microbiome that pose new questions as to how the GI tract effectively adapts to pregnancy, especially in conjunction with maternal obesity.

Lineker C, Kerr PM, Nguyen P, Bloor, I, **Astbury S**, Patel N, Budge H, Hemmings DG, Plane F, Symonds ME, Bell RC (2015) High fructose consumption in pregnancy alters the perinatal environment without increasing metabolic disease in the offspring. *Reproduction, Fertility and Development* (in press, doi: 10.1071/RD15119).

Maternal carbohydrate intake is one important determinant of fetal body composition, but whether increased exposure to individual sugars has long-term adverse effects on the offspring is not well established. Therefore, we examined the effect of fructose feeding on the mother, placenta, fetus and her offspring up to 6 months of life when they had been weaned onto a standard rodent diet and not exposed to additional fructose. Dams fed fructose were fatter, had raised plasma insulin and triglycerides from mid-gestation and higher glucose near term. Maternal resistance arteries showed changes in function that could negatively affect regulation of blood pressure and tissue perfusion in the mother and development of the fetus. Fructose feeding had no effect on placental weight or fetal metabolic profiles, but placental gene expression for the glucose transporter GLUT1 was reduced, whereas the abundance of sodium-dependent neutral amino acid transporter-2 was raised. Offspring born to fructose-fed and control dams were similar at birth and had similar post-weaning growth rates, and neither fat mass nor metabolic profiles were affected. In conclusion, raised fructose consumption during reproduction results in pronounced maternal metabolic and vascular effects, but no major detrimental metabolic effects were observed in offspring up to 6 months of age.

Appendix B: Selected conference abstracts

Physiology 2015, Cardiff, United Kingdom (poster presentation)

Supplementation of the maternal diet with palm oil affects intestinal permeability in offspring at 7 days but not 6 months.

Stuart Astbury[1], Alison Mostyn[2], Don Sharkey[1], Michael E Symonds[1]
[1]*Early Life Research Unit, Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham*
[2]*School of Veterinary Medicine and Science, University of Nottingham*

Background and aims: Excess caloric load or a proinflammatory diet may contribute to gut barrier dysfunction, leading to increased nutrient uptake and components of the microbiota passing into the systemic circulation. These effects have been linked to the development of obesity and the metabolic syndrome (1,2). As the maternal diet has been shown to have a significant effect on offspring development (3), we used a porcine model of a fat supplemented diet to test the effect of maternal diet on offspring gut development at two timepoints.

Materials and methods: 16 sows were randomly assigned (n=8 per group) to a control diet of commercial feed (maternal control, mC) or commercial feed supplemented with palm oil (maternal high fat, mHF) from the first day of breeding, and continued with a gradually increasing amount of palm oil throughout gestation. Diets were made isocaloric by reducing the starch content in the palm oil supplemented diet. Sows farrowed naturally and their offspring were fed standard commercial diets regardless of maternal diet. All offspring were grouped based on diet and size at birth. The first group were dissected at 7 days (mC median n=6, mC small n=7, mHF median n=8, mHF small n=7), and the second at 6 months (mC median n=6, mC small n=4, mHF median n=6, mHF small n=5). All animals were sedated with intramuscular injection of a ketamine-xylazine combination before being humanely euthanised using T-61.

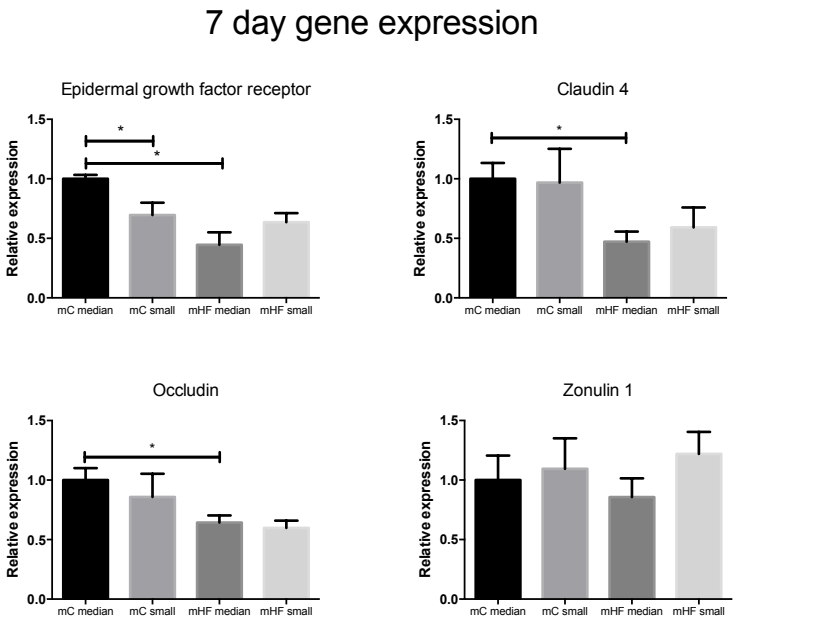
Three genes coding for proteins located on epithelial tight junctions were used as markers of gut barrier function: claudin-3 (CLDN3), occludin (OCLN), and zonulin-1 (ZO-1). Epidermal growth factor receptor (EGFr) was used as a marker of intestinal development. Quantitative PCR was used to measure mRNA abundance of the above genes in RNA extracted from the ileum. All data is presented as mean±SEM, with groups compared using Student's t-test or Mann-Whitney U test as appropriate.

Results: Birth weight and small intestine weight relative to total body weight were unaffected by maternal diet at both timepoints. Expression of CLDN3 and OCLN was significantly reduced in mHF median offspring at 7 days compared to mC median, EGFr was significantly reduced in both mC small and mHF median offspring compared to mC median at 7 days (see figure). ZO-1 expression at 7 days was unaffected by diet or size.

Expression of all four genes was not significantly different between groups at 6 months.

Conclusion: These findings demonstrate a high-fat diet during pregnancy may increase intestinal permeability in the offspring, and adversely affect intestinal development at an early age. However the changes observed at 7 days are no longer present in 6 month old pigs, suggesting that development of the small intestine taking place after 7 days, predominantly at weaning (4) may compensate for any adverse effects. Further work will explore whether other organs such as the liver are adversely affected by an increased intestinal permeability at 7 days.

Figure – 7 day gene expression



mC median n=6, mC small n=7, mHF median n=8, mHFsmall n=7
All values mean±SEM
***p<0.05, Student's t/Mann Whitney U test as appropriate**

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Obesity: A Physiological Perspective (Physiological Society Topic Meeting, 2014), Newcastle-upon-Tyne, United Kingdom (poster presentation)

Exposure to a high fructose diet during pregnancy remodels the intestinal and body composition in the near-term pregnant offspring.

Stuart Astbury, Aleida Song, Brent Nielsen, Nicole Coursen, Abha Hoedl, Rhonda C Bell
Division of Human Nutrition, Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada

Background and aims: Excess caloric load or a proinflammatory diet may contribute to gut barrier dysfunction, leading to increased nutrient uptake and components of the microbiota passing into the systemic circulation. These effects have been linked to the development of obesity and the metabolic syndrome1,2. Maternal diet and diabetes in pregnancy have been shown to adversely affect many aspects of offspring development. This study examined the effect of a high fructose diet during gestation on gut permeability in female, pregnant offspring.
Methods: Female Wistar rats were placed on either 10% fructose (f) or distilled water (c) at 8 weeks of age and were mated at 10 weeks, with diet continuing through gestation. The offspring were then maintained on the same diet as their dams (c, n=10 and f, n=10) starting from 1 week post-weaning. The offspring were then mated at 10 weeks of age, with tissue collected at gestational day 20 (GD20). Their body composition was determined by MRI directly prior to euthanasia. Ileum and jejunum sections were taken from the small intestine and snap-frozen followed by total RNA extraction for quantitative PCR. Four epithelial tight junction genes were used as markers of intestinal permeability: claudin-3 (CLDN-3), occludin (OCLN), junctional adhesion molecule A (JAMA) and zonulin-1 (ZO-1). Gene expression was measured relative to GAPDH and RPLP0. Groups were compared using t-test or Mann-Whitney U test as appropriate. All values are mean±SEM.
Results: Birth weight was significantly reduced in the f group (c=4.23g±0.5 vs f=3.94g±0.8,$p<0.05$). Adult weights were unaffected but lean mass reduced and fat mass raised (% lean c=75.8±0.3 vs f=71.4±1.0, $p<0.05$ and % fat c=11.4%±0.5 vs f=16.8%±1.3, $p<0.01$). Gut length was also reduced in the f group (c=128.5cm±1.8 vs f=123.8cm±0.4, $p<0.05$) and was accompanied with reduced gene expression ($p<0.05$) for JAMA (0.62±0.06), OCLN (0.44±0.08) and ZO-1 (0.39±0.05) in the jejunum, but not the ileum.
Conclusion: A high-fructose diet during pregnancy may adversely affect gut development in the offspring via a decrease in the expression of genes coding for a number of epithelial tight junction proteins. This response appears to be confined to the jejunum and may contribute to an increase

in energy uptake across the gut, thereby leading to an increase in fat mass at the expense of skeletal muscle.

Canadian Diabetes Association/Canadian Society of Endocrinology and Metabolism Annual Meeting 2014, Winnipeg, MB, Canada (poster presentation by Aleida Song)

Maternal high-fructose intake induces adverse effects on their metabolic profile and that of their female offspring in pregnancy.

Aleida Song, Stuart Astbury, Nicole Coursen, Abha Hoedl, Rhonda C Bell
Division of Human Nutrition, Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada

Background: High fructose (FR) consumption in non-pregnant rats has been linked to impaired glucose metabolism and insulin resistance, but its effects on pregnancy and offspring are not well studied. This study examined the effects of high FR intake on the metabolic profiles of rat dams and their female offspring during pregnancy.
Methods: Female Wistar rats received a 10% FR solution (FR1, n=9) or tap water (CONT1, n=8) for 3 weeks before and during pregnancy. Female offspring received the same treatment (FR2, n=8; CONT2, n=5) as their mother. Plasma glucose, insulin and triglycerides (TG) were determined in early and mid pregnancy. Glucose and insulin was measured as area under the curve (AUC) during OGTTs in late-pregnancy. $P<0.05$ is considered statistically significant.
Results: FR1 had higher TG in early pregnancy than CONT1 (FR1= 9.5±3.2; CONT1=5.2±1.1 mmol/L) and higher insulinAUC during the OGTT (FR1=532.9±215.6; CONT1=210.4±77.2 ng/mL/90 min). FR2 had higher glucose, insulin and TG in early pregnancy than CONT2 (glucose: FR2=7.7±0.5, CONT2=6.8±0.6 mmol/L; insulin: FR2=2.8±1.6, CONT2=1.3±1.0 ng/mL; TG: FR2=14.1±4.2, CONT2=4.5±1.3). Insulin and TG were higher in FR2 than FR1 in early pregnancy (insulin: FR1=1.1±0.2). FR2 glucose, insulin and TG were also elevated in mid pregnancy compared to CONT2 (glucose: FR2=6.7±0.8, CONT2=5.6±0.5; insulin: FR2=2.8±1.7, CONT2=1.0±0.5; TG: FR2=21.3±6.7, CONT2=10.4±2.7). InsulinAUC was higher in FR2 (995.6±250.4) than CONT2 (362.5±58.0), FR1 and CONT1. GlucoseAUC did not differ between groups.
Conclusion:High fructose intake has adverse metabolic effects in pregnancy that are more pronounced in their offsprings' pregnancies, suggesting that diet can contribute to poor metabolic profiles during pregnancy across multiple generations.

Experimental Biology 2014, San Diego, CA, USA (poster presentation)

The effect of a high fructose diet during pregnancy on markers of intestinal permeability in the offspring.

Stuart M Astbury[1,2], Aleida Song[2], Brent Nielsen[2], Nicole Coursen[2], Abha Hoedl[2], Rhonda C Bell[2]
[1]*Early Life Research Unit, Division of Child Health, Obstetrics and Gynaecology, School of Medicine, University of Nottingham*
[2]*Division of Human Nutrition, Department of Agricultural, Food and Nutritional Science, University of Alberta*

Maternal diet in pregnancy has been shown to affect offspring development but few studies have examined changes in the gut. Gut barrier dysfunction may allow components of the microbiota to pass into the circulation, which has been linked to the development of obesity and the metabolic syndrome. This study examined the effect of a maternal diet that is high in fructose on gut permeability markers in pregnant offspring.

Female Wistar rats were placed on 10% fructose (F1) or water (W1) at 8 weeks of age and were mated at 10 weeks; the intervention continued throughout pregnancy. Female offspring continued on the same diet as their dams (W2, n=10 and F2, n=10) from 4 weeks of age, were mated at 10 weeks, and tissue was collected at gestational day 20. Ileum and jejunum expression of occludin (OCLN), claudin 3 (CLDN3) and zonulin 1 (ZO1) were used as markers of intestinal permeability.

F2 pups had a lower birth weight but similar weights at 13 weeks of age compared with W2. F2 also had a significantly higher % fat mass and reduced gut length vs W2. Expression of ZO1 (W2=1.64±0.28; F2=0.64±0.09), OCLN (W2=1.27±0.15; F2=0.51±0.09), but not CLDN3 (W2=1.09±0.19; F2=2.15±0.45) was reduced in the jejunum ($p<0.05$) but not different in the ileum.

A high fructose diet during pregnancy increases intestinal permeability in pregnant offspring. Further studies will examine the effect of the fructose diet on the microbiome during pregnancy.

Women and Children's Health Research Institute Research Day 2013, Edmonton, AB, Canada (oral presentation)

A high fructose diet during pregnancy significantly affects intestinal development in the offspring.

Stuart Astbury, Aleida Song, Brent Nielsen, Nicole Coursen, Abha Hoedl, Rhonda C Bell
Division of Human Nutrition, Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada

Introduction: Excess caloric load or a proinflammatory diet may contribute to gut barrier dysfunction, leading to components of the microbiota passing into the systemic circulation. These effects have been linked to the development of obesity and metabolic syndrome. Maternal diet and diabetes in pregnancy have been shown to adversely affect many aspects of offspring development. This study examined the effect of a high fructose diet during gestation on gut permeability in female, pregnant offspring.

Methods: Female Wistar rats were placed on either 10% fructose or distilled water at 8 weeks of age and were mated at 10 weeks, with diet continuing through gestation. Offspring rats were then continued on the same diet as their dams (c, n=10 and f, n=10) starting from 1 week post-weaning, these offspring rats were then mated at 10 weeks of age, with tissue collected at gestational day 20. Body composition was determined by MRI directly prior to euthanasia.

Ileum and jejunum sections were taken from the small intestine and snap-frozen. Total RNA was extracted for qPCR. Three epithelial tight junction genes were used as markers of intestinal permeability: occludin (OCLN), claudin-3 (CLDN3) and zonulin-1 (ZO-1). Gene expression was measured relative to GAPDH and RPLP0. Groups were compared using t-test or Mann-Whitney U test as appropriate. All values are mean±SEM.

Results: Birth weight was significantly reduced in the f group (c=4.23g±0.5 vs f=3.94g±0.8,p<0.05) Weights at GD20 were not significantly different between diet groups, however percentage lean and percentage fat mass was significantly affected by diet (percentage lean c=75.8±0.3 vs f=71.4±1.0, p<0.05 and percentage fat c=11.4%±0.5 vs f=16.8%±1.3, p<0.01). Gut length was significantly reduced in the f group (c=128.5cm±1.8 vs f=123.8cm±0.4, p<0.05).

A significant downregulation (p<0.05) in ZO-1 (c=1.64±0.28 vs f=0.64±0.09), OCLN (c=1.27±0.15 vs f=0.51±0.09), but not CLDN3 (c=1.09±0.19 vs f=2.15±0.45), was observed in the jejunum. No differences were seen in the ileum.

Conclusions: A high-fructose diet during pregnancy may adversely affect gut development in the offspring via increasing intestinal permeability, potentially allowing components of the microbiota to pass into the systemic circulation. It is unclear whether this is caused by the fetal growth restriction, an effect of the fructose diet on the microbiota, or a combination of these effects. Further research will examine the role of diet in intestinal permeability in the mother, how the microbiota changes throughout pregnancy, and how a high-fructose diet may modulate these changes.

Experimental Biology 2013, Boston, MA, USA (poster presentation)

Supplementation of commercial pig feed with palm oil during pregnancy downregulates genes related to development and permeability in the offspring small intestine.

Stuart M Astbury[1,2], Rhonda C Bell[2], Alison Mostyn[3], Don Sharkey[1] and Michael E Symonds[1]

[1] Academic Child Health, University of Nottingham, Nottingham, United Kingdom

[2] Division of Human Nutrition, University of Alberta, Edmonton, AB, Canada

[3] School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington, United Kingdom

The passage of microbes from the gut flora into the systemic circulation via increased intestinal permeability has been linked to inflammation of adipose tissue and the onset of obesity. A porcine model of a maternal high-fat diet (mHF) consumed during pregnancy, compared to a control diet (mC) was used to test the hypothesis that a high-fat diet during pregnancy will adversely affect offspring gut development.

Expression of claudin-4 (CLDN-4), occludin (OCLN) and zonulin-1 (ZO-1) were used as markers of epithelial tight junction permeability. Epidermal growth factor receptor (EGFr) was used as a marker of intestinal development. mRNA expression was measured using qPCR. Tissue was taken at 7d, and offspring were grouped by maternal diet and birth weight: mC median (n=6), mC small (n=7), mHF median (n=8), mHF small (n=7).

Offspring birth weights were not affected by maternal diet, but mHF offspring had a significantly increased fractional growth rate from 0–7d ($p<0.05$). EGFr, CLDN-4 and OCLN mRNA expression was 50% lower in mHF piglets ($p<0.05$). ZO-1 expression was unaffected by maternal diet. These findings show that a high-fat diet during pregnancy may increase intestinal permeability in the offspring, and adversely affect intestinal development. These changes may predispose to the inflammation of adipose tissue and the onset of obesity.

Funding sources: NSERC, University of Nottingham.

Appendix C: Full composition of Schothorst study diets

	Control	Fat supplemented	Lactation
Feed stuff	**%/kg of diet**	**%/kg of diet**	**%/kg of diet**
Tapioca	28.1	3.1	-
Rapeseed meal	10.0	10.0	4.0
Lupins	-	-	-
Sunflower seed meal	4.0	4.0	2.0
Soybean hulls	13.0	17.0	2.0
Sugar beet pulp	10.0	10.0	2.0
Palm oil	-	6.6	3.1
Soybean oil	0.5	0.5	0.97
Maize	-	-	10.0
Soybean meal	-	-	11.7
Wheat	10.0	10.0	26.4
Barley	10.0	10.0	15.0
Wheat middling's	15.0	15.0	15.0
Molasses	4.0	4.0	4.0
Monocalciumphosphate	0.22	0.22	0.48
Salt	-	-	0.37
Limestone	0.50	0.60	1.56
Premix vit. + min.	0.5	0.5	0.5
Lysine (25%)	0.17	0.17	-
Lysine-HCl (L, 79%)	-	-	0.17
Threonine (L, 98%)	-	-	0.02
Phytase	0.5	0.5	0.5
Threonine 15% 2390*	0.01	0.01	-
Sodium bicarbonate	0.6	0.6	0.16
Diet composition	**g/Kg**	**g/Kg**	**g/Kg**
Ash	64	52	61
Crude protein	123	122	161
Crude fat	25	89	63
Crude fibre	120	121	52
ID starch + sugar	368	212	367
Sugar	61	60	59
Starch	316	161	319
AID LYS	4.03	4.12	7.10
AID MET	1.57	1.58	2.14
AID M+C	3.20	3.24	4.47
AID THR	2.62	2.68	4.29
AID TRP	0.98	0.99	1.53
LYS	5.92	5.98	8.58
MET	2.07	2.04	2.55

CYS	2.46	2.46	3.05
M & C	4.53	4.50	5.60
THR	4.54	4.50	5.87
TRP	1.48	1.46	1.96
ILE	4.37	4.35	6.13
ARG	7.10	7.04	9.83
FEN	5.05	5.02	7.35
HIS	3.16	3.13	4.09
LEU	7.81	7.76	11.46
TYR	3.81	3.86	5.15
VAL	5.84	5.79	7.49
ALA	5.39	5.27	7.07
ASP	9.57	9.53	13.53
GLU	22.04	21.74	32.66
GLY	6.17	6.26	7.09
PRO	7.66	7.64	11.08
SER	5.44	5.46	7.49
Ca	6.78	6.70	9.31
Na	2.00	1.98	2.17
Ferm CHO	235	234	145
Ind. CHO	122	110	80
Dig. P	2.47	2.41	3.27
NSP	343	330	209
dig. NSP	224	223	132
dEB	301	280	220
C18:2	7.7	14.6	15.7
C18:3	1.04	1.22	1.46
C18:2+3	8.8	15.8	17.1
C16+18>0	12.59	44.20	33.83
C16+18.0	2.42	30.26	16.92

Appendix D: Suppliers

Abcam Plc., 330 Cambridge Science Park, Cambridge, CB4 0FL, UK; www.abcam.com
Alfasan International B.V., Kuipersweg 9, JA 3440, Woerden, The Netherlands, PO Box 78 3440 AB; www.alfasan.com
ALPCO Diagnostics, 26-G Keewaydin Drive, Salem, NH 03079, USA
Ambion Inc. (Life Technologies), 850 Lincoln Centre Dr., Foster City, CA 94404, USA; www.ambion.com
Anatech Ltd., 1020 Harts Lake Rd., Battle Creek, MI 49037, USA; www.anatechusaltd.com
Applied Biosystems (Life Technologies), 850 Lincoln Centre Dr., Foster City, CA, 94404, USA; www.appliedbiosystems.com
AVEBE, 9640 AA Veendam, The Netherlands; www.avebe.com
Becton, Dickinson and Company, 1 Becton Dr., Franklin Lakes, NJ, 07417-1880, USA; www.bd.com
Bio-Rad Laboratories Inc., 2000 Alfred Nobel Dr., Hercules, CA 94547, USA; www.bio-rad.com
BIORON GmbH, Rheinhorstraße 18, 67071 Ludwigshafen, Germany; www.bioron.net
Carl Zeiss Canada, 45 Valleybrook Dr., Toronto, ON, M3B 2S6, Canada; www.zeiss.ca
Charles River Laboratories, 324 Rang Saint-Régis N, Saint-Constant, QC, J5A 2E7, Canada; www.criver.com
Echo Medical Systems LLC., 14871 Memorial Drive #222, Houston, TX 77079, USA; www.echomri.com
EMD Millipore, 290 Concord Rd., Billerica, MA, USA; www.emdmillipore.com
Eurovet Animal Health B.V., Handelsweg 25, 5531 AE Bladel, The Netherlands
Foss, Foss Allé 1, Postbox 260, DK-3400 Hillerød, Denmark; www.foss.dk
Fujifilm Europe GmbH, Heesenstraße 31, 40549 Düsseldorf, Germany; www.fujifilm.eu
GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK; www3.gehealthcare.co.uk
GraphPad Software Inc., 7825 Fay Ave., Suite 230, La Jolla, CA, USA; www.graphpad.com
IBM, 1 N Castle Dr., Armonk, NY 10504, USA; www.ibm.com
Integrated DNA Technologies, 1710 Commercial Park, Coralville, IA, 52241, USA; www.idtdna.com
Intervet, Wim de Körverstraat 35, 5831 AN Boxmeer, The Netherlands; www.intervet.com
Leica Microsystems Inc., 71 Four Valley Drive, Concord, ON, L4K 4V8, Canada; www.leicabiosystems.com
Mercodia AB, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden; www.mercodia.se
McGill University and Génome Québec Innovation Centre, 740 Doctor Penfield Ave., Montréal, QC, H3A 0G1, Canada; www.gqinnovationcenter.com

Microsoft Corp., 1 Microsoft Way, Redmond, WA 98052-7329, USA; www.microsoft.com
Miltenyi Biotec GmbH., Friedrich-Ebert-Straße 68, 51249 Bergisch Gladbach, Germany; www.miltenyibiotec.com
Molecular Devices, 1311 Orleans Dr., Sunnyvale, CA 94089, USA; www.moleculardevices.com
MP Biomedicals, 29525 Fountain Parkway, Solon, OH 44139, USA; www.mpbio.com
New England Biolabs, 240 County Rd., Ipswich, MA 01938, USA; www.neb.com
Purina, 2500 Royal Windsor Dr., Mississauga, ON, L5J 1K8, Canada; www.labdiet.com
Qiagen Ltd., Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK; www.qiagen.com
Randox Laboratories, 55 Diamond Road, Crumlin, Co. Antrim, BT29 4QY, United Kingdom; www.randox.com
Renco Corp., 116 3rd Ave. N. Minneapolis, MN 55401, USA; www.rencocorp.com
Santa Cruz Biotechnology Inc., Bergheimer Straße 89-2, 69115 Heidelberg, Germany; www.scbt.com
SAS Institute Inc., 100 SAS Campus Dr., Cary, NC 27513, USA; www.sas.com
Sekisui Diagnostics P.E.I. Ltd., 70 Watts Ave., Charlottetown, PEI, C1E 2B9, Canada; www.sekisuidiagnostics.com
Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK; www.sigma-aldrich.com
Simport Scientifique Inc., Rue Bernard Pilon, Saint-Mathieu-de-Beloeil, QC, J3G 4S5, Canada; www.simport.com
Techne (Cambridge) Ltd., Duxford, Cambridge, CB1 0SA, UK; www.techne.com
Thermo Fisher Scientific Inc., 81 Wyman St., Waltham, MA 02451, USA; www.thermofisher.com