Modelling maternal obesity:

Unravelling prolonged and dysfunctional labour

Ronan Davidson Muir BSc (Hons)

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Division of Nutritional Science

School of Bioscience

University of Nottingham

Sutton Bonington Campus

Loughborough

Leicestershire

LE12 5RD

Declaration

All the work presented within this thesis was completed by the author, except from the characterisation of uterine phosphorylated connexin-43 expression using western blotting, which was completed by Jean Ballan. Scientific Analysis Laboratories Ltd carried out the nutritional analysis of the HFHC diet. Dr Ge Liu and Dr John Carragher of the Waites Lipid Analysis Service at the University of Adelaide characterised the fatty acid profiles of the control and HFHC diets, along with the fatty acid profiles of the plasma, liver, and uterus collected from control and HFHC animals. All studies were carried out under the supervision of Dr Matthew Elmes, Division of Nutritional Science, School of Bioscience, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD.

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Publications

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Abstract

Maternal obesity significantly increases the risk of a prolonged and dysfunctional labour resulting in emergency caesarean delivery. As the mechanism(s) remain to be eludicated, a translatable animal model is warranted. Recently, Elmes et al (2011) illustrated the potential of the high-fat, high-cholesterol (HFHC) diet animal model to investigate maternal obesity associated uterine dystocia, but it remains to be fully evaluated. The aim of this thesis was to evaluate this animal model by assessing the effect of a HFHC diet upon uterine contractility, and to highlight the potential mechanism(s) responsible. All female Wistar rats were sustained on a control (CON) or HFHC diet prior to and during pregnancy and labour, with tissue collected from these animals upon term delivery of the 1st pup. Uterine contractility was assessed through *ex vivo* contractility studies and *in vivo* intra-uterine telemetry surgery. Chronic exposure to a HFHC diet resulted in an aberrant asynchronous multiphasic spike bundle contraction phenotype *ex vivo*, with HFHC animals displaying a prolonged duration of labour up to 12 hours longer than CON animal's *in vivo*. These results are reflective of human clinical studies, highlighting the efficacy and translatability of this animal model, but the mechanism(s) remain to be identified. Enzyme-linked immuno-sorbent assay and western blotting revealed that HFHC animals displayed significantly increased circulating progesterone at term, coupled with a decrease in uterine contractile-associated protein (CAP) expression including connexin-43 and caveolin-1 at the onset of term-labour (TL). Further to this, chronic exposure to a HFHC diet

significantly increased circulating cholesterol, which could alter uterine myocyte membrane fluidity and compromise the expression and function of their integral CAP's including the oxytocin receptor (OXTR); OXTR expression was increased at TL but HFHC animals displayed a protract response to this uterotonin *ex vivo*. Feeding of a HFHC diet significantly increased circulating oleic acid and decreased arachidonic acid, which could explain lower plasma concentrations of the utertonin prostaglandin $F_{2\alpha}$ identified during a previous pilot study, and offer another potential mechanism by which maternal obesity could compromising the outcomes of labour. This thesis has highlighted the efficacy and translatability of this animal model in the study of maternal obesity associated uterine dystocia, and offered insights into the mechanism(s) responsible.

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Abbreviations

%	Percentage
11β-HSD 1/2	11 beta-hydroxysteroid dehydrogenase 1/2
α	Alpha
AA	Arachidonic acid
ALA	Alpha linolenic acid
ACTH	Adrenocorticotrophin hormone
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Apo	Apolipoprotein
ASPA	Animals in Scientific Procedures Act
ATP	Adenosine triphosphate
β	Beta
BMI	Body mass index
°C	Degrees Celsius
C=C	Carbon carbon double bond
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine mono-phosphate
CAP's	Contractile associated proteins
Cav-1	Caveolin-1
cm	Centimetres
CO_2	Carbon dioxide
CON	Control
COX-1/2	Cyclooxygenase 1/2

CRH	Corticotropin releasing hormone
CRH-BP	Corticotropin releasing hormone – binding protein
CX-43	Connexin-43
DAG	Diacylglycerol
DEXA	Dual-energy X ray absorptiometry
DHA	Docosahexaenoic acid
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DPA	Docosapentaenoic acid
E_1	Oestrone
E_2	Oestradiol
E_3	Oestriol
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immune-sorbet assay
EP_{1-4}	Prostaglandin E_2 receptors 1-4
EPA	Eicosapentaenoic acid
$ER_{\alpha/\beta}$	Nuclear oestrogen receptor α/β
FH	Familial hypercholesterolemia
FP	Prostaglandin $F_{2\alpha}$ receptor
g	Grams
GC-FID	Gas chromatography flame ionised detection
GPCR	G protein coupled receptor
H_2O	Dihydrogen oxide/water
HCl	Hydrochloric acid
HDL	High density lipoprotein

HFHC	High fat high cholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPA	Hypothalamic-pituitary-adrenal
IDL	Intermediate density lipoprotein
IL	Interleukin
IP_3	Inositol-1, 4, 5-triphosphate
IOM	Institute of Medicine
IUP	Intra-uterine pressure
K^+	Potassium
Kcal	Calories
Kcal/g	Calories per gram
KCl	Potassium chloride
KDa	Killodalton
Kg	Killogram
KH_2PO_4	Potassium dihydrogen phosphate
L	Litre
LA	Linoleic acid
LDL	Low density lipoprotein
M	Molar
m^2	Metres squared
Mg	Milligrams
$MgSO_4$	Magnesium sulphate
Mins	Minutes
ml	Millilitre
MLC_{20}	Myosin light chain 20 Killodaltons

MLCK	Myosin light chain kinase
mM	Millimolar
mm	Milimetre
mmHg	Millimetres of mercury
mmol/L	Millimoles per litre
mN	Millinewton
mN/s	Milinewton per second
mV	Millivolts
MUFA	Monounsaturated fatty acid
n-3	Omega 3 fatty acid
n-6	Omega 6 fatty acid
n-9	Omega 9 fatty acid
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NF-κB	Nuclear factor – kappa B
ng	Nanograms
ng/ml	Nanograms per millilitre
NHS	National Health Service
NICE	National Institute for Clinical Excellence
nm	Nanometres
O_2	Oxygen
OXT	Oxytocin
OXTR	Oxytocin receptor
P_4	Progesterone

PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PK A/C	Protein kinase A/C
PLA_2	Phospholipase A_2
PR A/B	Nuclear progesterone receptor A/B
PUFA’s	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SEM	Standard error of mean
Sec’s	Seconds
SFA	Saturated fatty acid
SOCE	Store operated calcium entry
SR	Sarcoplasmic reticulum
SREBP	Sterol regulatory element binding protein
TAG	Triacylglycerol/triglycerides
TCA	Tricarboxylic acid
TL	Term labouring
TNF_{α}	Tumour necrosis factor α
TNL	Term non-labouring
Δ	Delta
µg	Microgram
µl	Microlitre
µm	Micrometre
UK	United Kingdom

USA	United States of America
vLDL	Very low density lipoprotein
WHO	World Health Organisation

1. Introduction

Obesity is a chronic disease characterised by a significant accumulation of subcutaneous and visceral white adipose tissue, resulting in serious health issues including cardiovascular disease, metabolic dysfunction, cancer, musculoskeletal disorders, and reproductive dysfunction (Butland et al., 2007).

Since 1975, global obesity rates have significantly increased from 105 million to 641 million people in 2014 (NCD Risk Factor Collaboration, 2016), placing a severe strain upon social and health services, globally. The rates of female obesity within the British population are currently at 20%, and are estimated to reach 50% by 2050 (Butland et al., 2007). This has translated to increasing rates of obesity during pregnancy and labour, referred to as maternal obesity (Centre for Maternal and Child Enquiries (CMACE), 2010).

Maternal obesity significantly increases the risk of complications occurring during pregnancy and labour including gestational diabetes, pre-eclampsia, post-partum haemorrhaging, and prolonged and dysfunctional labour resulting in emergency caesarean delivery (Bogaerts et al., 2013; Centre for Maternal and Child Enquiries (CMACE), 2010; Kominiarek et al., 2011; Poobalan et al., 2009). As a result, the rates of emergency caesarean delivery are nearly double in nulliparous singleton obese pregnancies compared to that in nulliparous singleton normal weight pregnancies (29% vs 15%)

(Poobalan et al., 2009). The increased rates of emergency caesarean delivery associated with maternal obesity are placing the National Health Service (NHS) under financial strain. This is a result of a caesarean section costing £1500 more per delivery compared to a spontaneous vaginal birth (NICE, 2011). If the national caesarean delivery rate could be decreased by 1%, it would save £5 million annually for the NHS (NICE, 2011).

Though the risks of maternal obesity upon the outcomes of labour are clear, few studies have attempted to eludicate the mechanism(s) behind maternal obesity associated uterine dystocia. *Ex vivo* and *in vitro* studies have highlighted the significant utero-relaxant effect of increasing body mass and its associated hypercholesterolemia (Farias et al., 2015) upon human and rat uterine tissue (Smith et al., 2005; J Zhang et al., 2007; Jie Zhang et al., 2007).

Despite this evidence, there is clear limitation in our knowledge and understanding of the mechanism(s) governing maternal obesity associated uterine dystocia. One reason for this is the inability to obtain term-labouring human uterine biopsies from women of ranging body mass. As a result, Elmes et al. (2011) have developed a high fat, high cholesterol (HFHC) dietary animal model to mimic maternal obesity, and to investigate the mechanism(s) governing maternal obesity associated uterine dystocia. The aim of this project is to evaluate the efficacy and translatability of this HFHC dietary animal model for the

study of maternal obesity associated uterine dystocia, and through this unravel the mechanism(s) responsible.

2. Literature review

2.1. Obesity

2.1.1. Trends in obesity

Global obesity rates have increased to pandemic proportions, with many nations on the cusp of a public health crisis. From 1975 to 2014, global obesity rates have significantly increased from 34 million to 266 million in males, and 71 million to 375 million in females (NCD Risk Factor Collaboration, 2016).

2.1.2. Aetiology

Obesity is a disease characterised by the significant accumulation of subcutaneous and visceral white adipose tissue, and a change in circulating lipid concentrations (dyslipidemia), consequential of an imbalanced energy dense diet and sedentary lifestyle (Butland et al., 2007; Insel et al., 2011). In a small number of cases, this energy imbalance that commonly characterises obesity can be caused by genetic induced neuroendocrine dysfunction of appetite regulation (Sainz et al., 2015; Wang et al., 2015).

Excess energy is stored within the lipid droplets of adipocytes as triacylglycerol (TAG) (Feng et al., 2013), resulting in adipocyte hypertrophy once the lipid droplet has reached capacity (Coelho et al., 2013). Once capacity is reached, adipocytes undergo hyperplasia to

provide further storage for excess TAG, resulting in the increase in subcutaneous and visceral white adipose tissue (Rosen and Spiegelman, 2014).

White adipose tissue does not only act as storage for excess energy, it is also an active endocrine organ capable of releasing a range of hormones known as adipokines (Leal and Mafra, 2013). Adipokines released from white adipocytes including leptin, adiponectin, and visfatin are able to regulate appetite, insulin sensitivity/glucose homeostasis, adipogenesis, and lipolysis (Leal and Mafra, 2013). Leptin is released from adipocytes in response to TAG storage induced hypertrophy, and acts as the endogenous signal of satiety through the central nervous system (Leal and Mafra, 2013). The neurons receptive to leptin become insensitive to this adipokine during obesity, contributing to hyperphagia and weight gain (Tessier et al., 2013). Genetic induced obesity can result from the inheritance of a pathological leptin allele, resulting in hypoleptinemia, which will contribute to hyperphagia and the accumulation of white adipose tissue (Sainz et al., 2015; Wang et al., 2015). This increase in white adipocytes can play a central role in promoting the chronic low-grade inflammatory state of obesity through an increase in oxidative stress (Fernández-Sánchez et al., 2011).

Lipolysis is an oxidative process resulting in the release of reactive oxygen species (ROS) when fatty acids are freed from the glycerol

backbone of TAG (Fernández-Sánchez et al., 2011). Further to this, white adipocytes actively synthesis and secrete pro-inflammatory cytokines [interleukin-6 (IL-6) and tumour necrosis factor α (TNFα)] that can promote the synthesis of ROS (Fernández-Sánchez et al., 2011; Leal and Mafra, 2013). Generally, ROS are neutralised by the release of anti-oxidants, but there is insufficient activity/quantities of anti-oxidants to mediate this in obesity, resulting in the development of obesity associated inflammatory co-morbidities (Ozata et al., 2002).

Category	**BMI Range (kg/m^2)**
Underweight	≤18.5
Normal weight	18.50 – 24.99
Overweight	25.00 – 29.99
Obese Class I	30.00 – 34.99
Obese Class II	35.00 – 39.99
Obese Class III	≥40.00

Table 2.1 – WHO BMI scale (Scott-Pillai et al., 2013)**.**

Pre-pregnancy BMI	**BMI (kg/m^2 (WHO)**	**Total weight gain range (lbs)**	**Rates of weight gain 2nd and 3rd trimester (mean range in lbs/week)**
Underweight	<18.5	28-40	1 (1 - 1.3)
Normal weight	18.5 – 24.9	25-35	1 (0.8 – 1)
Overweight	25.0 – 29.9	15-25	0.6 (0.5 – 0.7)
Obese (includes all classes)	≥30.0	11-20	0.5 (0.4 – 0.6)

Table 2.2 – IOM pregnancy weight gain recommendations for WHO BMI classification (IOM (Institute of Medicine), 2009).

2.1.3. Diagnosis and treatment

Obesity is commonly diagnosed through the measurement of a patient's body mass index (BMI: kg/m^2). The patient's BMI is calculated by dividing their body mass (kg) by their height squared (m^2), which is then compared to the World Health Organisation (WHO) scale to determine their BMI classification e.g. normal weight or obese (Table 2.1) (James, 2008). Despite this, BMI is not the most effective method for diagnosing obesity, as it cannot distinguish between fat and fat-free mass e.g. muscle (Baumgartner et al., 1995). To overcome this, methods such as hydrodensitometry, air-displacement plethysmography, and dual-energy X ray absorptiometry (DEXA) are employed, as they are capable of distinguishing between fat and fat-free mass (Insel et al., 2011).

The class of obesity or the degree of adiposity will directly dictate the patient's treatment options. Many obese patients are referred to dietitians for dietary and lifestyle assessment. Through this assessment, the patients are encouraged and supported to decrease their energy intake and increase their energy expenditure, through a balanced diet and moderate exercise (Butland et al., 2007). In many cases this is successful and the patients return to a healthy weight before the onset of any co-morbidities. If this treatment option is not successful or it cannot be implemented, bariatric surgery is an option. Bariatric surgery is the laparoscopic bypass or partial removal of the

stomach to restrict the patient's energy intake, and facilitate weight loss (McGohan and Caflisch, 2004). Treatment of obesity is further complicated by the onset of its co-morbidities including cardiovascular disease, diabetes mellitus, musculoskeletal disorders, and reproductive dysfunction, which can all require specialist life-long treatment and management (Butland et al., 2007).

2.1.4. Maternal Obesity

Maternal obesity is increasing in conjunction with female obesity. In the UK, 20% of the female population is diagnosed as obese (Bhattacharya et al., 2007), with this figure estimated to reach 50% by 2050 (Butland et al., 2007). The increasing rate of female obesity is occurring within women of reproductive age, doubling the rate of maternal obesity within the UK since 1989 (Heslehurst et al., 2010).

Maternal obesity is diagnosed through the measurement of BMI at an antenatal visit, where it can be classified as having a pre-gestational or gestational onset. Pre-gestational onset of maternal obesity is where the woman was obese at conception, while gestational onset is when the expecting mother becomes obese during pregnancy (Bogaerts et al., 2013). Lifestyle management is the only treatment currently prescribed to women diagnosed with maternal obesity. They are encouraged to balance their diet and take up mild exercise to limit their weight gain to the institute of medicines recommendations (Table 2.2) (IOM (Institute of Medicine), 2009). Currently, no UK government body supports weight loss during pregnancy, as they do not know how it will

affect maternal health, foetal development, and labour outcomes (NICE, 2010). Though the effects of weight loss during pregnancy are not well established, the effects of maternal obesity upon gestation and parturition are well founded.

Maternal obesity significantly increases the risk of the expecting mother developing gestational diabetes mellitus, pre-eclampsia, post-partum haemorrhaging, and prolonged and dysfunctional labour resulting in emergency caesarean delivery (Bogaerts et al., 2013; Centre for Maternal and Child Enquiries (CMACE), 2010; Kominiarek et al., 2011; Poobalan et al., 2009). Specifically, singleton nulliparous obese parturients (29%) are nearly twice as likely to require an emergency caesarean delivery compared to their singleton nulliparous normal weight counterparts (15%) (Poobalan et al., 2009). The prolonged and dysfunctional labour of obese parturients is a result of uterine dystocia, characterised by poor uterine contractility and/or a insufficient rate of cervical dilation (Karaçam et al., 2014). *Ex vivo* human uterine contractility studies have illustrated the significant utero-relaxant effect of increasing body mass (J Zhang et al., 2007), but the aetiology and mechanism(s) governing maternal obesity associated uterine dystocia remain to be determined. An number of hypotheses have been proposed to explain maternal obesity associated uterine dystocia (Bogaerts et al., 2013; Carlson et al., 2015), but these will be discussed in detail later within this review.

2.2. Lipids

Lipids are a class of aliphatic and cyclic hydrophobic or amphipathic hydrocarbons that form fats, oils, and sterols. Lipids are essential to biological function by forming the cell membrane, being the raw material for the synthesis of sex steroids, and a source of storable energy (Glatz, 2015; Miller, 2013). Lipids including cholesterol, triacylglycerol, fatty acids, and phospholipids, are synthesised endogenously or obtained from the diet (Insel et al., 2011). Endogenous concentrations of these lipids can be altered by obesity-induced dyslipidaemia (Feng et al., 2013). Through this section we will review the nomenclature, structure, synthesis, function, pathologies, and the effects of these lipids on parturition.

2.2.1. Lipid transport

Lipids are transported throughout the body by a group of macromolecules known as lipoproteins; lipids are also able to circulate free within the blood. Lipoproteins are composed of a cholesterol ester and triglyceride core, which is encapsulated by a phospholipid shell that supports a range of integral proteins known as apolipoproteins (apo) (Thaxton et al., 2016); apo act as signals for lipase activity and cellular uptake e.g. ApoB facilitating LDL uptake via its receptor (Costet, 2010; Ramasamy, 2013). Lipoproteins are classed according to their density (Figure 2.1) from chylomicrons, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein (LDL), to high-density lipoprotein (HDL).

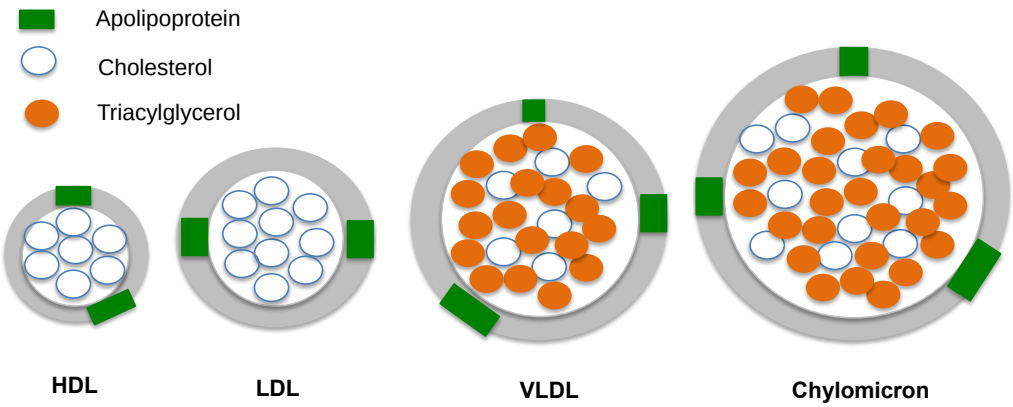


Figure 2.1 – Structure of lipoproteins. Adapted from (Thaxton et al., 2016).

2.2.1.1. Chylomicrons

Chylomicrons are the lowest density lipoprotein, responsible for transport of dietary absorbed TAG and cholesterol from the intestines to the liver (Ramasamy, 2013). While circulating to the liver, lipoprotein lipases release triglycerides from its core, with the remaining cholesterol taken up by the liver (Ramasamy, 2013).

2.2.1.2. Very low-density lipoprotein

VLDL is formed within the liver, with its primary function to transport endogenously synthesised lipids to peripheral tissue (Ramasamy, 2013). Circulating lipoprotein lipase induced TAG release converts VLDL to IDL (Costet, 2010).

2.2.1.3. Intermediate density lipoprotein

IDL will return to the liver where it will be dissociated or converted to LDL for cholesterol transport to peripheral tissue (Insel et al., 2011).

2.2.1.4. Low-density lipoprotein

LDL is responsible for supplying cholesterol to peripheral tissue for cell-membrane integration or sex steroid synthesis (Insel et al., 2011).

2.2.1.5. High-density lipoprotein

HDL is responsible for the collection and transport of excess circulating cholesterol or cholesterol from lower density lipoproteins back to the liver (Ramasamy, 2013). Once in liver, this cholesterol can be recycled back into circulation, utilised to synthesise bile salts, or excreted from the body (Ramasamy, 2013).

2.2.2. Cholesterol

2.2.2.1. Structure

Cholesterol is an amphipathic molecule (Figure 2.2) composed of a hydrophilic hydroxyl head group, a hydrophobic tetracyclic steroid ring, and a hydrocarbon tail (Marquardt et al., 2016; Pucadyil and Chattopadhyay, 2006).

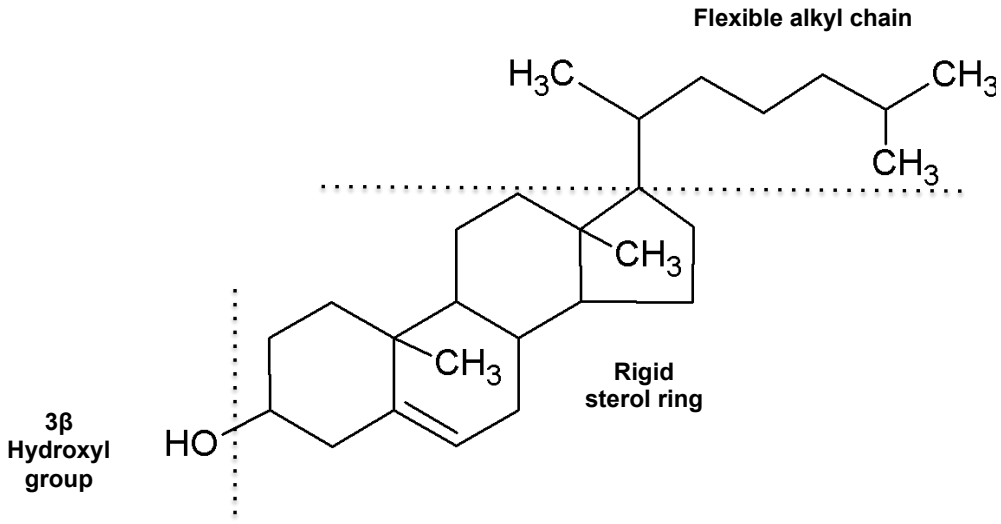


Figure 2.2 – Structure of cholesterol. Adapted from (Pucadyil and Chattopadhyay, 2006).

2.2.2.2. Cholesterol Synthesis

2.2.2.2.1 Exogenously sourced cholesterol

Dietary advice stipulates a maximum intake of 300mg/day of cholesterol (Insel et al., 2011), with excessive intake adversely effecting long-term health. Cholesterol is absorbed from the diet through the intestinal lumen into gastrointestinal enterocytes, with bile salts facilitating this process (Ramasamy, 2013). Within these cells, cholesterol is esterified and incorporated into chylomicrons, where it is transported through the lymphatic and cardiovascular system to the liver (Thaxton et al., 2016). Within the liver, cholesterol can be used to synthesise bile salts or it can be used to form LDL, where it will be transported to peripheral tissues around the body for storage or processing (Insel et al., 2011). LDL enters the cell through ApoB LDL-receptor mediated endocytosis, where it is subsequently released and de-esterified as free cytosolic cholesterol (Costet, 2010). Free cytosolic

cholesterol will regulate endogenous cholesterol synthesis and extracellular uptake of LDL (Goldstein and Brown, 1990). Increased cytosolic cholesterol concentrations will down regulate LDL-receptor expression, limiting peripheral tissue uptake of cholesterol (Costet, 2010). Hepatic clearance of excess circulating cholesterol will occur through uptake of LDL or HDL, where it can be converted to bile salts or excreted via the intestines (Ramasamy, 2013).

2.2.2.2.2 Endogenous synthesis of cholesterol

Endogenous cholesterol is predominantly synthesised *de novo* through the mevalonate pathway within the smooth endoplasmic reticulum of hepatocytes (Figure 2.3) (Goldstein and Brown, 1990). Acetate is converted to mevalonate via 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase and reductase (Miller, 2013; Porter and Herman, 2011). Mevalonate is then further converted to cholesterol through the remainder of this pathway (Pucadyil and Chattopadhyay, 2006). Cytosolic free-cholesterol and mevalonate concentrations regulate the activity of this pathway, to limit the toxic accumulation of cholesterol within the liver. They achieve this through transcriptional regulation of HMG-CoA reductase activity, mediated through activation/inactivation of sterol regulatory element binding protein (SREBP) (Berg et al., 2011; da Silva Afonso et al., 2014).

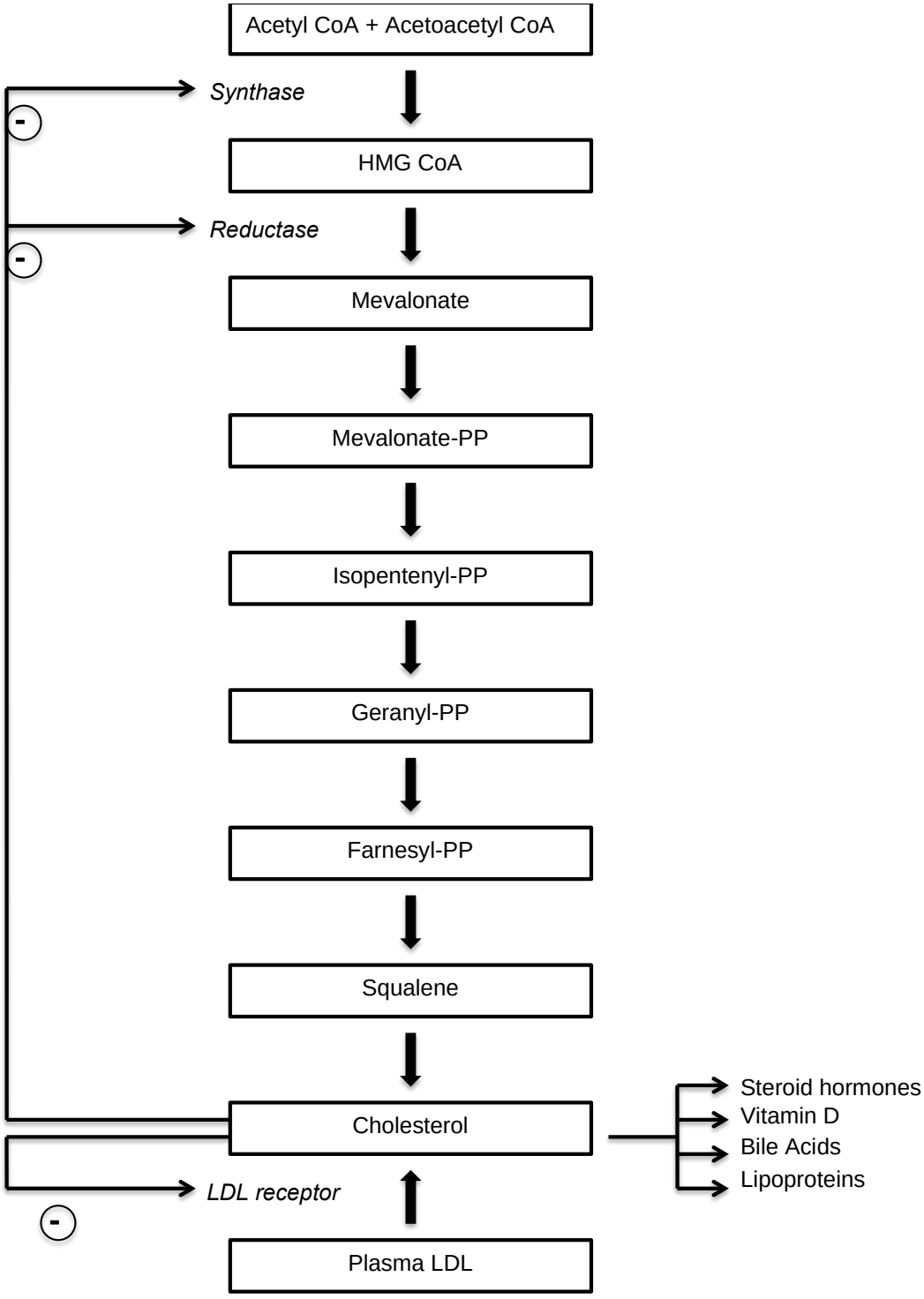


Figure 2.3 – Mevalonate pathway Adapted from (Goldstein and Brown, 1990).

2.2.2.3. Function

Cholesterol has a diverse but vital role in our physiology. The unique structure of cholesterol has facilitated it to become a key constituent of the plasma membrane, which contains 90% of our body's total cholesterol (Pucadyil and Chattopadhyay, 2006). Commonly, cholesterol constitutes 45% of all membrane lipids (Marquardt et al., 2016), with change in membrane cholesterol concentrations significantly altering its fluidity (Pucadyil and Chattopadhyay, 2006). Change in membrane fluidity has shown to effect peripheral and integral membrane protein expression, compromising downstream activation of cellular pathways and signal transduction (Grouleff et al., 2015; Pucadyil and Chattopadhyay, 2006). Further to this, cholesterol is essential to the formation of the omega-shaped invaginations of the plasma membrane known as caveolae through their cholesterol-rich structural protein caveolin-1 (Cav-1) (Noble et al., 2006; Schlegel et al., 1998). Caveolae increase the surface area of the membrane by up to 75% (Taggart, 2001), where ion channels (Brainard et al., 2009, 2005; A Shmygol et al., 2007), receptors (Gimpl and Fahrenholz, 2000; Klein et al., 1995; Turi et al., 2001), and gap junctions (Defamie and Mesnil, 2012) essential to cellular function and homeostasis can localise. Cholesterol is the homologous structure of all sex steroids, acting as the raw material for their synthesis via the steroid biosynthesis pathway (Figure 2.4) in the liver, adrenal glands, placenta, and the gonads (Miller, 2013). Cholesterol is converted to pregnenolone by cholesterol side-chain cleavage enzyme, with pregnenolone either being converted

to progesterone by 3β-hydroxysteroid dehydrogenase (HSD) or to dehydroepiandrosterone (DHEA) by 17,20 lyase (Wang et al., 2009). DHEA can be further converted to testosterone by 3β- and 17β-HSD, which can act as the precursor for the synthesis of oestrogens by aromatase (Wang et al., 2009). Sex steroids including progesterone, testosterone, and oestrogens are vital to male and female sexual maturation and reproduction.

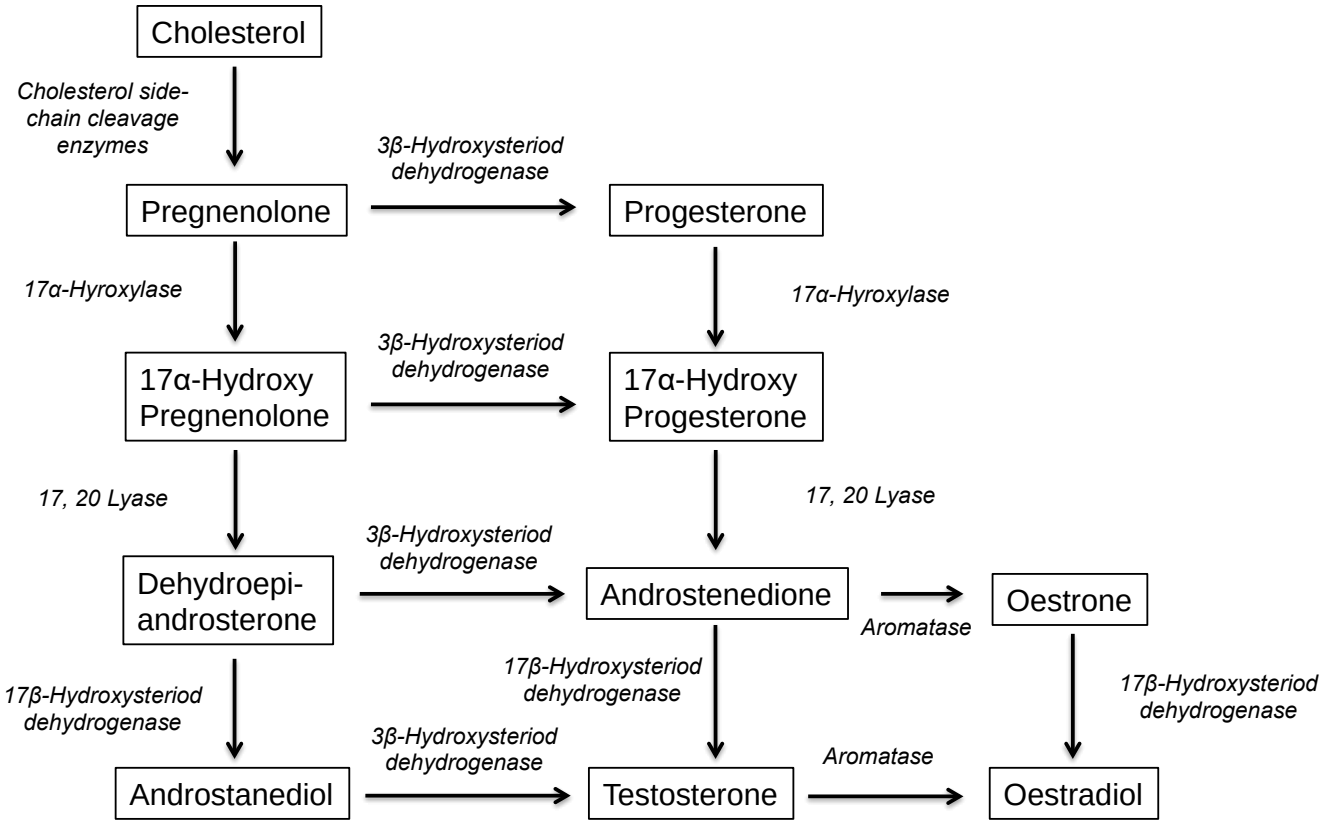


Figure 2.4 – Steroid biosynthesis pathway. Adapted from (Wang et al., 2009).

2.2.2.4. Hypercholesterolemia

Hypercholesterolemia is a disease characterised by increased serum total cholesterol concentrations beyond 5.0 mmol/L (NHS, 2015). Hypercholesterolemia is also diagnosed by an increase in LDL-cholesterol (>3 mmol/L) and a decrease in HDL-cholesterol concentrations (<1 mmol/L) (NHS, 2015).

Increased circulating cholesterol concentrations can be caused by excessive dietary consumption of the sterol (Rang et al., 2007). This is a consequence of cellular cholesterol concentrations reaching capacity, resulting in the decrease in LDL receptor mediated uptake of LDL-cholesterol, increasing circulating concentrations (Berg et al., 2011).

Hypercholesterolemia can also result from autosomal dominant familial hypercholesterolemia (FH) (Fahed and Nemer, 2011). FH is an genetic disease caused by the inheritance of aberrant alleles for the genes that transcribes the LDL-receptor and a range of apolipoproteins (Fahed and Nemer, 2011). The resulting increase in cellular and circulating cholesterol through excessive dietary intake or FH can significantly affect a patient's health.

Hypercholesterolemia will contribute to the pathogenesis of atherosclerosis, characterised by lipid-rich plaque occlusion of major blood vessels (da Silva Afonso et al., 2014; Parodi, 2009). Lipid-rich plaques are caused by the uptake of oxidised LDL-cholesterol by

macrophages, resulting in the formation of lipid-rich macrophage foam cells under the endothelium of major blood vessels (Toescu et al., 2004). The development of atherosclerotic plaques will reduce the elasticity and blood flow of major blood vessels, contributing to the development of cardiovascular disease (Boesen et al., 2015).

To treat hypercholesterolemia and limit the onset of atherosclerosis, patients are commonly prescribed statins to lower their circulating total cholesterol concentrations. Statins inhibit HMG-CoA reductase activity, halting endogenous cholesterol synthesis (Böhm et al., 2004; Costet, 2010). As a result, this promotes clearance of circulating LDL-cholesterol by increasing LDL-receptor mediated endocytosis, decreasing circulating cholesterol concentrations (Thaxton et al., 2016).

Further to this, hypercholesterolemia has been diagnosed in obese parturients (Farias et al., 2015) and may be responsible for their poor labour outcomes, as exposure of rat and human myometrium to increasing cholesterol concentrations significantly depresses contractility *ex vivo* (Smith et al., 2005; Jie Zhang et al., 2007). Unlike atherosclerosis, statins cannot be prescribed to overcome the significant utero-relaxant effect of cholesterol, as they delay the onset of murine lipopolysaccharide induced pre-term labour and depress the frequency of C5a stimulated uterine contractions, potentially by dulling the pro-inflammatory milieu of parturition (Basraon et al., 2012; Gonzalez et al., 2014). Statins are also not prescribed to pregnant

women as they are teratogenic and can increase the risk of stillbirth (Edison and Muenke, 2004). The effect of hypercholesterolemia upon uterine contractility will be discussed in detail later in this literature review.

2.2.3. Fatty acids

2.2.3.1. Nomenclature and structure

Fatty acids are small (≤ 6 carbons), medium (6-10 carbons) and long (≥ 12 carbons) aliphatic hydrocarbons (Insel et al., 2011), containing methyl (CH_3) and carboxyl (COOH) end functional groups. The nomenclature of fatty acids can be further determined by their saturation. Saturated fatty acids (SFA) are aliphatic hydrocarbons that do not contain a carbon-carbon double bound (C=C), while mono-unsaturated (MUFA) have one and poly-unsaturated fatty acids (PUFA) have two (or more) C=C, respectively. MUFA's and PUFA's can be further classified according to the position of the 1st C=C relative to the methyl functional group bound to the Ω-carbon e.g. n-9 MUFA, n-6 or n-3 PUFA (Lottenberg et al., 2012), as illustrated within Figure 2.5. Furthermore, unsaturated fatty acids can be separated into Cis (hydrogen molecules are on opposing sides of the carbon-carbon double bond) and Trans (hydrogen molecules are on the same side of the carbon-carbon double bond) (Cook and McMaster, 2002). The length of the fatty acid chain, the presence/number of carbon-carbon double bonds, and Cis or Trans formation will dictate the chemistry and physical properties of the fatty acid at room temperature.

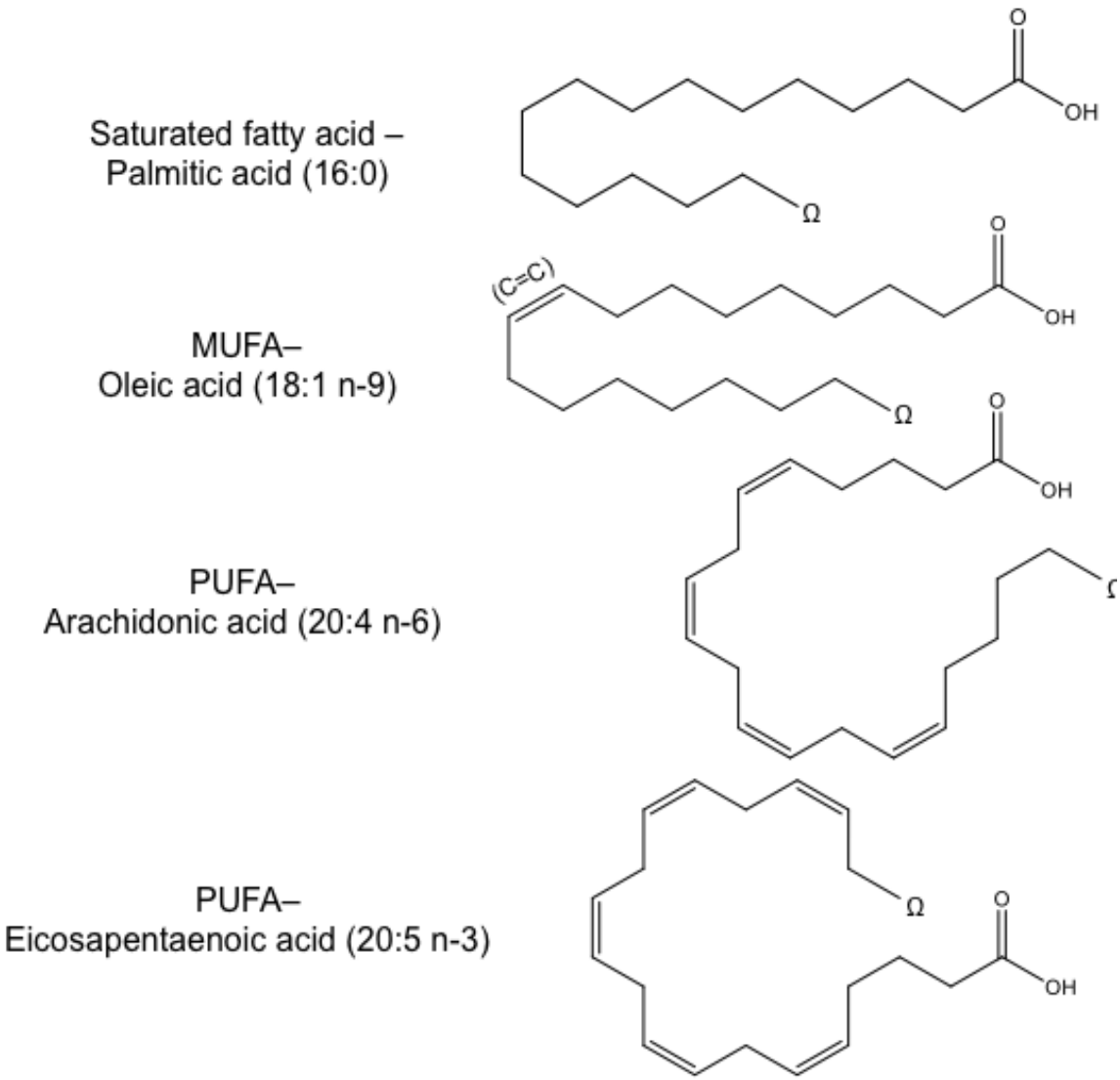


Figure 2.5 – Nomenclature and structure of fatty acids.

2.2.3.2. Synthesis and dietary source

Fatty acids (SFA, MUFA, and PUFA) can be obtained through the diet or synthesised endogenously *de novo*. As a result, fatty acids can be viewed as essential or non-essential. Essential fatty acids are those that cannot be synthesised endogenously, and thus must be obtained from the diet (Berg et al., 2011). While, non-essential fatty acids are those that can be synthesised endogenously or obtained through the diet (Insel et al., 2011). Essential fatty acids cannot be synthesised endogenously, as humans lack the specific desaturase enzymes capable of inserting a C=C greater than carbon-9 (Wathes et al., 2007). The dietary sources and endogenous pathways of SFA, MUFA, and PUFA will be reviewed separately within this section.

2.2.3.2.1 Saturated fatty acids

SFA's can be obtained through the consumption of dairy and meat products or they can be synthesised endogenously from acetyl-CoA (Medina-Gómez, 2012; Morio et al., 2016; Sanders, 2015). Pyruvate dehydrogenase irreversibly converts acetyl-CoA to pyruvate, which would ordinarily react with oxaloacetate to form citrate for the tri-carboxylic acid (TCA) cycle, but instead is utilised to synthesise palmitic acid (Berg et al., 2011). This is achieved by acetyl-CoA carboxylase converting acetyl-CoA to malonyl-CoA, with palmitic acid synthesised from malonyl-CoA by fatty acid synthetase (Bloch and Vance, 1977; Volpe and Vagelos, 1976a, 1976b). Palmitic acid acts as the raw

material for the endogenous synthesis of all other SFA's (Volpe and Vagelos, 1976a).

2.2.3.2.2 Mono-unsaturated fatty acids

MUFA's as like SFA's, can be obtained through the diet or synthesised endogenously. The human diet is a rich source of MUFA's (Lottenberg et al., 2012), with meat products supplying copious amounts of the n-9 MUFA oleic acid (Meyer et al., 2003). Palmitic acid and other SFA's can be converted to oleic acid in liver by Δ^9 desaturase inserting a C=C at carbon-9, with elongase adding two carbons onto the chain when necessary (Cook and McMaster, 2002; Mattos et al., 2000).

2.2.3.2.3 Poly-unsaturated fatty acids

The essential n-6 PUFA linoleic acid (LA) is primarily obtained through consumption of vegetable protein and oils (Wathes et al., 2007). LA is desaturated (addition of a C=C) and elongated (addition of two carbons to the chain) (Figure 2.6) to arachadonic acid (AA), which can be further converted to docosapentanenoic acid (DPA) (Cook and McMaster, 2002; Mattos et al., 2000). Fish and leafy vegetables are rich sources of the essential n-3 PUFA α-linolenic acid (ALA) (Abayasekara and Wathes, 1999). ALA is converted to eicosapentaenoic acid (EPA), which can be further converted to docosahexaenoic acid (DHA) by hepatic desaturases and elongases (Figure 2.6) (Abayasekara and Wathes, 1999; Cook and McMaster, 2002).

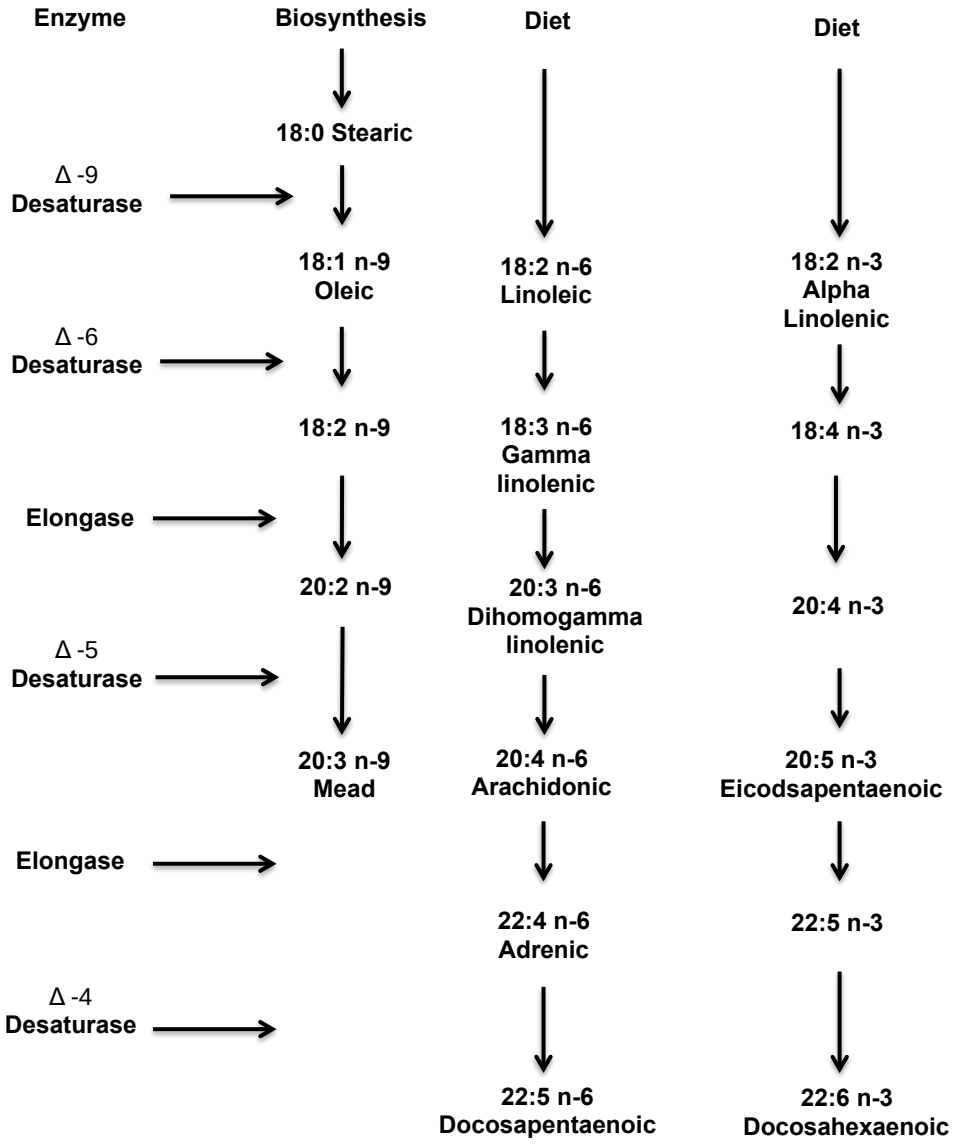


Figure 2.6 – Pathways for the metabolism of essential n-3 and n-6 PUFA. Adapted from (Abayasekara and Wathes, 1999).

2.2.3.3. Transport and storage

Fatty acids must be transported and stored throughout the body so they can meet their various biological functions. Fatty acids can circulate throughout the body in a free manner bound to albumin (Coelho et al., 2013), but are commonly integrated within TAG of the lipoprotein core (Thaxton et al., 2016). TAG is synthesised from the condensation reaction of glycerol and three fatty acids, forming ester-link functional groups between the glycerol and each single fatty acid. Following this, the TAG can be incorporated into a lipoprotein particle for transport to peripheral tissue or be stored within a lipid droplet as an energy reserve (Feng et al., 2013; Thaxton et al., 2016). Fatty acids can also be stored within the phospholipids of the cell membrane (Hac-Wydro and Wydro, 2007). Phospholipids are also formed through a condensation reaction between a glycerol, two fatty acids, and a phosphate.

2.2.3.4. Function

Fatty acids are a source of storable energy released from their lipid droplet and TAG backbone through the oxidative process known as lipolysis (Coelho et al., 2013; Feng et al., 2013).

The mobilisation and breakdown of fatty acids for energy is regulated by a range of endocrine signals including adipokines, insulin, and adrenaline, which can change in response to consumption of food, stress or exercise (Duncan et al., 2008). Binding of these hormones to their receptors e.g. adrenaline to its β-adrenoceptor will result in protein

kinase A induced remodelling of the lipid droplet, providing the lipases access to TAG (Duncan et al., 2008). Three enzymes are responsible for the breakdown of TAG to glycerol and free fatty acids. Adipose triglyceride lipase is the enzyme responsible for the first stage of TAG catabolism, converting TAG to DAG and freeing a fatty acid (Rosen and Spiegelman, 2014). DAG is then broken down by hormone sensitive lipase, freeing the second fatty acid from the glycerol backbone (Duncan et al., 2008). Monoglyceride lipase catabolises the release of the final fatty acid from the glycerol backbone, completing the oxidation of TAG within the adipocyte (Rosen and Spiegelman, 2014). These fatty acids are released into the circulation, where they are bound to serum albumin, and transported to peripheral tissue e.g. skeletal muscle (Coelho et al., 2013).

Once taken up by the peripheral tissue, the fatty acids are oxidised to acetyl-CoA within the mitochondria, where it can be fed directly into the tricarboxylic acid (TCA) cycle to generate energy (Berg et al., 2011; Coelho et al., 2013; Medina-Gómez, 2012). Excess glycerol is transported to the liver where it can be converted to glucose or pyruvate for glycolysis (Berg et al., 2011).

Another important function of fatty acids is in the synthesis of phospholipids, which are vital to the formation of the cell membrane lipid bilayer that encapsulates the cell (Hac-Wydro and Wydro, 2007). As mentioned previously, phospholipids are synthesised through the

esterification of two fatty acids and a phosphate with a glycerol, which is subsequently inserted into the plasma membrane. The type of fatty acid incorporated into the phospholipid will directly modulate the fluidity of the membrane (Murphy, 1990). Change in membrane fluidity can alter membrane integral protein expression and function, impacting upon the activation of intracellular pathways (Murphy, 1990). For example, rats fed a diet deficient in essential fatty acids reduces adenylyl cyclase activity, compromising the downstream signalling of the β-adrenergic receptor within the cardiovascular system (Murphy, 1990).

Fatty acids sequestered within the phospholipids of the cell membrane can be released by cytoplasmic phospholipases for the synthesis of eicosanoids (Abayasekara and Wathes, 1999; Wathes et al., 2007). PUFA's esterified in carbon 2 position of the phospholipid are commonly released for the synthesis of eicosanoids, including prostaglandins (PG's), leukotrienes, and thromboxanes (Mattos et al., 2000). For example, the n-6 PUFA AA can be released from this position by phospholipase A_2 for the synthesis of 2-series inflammatory associated PG's (Trujillo and Broughton, 1995; Wathes et al., 2007). Eicosanoids can stimulate contraction or relaxation of smooth muscle, modulate inflammation, and clotting factor expression. Further to this, PG's have been shown to play a vital role in parturition, which will be discussed later in this review.

2.3. The uterus

The uterus is the female reproductive organ that allows mammals to carry and deliver live young. The anatomy of the uterus differs between mammalian species, but its biological function inherently remains the same. The human uterus (Figure 2.7A) is a simplex muscular hollow organ maintained in position of anterversion and anteflexion within the pelvis via the round and broad ligaments (Verralls, 1993). The human uterus is composed of lower and upper segments, which are anatomically distinct. The supravaginal and infravaginal sections of the cervix compose the narrow lower segment of the uterus that dilates and effaces during labour, allowing delivery of the foetus (Verralls, 1993). The upper segment of the uterus is composed of the corpus and fundus, and is a major site of contraction during labour (Gray, 1974); the fundus is also the site of implantation during a healthy pregnancy. The uterus of the rat (*Rattus rattus*) is anatomically distinct but physiologically similar to the human uterus (Figure 2.7B). The rat has a duplex uterus, meaning it has two distinct uteri or uterine horns shared by a single cervix and vagina. Each horn has an exclusive ovary located at the distal end of the horn providing the ovum(s) through a single fallopian tube. The duplex uterus of the rat is also housed within the pelvis via the round and broad ligaments, with blood supply maintained via the ovarian and uterine arteries (Plant and Zeleznik, 2015).

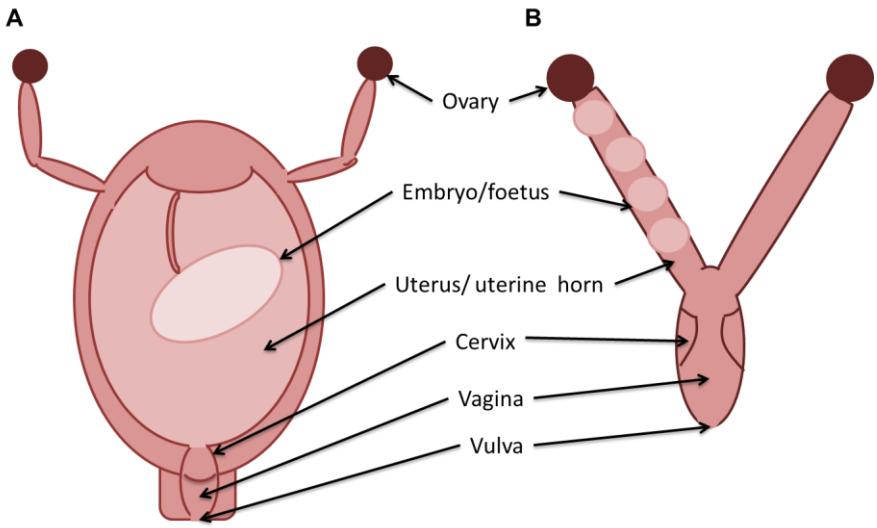


Figure 2.7- The anatomy of the human (**A**) and rat (**B**) uterus.

Regardless of species, the uterus is composed of three distinct tissue layers – the endometrium, the myometrium, and the perimetrium (Figure 2.8). The endometrium is the inner most tissue layer of the uterus, lining the uterine cavity. It supports a range of mucous membranes and receptors ideal to support the changing uterine environment that occurs during the menstrual cycle, gestation and parturition. The myometrium is the median layer of the uterus composed of interlacing layers of smooth muscle bundles and vasculature; this will be discussed further later in this review. The perimetrium is actually just the peritoneum that covers and isolates the uterus from the rest of the organs within the abdominal cavity. The perimetrium does provide the bedrock for the formation of the broad ligaments that fix the uterus in place within the abdomen (Verralls, 1993).

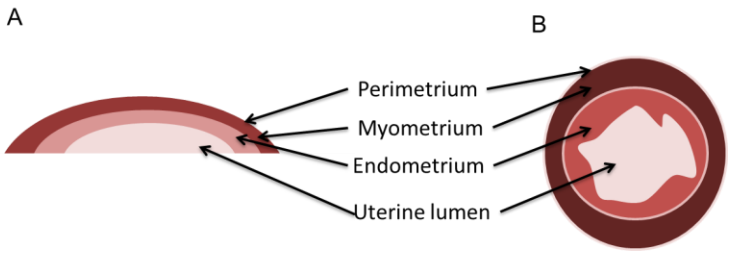


Figure 2.8 – Cross-sections of the (**A**) human and (**B**) rat uterus tissue layers.

2.3.1. The myometrium

The myometrium is the median tissue layer of the uterus, producing contractions essential to the delivery of the foetus during labour. The myometrium is composed of bundles of uterine myocytes that form three distinct myometrial layers of smooth muscle (Figure 2.9). The innermost layer is connected to the endometrium and is formed of circular smooth muscle. The middle oblique layer is formed of interweaving smooth muscle fibres of the inner circular and outer longitudinal layers of the myometrium, which is highly vascularised (Verralls, 1993). The outermost layer of the myometrium is composed of smooth muscle bundles organised in a longitudinal manner. These distinct layers of smooth muscle function collectively to apply force along the *x, y* and *z* axes, which is essential to deliver the foetus into the extra-uterine environment.

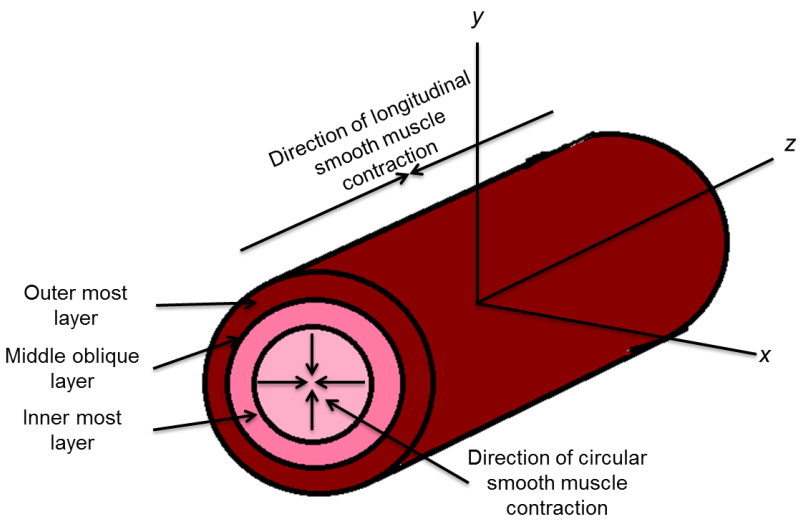


Figure 2.9 – Orientation and direction of contraction of the myometrial smooth muscle layers in the rat.

2.3.2. Uterine myocytes

Uterine myocytes are a type of smooth muscle cells that populate and arrange into indistinct bundles to form the layers of the myometrium. These bundles consist of uterine myocytes organised in a series or parallel order, up to 300-600µm in length (Sims et al., 1982). Uterine myocytes are 5-10µm in size, predominantly composed of filamentous cytoskeletal proteins and contraction machinery (Aguilar and Mitchell, 2010). Further to the normal organelles present within the cell cytoplasm, uterine myocytes like other muscle cells contain a sarcoplasmic reticulum (SR). The SR is a distinct organelle essential to storage and release of intracellular calcium ions (Ca^{2+}) (Wray and Shmygol, 2007). Uterine myocytes express a diverse range of membrane-bound and cytosolic proteins essential to contraction and relaxation of the myometrium including, G protein coupled receptors (GPCR's), ion channels, and gap junctions (Brenninkmeijer et al., 1999;

A Shmygol et al., 2007; Wray et al., 2003). Uterine myocytes are sensitive to the ever-changing uterine milieu of gestation and parturition. As a result, uterine myocytes progress from a state of quiescence to a state of stimulation, characterised by hyperplasia, hypertrophy, and an increase in contractile associated proteins (CAP's) and cellular communication (Challis et al., 2000; Shynlova et al., 2010).

2.4. Physiology of myometrial contractions

Uterine contractions are events that resonate from the myometrium, capable of increasing intra-uterine pressure sufficiently to deliver the foetus to the extra-uterine environment. These events result from synchronous contraction and relaxation of myometrial smooth muscle bundles, commonly propagated by a significant electro-chemical change (Challis et al., 2000). Throughout gestation, the uterus is maintained in a state of relative quiescence to facilitate implantation and development of the growing foetus. This is known as the brake effect, whereby a range of endocrine signals limit uterine contractile mechanisms and myocyte communication (Smith et al., 2015). Despite this effect, the uterus can undergo bouts of contractility during gestation. These are known as Braxton-Hicks contractures, which are small sporadic uncoordinated events that commonly have no clinical outcome or significance (Verralls, 1993).

Two dominant uterine contraction phenotypes have been identified during term labour, and they can illustrate the potential outcome of

labour and if obstetric intervention is required. The plateau contraction phenotype is associated with positive labour outcomes, and is characterised by high amplitude, biphasic synchronous contraction and relaxation of myometrial smooth muscle bundles at regular intervals (Anatoly Shmygol et al., 2007) (Figure 2.10A). Unlike the plateau contraction phenotype, the spike bundle contraction phenotype is associated with negative labour outcomes. The spike bundle contraction phenotype consists of irregular multiphasic contractions of fluctuating frequency and amplitude, characterised by asynchronous contraction and relaxation of uterine smooth muscle bundles (Anatoly Shmygol et al., 2007) (Figure 2.10B).

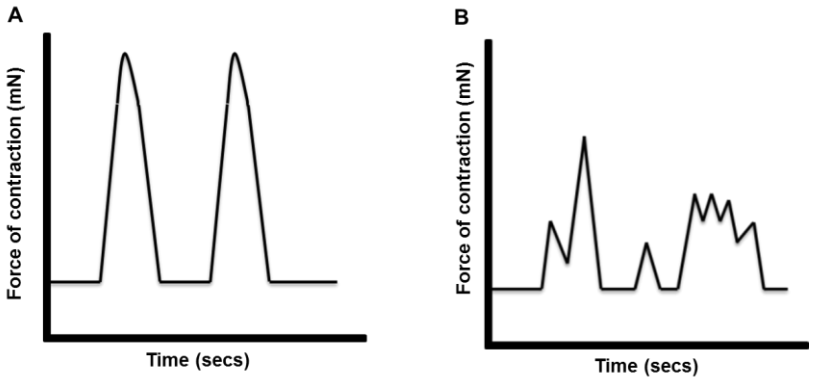


Figure 2.10 – Illustration of a (**A**) Plateau and (**B**) Spike Bundle contraction phenotype.

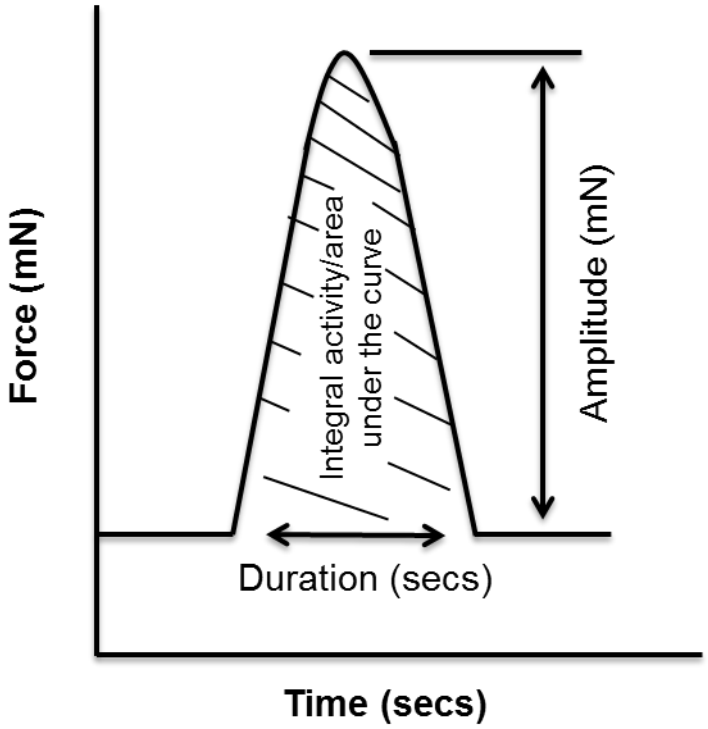


Figure 2.11 – Parameters of a uterine contractile event.

The quality of term-labouring uterine contractions can be further assessed by quantification of 4 distinct parameters including, integral activity, frequency, amplitude, and duration of contraction (Figure 2.11) (Aguilar and Mitchell, 2010). Integral activity, also referred to as area under the curve, is a measurement of the rate of change in force (mN) per second. The frequency of uterine contractions is defined by the number of contractions within a given time frame. Amplitude is the maximal force attained during a single contractile event relevant to its baseline. The duration of a uterine contraction is defined by the time it takes from its initiation till the event returns back to baseline. Many factors (discussed later) can directly or indirectly modulate these contractile parameters including the number of myocytes and their communication with other myocytes (Aguilar and Mitchell, 2010; Challis et al., 2000).

The quality of uterine contractions and its quantitative parameters are measured through a range of methods including intra-uterine pressure catheter, electromyography, or force measurement via a tocodynamometer (Euliano et al., 2013). These methods are effective within a clinical setting, but artefacts are commonplace when the foetus descends through the uterus into the vaginal canal. As a result, groups are developing systems to measure the electrical activity emitted by the contracting uterus via abdominal sited electromagnets (Eswaran et al., 2010; Ramon et al., 2005). In rats, uterine contractility can be measured via intra-uterine pressure catheter or uterine surface

electrodes, both of which can be implanted during terminal or recoverable surgical procedures (Buhimschi et al., 1998; Moustafa et al., 1999; Pierce et al., 2010). Qualitative and quantitative assessment of uterine contractions is essential in determining if a parturient will progress through labour to delivery or if they will require obstetric intervention.

2.5. Regulation and coordination of myometrial contractions

Myometrial contraction and relaxation is propagated by a significant change in intracellular ion concentrations via extracellular uptake or release from intracellular stores (Wray, 1993). The proteins and mechanisms responsible for the uptake and release of ions will be discussed to highlight how the uterus can shift from relative quiescence to a state of excitability at term.

2.5.1. Membrane potential

The cell membrane is the amphipathic bilayer composed of cholesterol, phospholipids, fatty acids, and proteins that encapsulates and segregates the cytoplasm/cytosol from the extracellular environment. Ion channels, receptors, and gap junctions essential to the contraction and relaxation of the uterus are supported within the cell membrane, and within the cholesterol-rich Ω-shaped invaginations of the membrane, known as caveolae (Okamoto et al., 1998; Schlegel et al., 1998). These invaginations of the cell membrane are supported by their cholesterol-rich structural proteins caveolins, of which Cav-1 is the

predominant uterine isoform (Brainard et al., 2005). To understand how these membrane proteins function and elicit a contractile event, the basis of membrane potential and excitability must be explained.

Membrane potential is the value defined by the electrical potential of the plasma membrane, resulting from the difference between the extracellular and intracellular ion concentrations; this difference in the extracellular and intracellular ion concentrations is the electrochemical gradient.

The resting membrane potential is the basal electrical potential of the plasma membrane when the electrochemical gradient has reached equilibrium. Resting membrane potential is commonly -80mV but this can increase depending on the physiological state e.g. parturition where is can increase to -50mV (Wray, 1993). Action potential is an event triggered by depolarisation of the membrane (+30mV) resulting from a positive shift in the electrochemical gradient (Berg et al., 2011).

An action potential is characterised by a rapid intracellular influx of ions, increasing the membrane potential. Depolarisation of the membrane leads to hyperpolarisation characterised by an efflux of intracellular ions sufficient to return the cell to its resting membrane potential. The magnitude and duration of the transient change in the membrane potential can occur spontaneously, or by ion channels and ion pumps that passively and actively diffuse ions across the plasma membrane,

respectively (Smith et al., 2015). In relation to uterine contractions, the magnitude and duration of the action potential will directly dictate if the resulting contractile phenotype forms a plateau or spike (Anatoly Shmygol et al., 2007; Wray, 1993).

2.5.2. Ion channels

Ion channels are a family of pore forming trans-membrane proteins that facilitate passive diffusion of ions across the cell membrane with the electrochemical gradient. Predominantly, potassium (K^+) and calcium (Ca^{2+}) ion channel types are responsible for the depolarisation and hyperpolarisation of the plasma membrane (Wray et al., 2015). Activation of Ca^{2+} ion channels provides an influx and increase in cytosolic Ca^{2+} capable of triggering the contractile pathway (Challis et al., 2000). In response to the sudden increase in cytosolic Ca^{2+}, K^+ channels are activated causing an efflux in K^+, returning the membrane to its basal potential and relaxing the myocyte (Brainard et al., 2007). Expression and activation of these channels are vital in determining the quiescent or stimulatory state of the uterus throughout gestation and parturition.

2.5.2.1. Potassium Channels

K^+ channels maintain relative uterine quiescence through repolarisation of the membrane via K^+ efflux. Expression of K^+ channels remain high throughout gestation to depress the magnitude and duration of the action potential (Aguilar and Mitchell, 2010; Smith et al., 2015).

There is a diverse subset of K^+ channels all activated through different mechanisms, with varying levels of expression. These include voltage-gated, Ca^{2+} sensitive, and adenosine triphosphate (ATP) sensitive K^+ channels (Brainard et al., 2007). The subset of K^+ channels dictates by which mechanism(s) it is activated. Voltage-gated K^+ channels are activated by the positive shift in membrane potential caused by depolarisation of the membrane (Khan et al., 2001).

Ca^{2+} sensitive K^+ channels are only activated upon a sufficient increase in the global cytosolic Ca^{2+} concentrations, resulting from a substantial influx or intracellular release of Ca^{2+} (Ghatta et al., 2006). ATP sensitive K^+ channels are activated by adenosine diphosphate (ADP), and maintained in an inactive state by ATP (Dunne and Petersen, 1986). ADP results from the dephosphorylation of ATP, required to generate myosin-actin cross bridging for the contraction of the myocyte (Wray, 1993); myosin-actin cross bridging is a Ca^{2+} sensitive mechanism (Taggart and Morgan, 2007). Thus if ADP is generated, it will activate the ion channel, allowing K^+ efflux from the cell, contributing to the relaxation of the myocyte. K^+ channel activity is also regulated through a range of 2^{nd} messenger activated protein kinases (A and C), these kinases are active through the contractile and relaxation pathways (Jonas and Kaczmarek, 1996).

Expression of these K^+ channels is found to be high during gestation, but they progressively decrease upon activation of the uterus,

facilitating a greater duration of Ca^{2+} influx during depolarisation (Smith et al., 2015). This will contribute to the formation of biphasic, plateau, high amplitude contractions during term labour.

2.5.2.2. Calcium channels

Ca^{2+} is an electrolyte essential to maintenance of basal tone and contraction of uterine smooth muscle, entering the cell via Ca^{2+} ion channels. Voltage and ligand gated Ca^{2+} ion channels are the two major subsets responsible for the influx of extracellular Ca^{2+} during depolarisation of the cell (Wray and Arrowsmith, 2012).

The voltage-gated Ca^{2+} ion channel class consist of low voltage transient (T) type and high voltage long-lasting (L) type channels (Anatoly Shmygol et al., 2007). T-type channels can be opened via a small action potential, but only for a short period of time creating a spike contraction phenotype (Anatoly Shmygol et al., 2007). Unlike the T-type channels, high-voltage L-type channels are opened upon full depolarisation of the plasma membrane that facilitates a sufficient influx of Ca^{2+} into the cytosol, capable of activating contractile mechanisms (Wray, 1993). L-type Ca^{2+} channels are associated with formation of a plateau contraction phenotype (Anatoly Shmygol et al., 2007).

Ligand-gated channels open only upon binding of their target ligand, and remain open only when their agonist is bound (Noble et al., 2009). The expression of Ca^{2+} ion channels differs between the layers of the

myometrium, and is under endocrine control, with L-type channel expression increasing at term (Challis et al., 2000; Helguera et al., 2002). The activity of these receptor-gated channels can also be regulated through 2nd messenger activated protein kinases (Wray, 1993). The activity, expression, and regulation of Ca^{2+} ion channels are vital to the production of contractions during term labour.

2.5.3. Gap junctions and contraction synchronisation

Synchronisation of myometrial smooth bundles is imperative for efficient effective contraction of the uterus during labour. Lack of synchronisation could promote an aberrant spike bundle contraction phenotype, compromising the outcome of the labour.

In the heart, synchronisation and rhythm of myocardial contraction originates from pacemaker cells located within the sinoatrial node, and propagated throughout the heart via intracellular conduits known as gap junctions (Smith et al., 2015). Despite being hypothesised, pacemaker cells have never been identified within the myometrium (Smith et al., 2015; Wray and Arrowsmith, 2012), but the essential role of gap junctions in synchronising uterine myocyte communication has been highlighted (Döring et al., 2006; Sims et al., 1982; Tong et al., 2009).

Gap junctions are inter-myocyte protein channels that facilitate cell-cell electro-coupling (synchronisation), metabolite (Ca^{2+}), and 2nd

messenger transfer (Challis et al., 2000) (Figure 2.12). These intracellular channels are formed from the connexin family of proteins, arranged into a hexameric pore forming hemi-channel or connexon (Oshima, 2014; Yeager and Nicholson, 1996). Two membrane-integrated connexons adjoin between neighbouring cells leaving a gap of only 2-4nm to form the gap junction conduit (Oshima, 2014). The 43KDa connexin-43 (CX-43) protein has been identified as the predominant isoform in the formation of myometrial gap junctions (Su and Lau, 2014), and is directly responsible for the syncytium of the labouring uterine environment (Smith, 2007). A range of studies have highlighted this through ablation of CX-43, which significantly decrease cellular communication, and prolongs labour in genetically modified animal models (Döring et al., 2006; Tong et al., 2009).

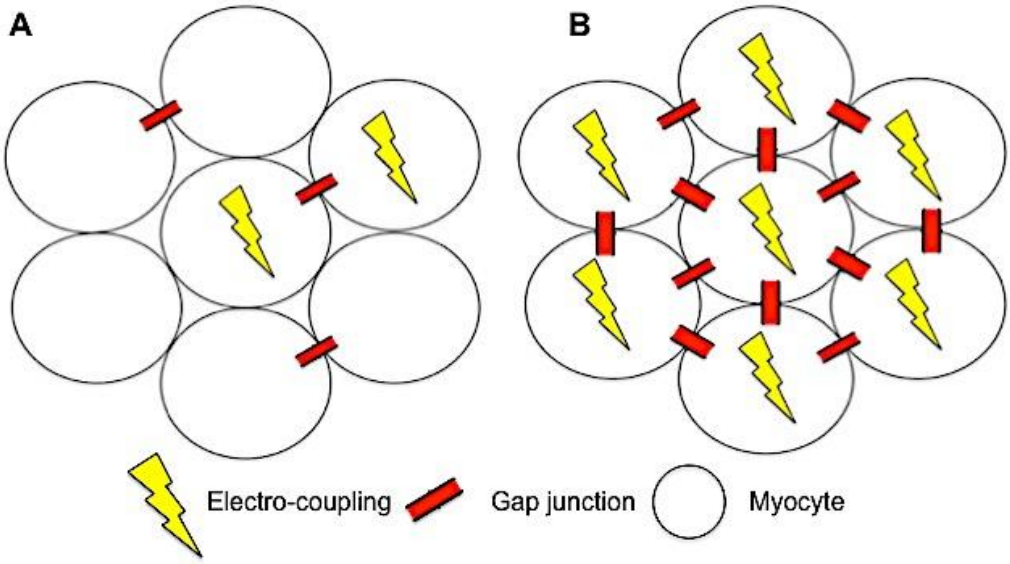


Figure 2.12 – Electro-coupling and gap junction expression of the (**A**) early pregnant (**B**) labouring myometrium.

G protein coupled receptors

GPCR's are one of the largest families of cell-surface bound receptors that transmit the intracellular response of extracellular stimuli across the cell-membrane via their seven transmembrane domains (Pucadyil and Chattopadhyay, 2006). GPCR's internalise upon agonist binding, resulting in the dissociation of the heterotrimeric G protein complex, activating downstream mechanisms (Pucadyil and Chattopadhyay, 2006). Endogenous tocolytics (myometrial relaxants) and uterotonins (myometrial stimulants) transmit their signals via GPCR's, activating downstream mechanisms capable of regulating the release of intracellular Ca^{2+} and myosin-actin cross-bridging (Arrowsmith and Wray, 2014; Challis et al., 2000; Yuan and López Bernal, 2007).

2.5.4. Adenylyl cyclase – cyclic adenosine monophosphate – protein kinase A pathway

Endogenous tocolytics including prostacyclin, corticotrophin releasing hormone (CRH), relaxin, and PGE_2 act through GPCR's to maintain the uterus in a relative state of quiescence via the adenylyl cyclase - cyclic adenosine monophosphate (cAMP) - protein kinase A (PKA) pathway (Figure 2.13) (Yuan and López Bernal, 2007). Binding of the agonist to its target GPCR leads to dissociation of the Gsα subunit from the intracellular heterotrimeric G-protein complex (Challis et al., 2000; Pucadyil and Chattopadhyay, 2006). Gsα increases PKA expression through activation of adenylyl cyclase, promoting synthesis of the cAMP from ATP (Challis et al., 2000). PKA directly maintains uterine

quiescence through phosphorylation of phospholipase C and myosin light chain kinase (MLCK), limiting intracellular Ca^{2+} release and actin-myosin cross-bridging (Yuan and López Bernal, 2007).

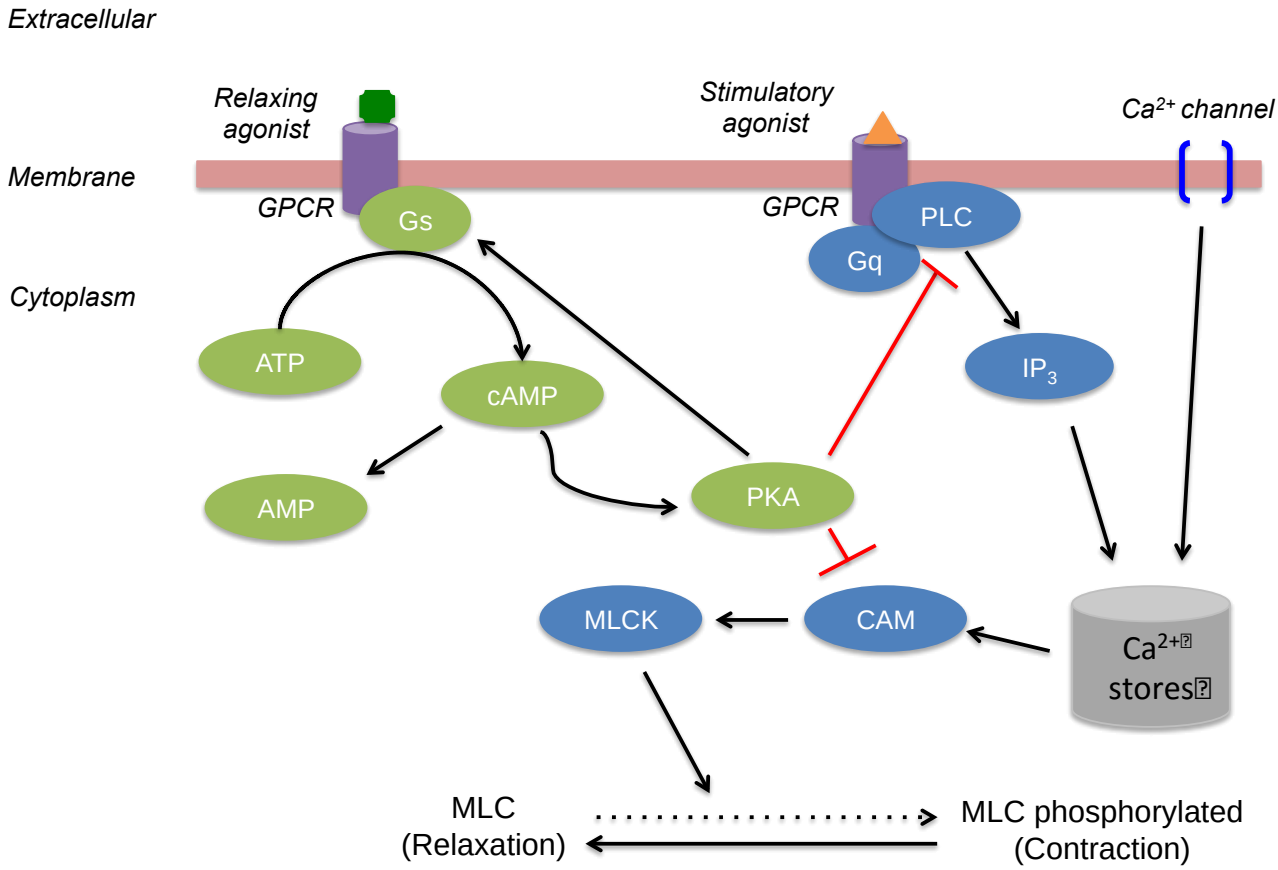


Figure 2.13 – Illustration of the adenylyl-cAMP-PKA and Ca^{2+}-calmodulin-MLCK pathway. Adapted from (Yuan and López Bernal, 2007).

2.5.5. Calcium – calmodulin – myosin light chain kinase pathway

Contraction of the uterus is achieved through GPCR activation of the Ca^{2+}-calmodulin-myosin light chain kinase (MLCK) pathway (Figure 2.13) (Challis et al., 2000; Wray, 2007).

Endogenous uterotonins (oxytocin and $PGE_2/F_{2\alpha}$) dissociate the intracellular heterotrimeric G protein complex, allowing $G_{\alpha q/11}$ to activate phospholipase C (Aguilar and Mitchell, 2010). Phospholipase C hydrolyses phosphatidylinositol bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and releases diacylglycerol from the plasma membrane (Challis et al., 2000; Kim et al., 2012). AA is cleaved from the DAG, providing substrate for prostaglandin synthesis (Wray, 1993). IP_3 binds to its receptor on the surface of the sarcoplasmic reticulum, stimulating release of Ca^{2+} from this organelle (Noble et al., 2009). A sufficient increase in global cytosolic Ca^{2+} concentrations will lead to calmodulin induced activation of MLCK; calmodulin activated upon binding of four Ca^{2+} ions (Challis et al., 2000). Calmodulin activates phosphodiesterase, leading to breakdown of cAMP, dampening PKA induced smooth muscle relaxation (Challis et al., 2000). Increased cytosolic Ca^{2+} concentrations induce conformational changes to the tropomyosin-troponin complex, freeing the actin-binding site (Barua et al., 2012). Activated MLCK stimulates ATPase (O Brien et al., 2013), allowing it to phosphorylate Serine19 of the myosin 20KDa light chain (MLC_{20}) (Aguilar and Mitchell, 2010; Taggart, 2001), resulting in

contraction of the smooth muscle by myosin-actin cross-bridging (Barua et al., 2012; Taggart and Morgan, 2007). Myosin phosphatase dephosphorylates the myosin light chain, relaxing the smooth muscle and forming ATP (O Brien et al., 2013). It should be noted, only a sufficient influx of Ca^{2+} ions via ion channels will promote myometrial contraction through this mechanism (Wray, 1993). Store operated Ca^{2+} entry (SOCE) and Ca^{2+}/ATPase help in maintaining basal cytosolic Ca^{2+} concentrations and sarcoplasmic reticulum stores (Noble et al., 2009).

2.6. Gestation and parturition

Gestation and parturition is a complex multifactorial process regulated by an array of endogenous signals resulting in the delivery of the foetus to the extra-uterine environment. This section aims to review and highlight the complex mechanisms regulating gestation and labour, and to highlight the limitations in our current knowledge.

2.6.1. Phases of parturition

2.6.1.1. Phase 0 - gestation

Phase 0 covers the majority of gestation, where by the uterus is maintained in a state of relative quiescence to facilitate foetal growth and development (Challis et al., 2000). Relative uterine quiescence is predominantly maintained through the pro-gestational effects of progesterone (Smith et al., 2012).

2.6.1.2. Phase 1 – uterine activation

This is the phase where the uterus is primed and prepared into a stimulatory phenotype. As gestation progresses, increasing oestrogen and uterine stretch (mechanical stimuli) begin to overcome the sedentary effect(s) of progesterone, preparing the uterus for labour (Challis et al., 2000); the functional withdrawal of progesterone also contributes to this (Mesiano and Welsh, 2007). This results in an increase in the cassette of contractile-associated proteins (CAP's) including CX-43, Cav-1, oxytocin receptor (OXTR), cyclooxygenase-2 (COX-2), and L-type Ca^{2+} ion channels priming the uterus for labour (Bogaerts et al., 2013). Oestrogen will also up-regulate expression of myosin light chain kinase (MLCK) and calmodulin increasing myometrial sensitivity to Ca^{2+} (Snegovskikh *et al.*, 2006).

2.6.1.3. Phase 2 – stimulation

Phase 2 is the stimulatory phase, where by which the uterus contracts in a synchronous biphasic manner to deliver the foetus into the extra-uterine environment (Challis et al., 2000). This is achieved through the increase in uterine CAP expression, change in the hormonal milieu, uterine stretch, and the activation of the pro-inflammatory immune response (Smith, 2007).

The progress of the parturient through labour is monitored by the rate of cervical dilation over time (Figure 2.14). This progress is illustrated within the Friedman curve, where labour is split into two distinct stages.

Stage 1 consists of a latent and active phase where by the cervix dilates from 0-10cm over the course of a 14-hour period (Friedman, 1955). Once the cervix is fully dilated (10cm), the parturient will enter the 2nd stage of labour where they can actively push in concert with their contractions to deliver the foetus vaginally (Friedman, 1955).

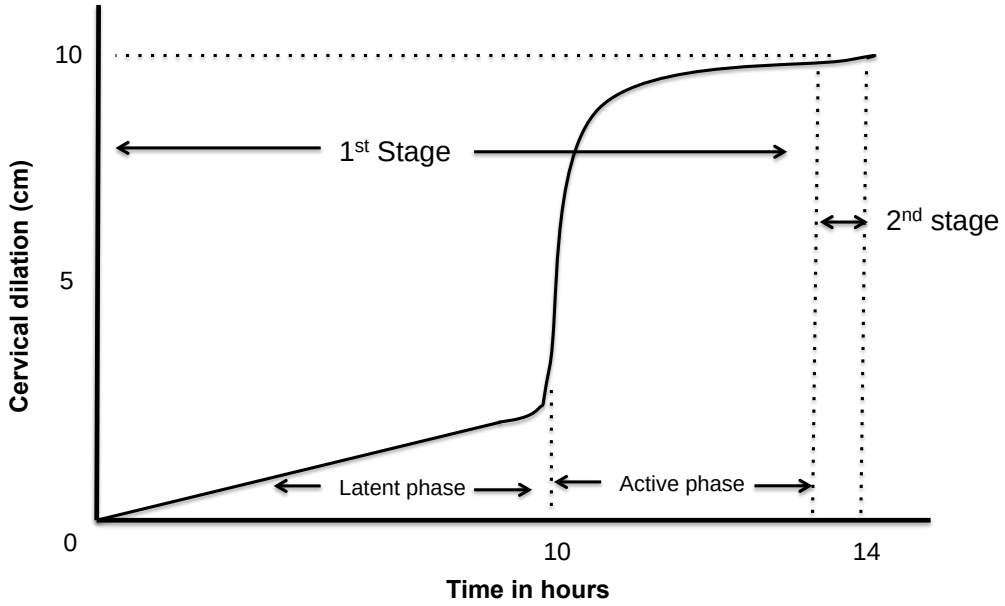


Figure 2.14 – The Friedman curve illustrating the rate of cervical dilation of 500 nulliparous parturients during labour. Adapted from (Friedman, 1955)

2.6.1.4. Phase 3 - postpartum

Phase 3 is the postpartum period that consists of placental delivery, uterine involution, and lactation (Challis et al., 2000). Six weeks from the beginning of the postpartum period, the woman should return to a normal menstrual cycle, able to conceive soon after (Shynlova et al., 2009).

2.6.2. Endocrinology of gestation and parturition

Pregnancy and labour is tightly regulated through an array of endocrine signals (Figure 2.15) that maintain the uterus in a relative state of quiescence, promote foetal organ maturation, and uterine activation.

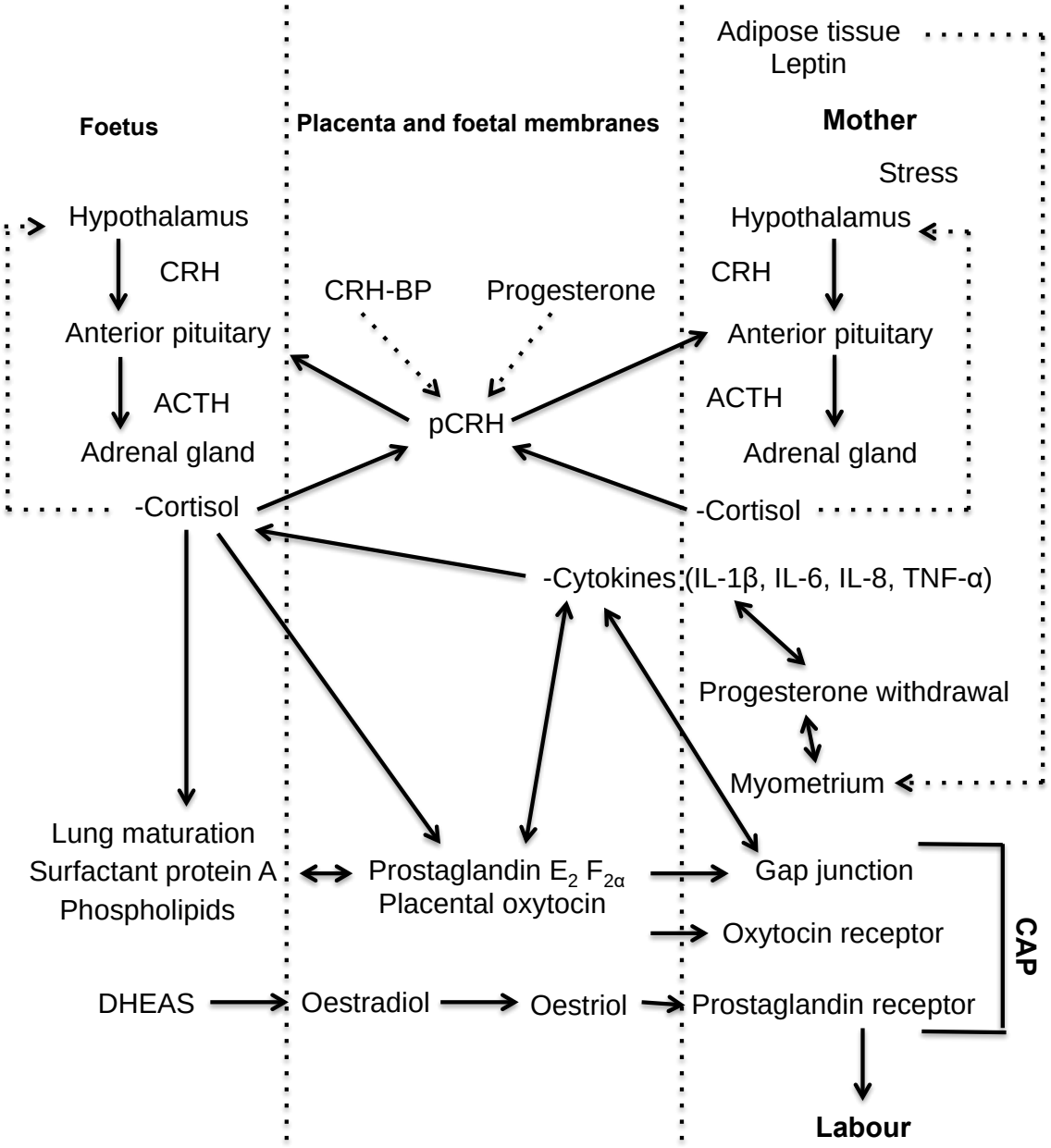


Figure 2.15 – Overview of the endocrine pathways that govern gestation and parturition. Adapted from (Bogaerts et al., 2013).

2.6.2.1. Progesterone

Progesterone is the pro-gestational sex-steroid hormone synthesised from cholesterol via the steroid biosynthesis pathway (Miller, 2013). Its pro-gestational role has been demonstrated through numerous studies where by the administration of its antagonist promotes abortion or premature labour (Shynlova et al., 2009).

During ovulation, progesterone is synthesised by the corpus luteum, promoting differentiation of the functionalis (Henriet et al., 2012). In rats, progesterone is continuously synthesised by the corpus lutuem until lutolysis at term (Figure 2.16) (Mitchell and Taggart, 2009). Unlike rats, *Homo sapiens* generate progesterone via the corpus luteum until week 7 of gestation, where the placenta then takes over until term (Snegovskikh et al., 2006).

Progesterone promotes uterine quiescence through genomic pathways, maintaining the brake effect (Mesiano and Welsh, 2007; Smith et al., 2015). Progesterone elicits its pro-gestational genomic effect(s) by activating its nuclear receptor PRB and to a lesser extent PRA (Mesiano and Welsh, 2007). PRB is the active isoform that sustains the genomic pathways responsible for uterine quiescence (Kowalik et al., 2013). PRA is the truncated isoform lacking the N-terminal activating domain, which is only capable of inactivating PRB and its downstream effect(s) (Smith, 2007). Through PRB, progesterone is able to supress

oestrogen and stretch induced contractile-associated protein (CAP) gene transcription, sustaining the uterus in a quiescent state (Bogaerts et al., 2013; Challis et al., 2000).

Further to this, progesterone maintains the maternal and foetal tissues in an anti-inflammatory state by depressing nuclear factor kappa B (NF-κB) activation, and the synthesis of PG's by sustaining PG dehydrogenase (PGDH) activity (Challis et al., 1997; Tan et al., 2012). As stated previously, progesterone will supress CX-43 expression, but it will also inhibit its translocation to the plasma membrane and thus the formation of functioning gap junctions (Hendrix et al., 1995).

Progesterone also impedes the activity of the Ca^{2+}-calmodulin-MLCK pathway, by lowering A-Kinase anchoring protein expression, and thus increasing PKA activity (Mesiano and Welsh, 2007). Progesterone continues to sustain its pro-gestational effects until its withdrawal at term. In rats, term labour is initiated by the sterile pro-inflammatory synthesis of $PGF_{2\alpha}$, causing luteolysis and withdrawal of progesterone (Mitchell and Taggart, 2009).

In humans, no progesterone withdrawal occurs at term, but its functional withdrawal has been described (Bogaerts et al., 2013; Snegovskikh et al., 2006). It is hypothesised, that the promotion of sterile inflammation via the hypothalamic-pituitary-adrenal (HPA) axis is capable of increasing PRA expression, which will impede the PRB mediated pro-gestational genomic effects of progesterone (Mesiano and Welsh, 2007). Sterile inflammation will also drive the synthesis of

PG's capable of dilating the cervix and stimulating contraction of the uterus (Petraglia et al., 2010). Decreased PRB activity facilitates oestrogen and stretch induced CAP gene transcription, providing the uterus with the necessary machinery to contract in a biphasic synchronous manner (Bogaerts et al., 2013; Challis et al., 2000). Further to this, both PG's and oestrogen will promote the synthesis of oxytocin, which will contribute to the development of uterine contractions during labour (Arrowsmith and Wray, 2014).

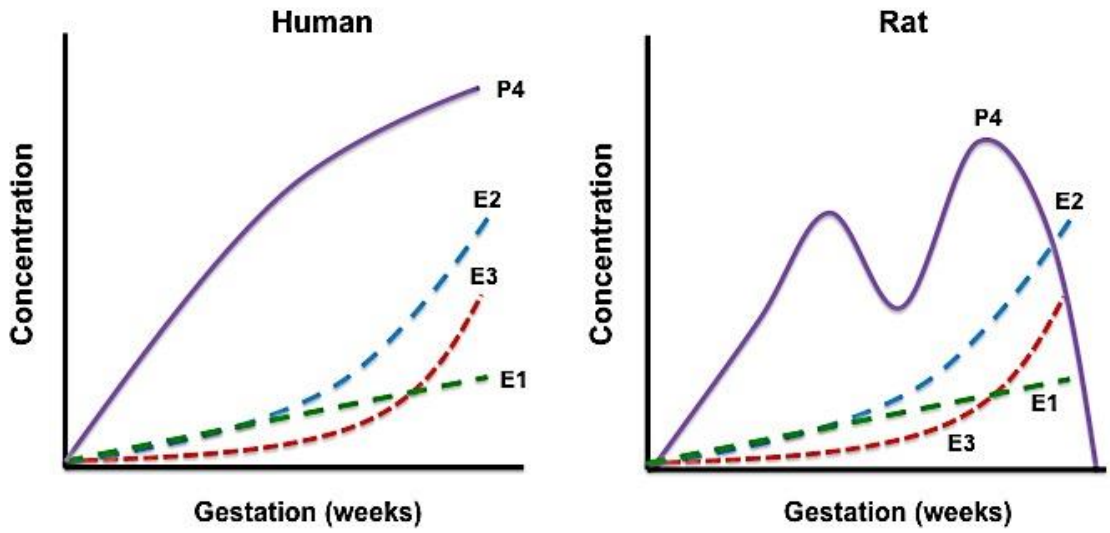


Figure 2.16 – Representative change in maternal progesterone (P_4) and oestrogens [oestrone (E_1), oestradiol (E_2), and oestriol (E_3)] as gestation progress towards term. Adapted from (Derbyshire, 2011; Mitchell and Taggart, 2009).

2.6.2.2. Oestrogen

Oestrogen is a collective term for the sex steroids oestrone, oestradiol, and oestriol synthesised from cholesterol via the steroid biosynthesis pathway (Wang et al., 2009). Oestrogen concentrations remain low during early gestation, but then increase from mid gestation to delivery

(Figure 2.16) (Bogaerts et al., 2013). The primary function of oestrogen is to activate the uterus at term (phase 1), beginning the transition from phase 0 to phase 2 of parturition (Challis et al., 2000). Oestrogen successfully directs this transition by promoting the transcription of uterine CAP's including CX-43, Cav-1, COX-2, OXTR, PG receptors and Ca^{-2+} ion channels through its nuclear receptors ER_{α} and ER_{β}, which remain low throughout gestation until the onset of parturition (Challis et al., 2000; Mesiano and Welsh, 2007). This in conjunction with the functional withdrawal of progesterone will successfully activate the uterus for parturition.

2.6.2.3. Hypothalamic-pituitary-adrenal axis

The HPA axis (Figure 2.15) is a maternal and foetal endocrine pathway essential to foetal pulmonary maturation, uterine activation, and potentially the timing of labour (Bogaerts et al., 2013).

2.6.2.3.1 Overview

The maternal axis constitutes the HPA activity of early gestation, until the foetal HPA axis takes over for the remainder of the pregnancy (Challis et al., 2000). The HPA axis commences with the hypothalamic synthesis and release of corticotropin-releasing hormone (CRH), though the placenta will become the primary site of CRH synthesis as gestation advances (Bogaerts et al., 2013). CRH stimulates the synthesis and release of adrenocorticotropin hormone (ACTH) from the anterior lobe of the pituitary gland (Challis et al., 2000). ACTH promotes the conversion and release of cortisol from cortisone [via

11β-hydroxysteroid dehydrogenase-1 (11β-HSD-1)] within the adrenal cortex (Snegovskikh et al., 2006). As the final product of this pathway, cortisol will promote foetal lung development by increasing lung surfactant protein A and phospholipid production (Bogaerts et al., 2013). Cortisol will also act as a positive feed-forward signal, promoting placental and hypothalamic synthesis of CRH (Challis et al., 2000). Throughout gestation, CRH-binding-protein (CRH-BP) will bind and decrease the bioavailability of CRH, dampening the feed-forward mechanism (Golightly et al., 2011). At term, placental CRH-BP expression decreases, increasing the bioavailability of CRH and driving the HPA axis forward (Jeschke et al., 2005). The HPA axis has important roles in both sustaining uterine quiescence, but also in uterine activation, and potentially the timing the onset of labour.

2.6.2.3.2 The role of the Hypothalamic-pituitary-adrenal axis in uterine quiescence

Throughout gestation, the HPA axis plays a contributory role in sustaining uterine quiescence. CRH is able to contribute to this through activation of the adenylyl cyclase-cAMP-PKA relaxation pathway via its receptors, CRH-R1 and -2 (Whittle et al., 2001). Further to this, CRH is able to effect T-and L-type Ca^{2+} channel activation, and increase ATP sensitive K^{+} channel expression (Petraglia et al., 2010). This will contribute to maintaining a quiescent contraction phenotype during gestation.

2.6.2.3.3 The role of the Hypothalamic-pituitary-adrenal axis in uterine activation

Despite CRH sustaining quiescent mechanisms, it also contributes to the activation of the uterus by promoting ACTH synthesis. While promoting the synthesis of cortisol, ACTH promotes the release of DHEA and dehydroepiandrosterone sulphate (DHEAS) from the maternal and foetal adrenal glands, respectively (Petraglia et al., 2010). DHEA and DHEAS are sex steroid precursors, which are converted within the placenta to oestrogens, namely oestradiol and oestriol, respectively (Snegovskikh et al., 2006). Positive feed-forward activation of the HPA axis increases the release of the sex steroid precursors, with the resulting oestrogens activating the uterus (Challis et al., 2000). Cortisol will also directly contribute to uterine activation by driving the pro-inflammatory production of PG's through the synthesis of surfactant protein A and phospholipids (Reinl and England, 2015). These PG's are capable of nulling the pro-gestational effect of progesterone, dilating the cervix, and promoting myometrial contractility (Bogaerts et al., 2013). Further to this, CRH may also directly contribute to the inflammatory milieu of parturition, as it has been identified to increase cytokine production and uterine CAP expression through NF-κB *in vitro* (You et al., 2014). As a result, CRH is hypothesised to be central to the timing of labour (You et al., 2014), but further work is required to fully evaluate this.

2.6.2.3.4 Summary of the Hypothalamic-pituitary-adrenal axis

The HPA axis plays an essential role in gestation and parturition, through supporting uterine quiescence, activation, and foetal lung maturation. In other mammals, namely *Ovis aries*, foetal cortisol derived from the HPA axis plays a central role in triggering parturition (Challis et al., 2001). Unlike sheep, human parturition is not triggered by cortisol alone, but cortisol and the HPA axis plays a vital role in this multi-factorial process (Challis et al., 2000, 2001).

2.6.2.4. Relaxin

Relaxin is a peptide hormone that contributes to sustaining relative uterine quiescence through activation of the adenylyl cyclase-cAMP-PKA pathway (Challis et al., 2000). Concentrations of relaxin are at their peak during the first trimester, where they decrease and remain low throughout the rest of gestation (Petraglia et al., 2010). Relaxin's role is not vital to sustaining gestation, as pregnancies where relaxin is low still progress to term without complication (Petraglia et al., 2010).

2.6.2.5. Prostaglandins

Prostaglandins are a group of eicosanoids synthesised from membrane-released PUFA's that can modulate smooth muscle contractility (Challis et al., 1997).

Three distinct series of PG's can be synthesised from PUFA's, of which the pro-inflammatory associated 2-series have the greatest effect upon

the myometrium (Myatt and Lye, 2004). 2-seris PG's (PGE_2 and $PGF_{2\alpha}$) are synthesised from PLA_2 released non-esterified AA within maternal and foetal tissues via COX-1 and -2 (Vichai et al., 2005). COX-1 is the constitutively active PG synthesising enzyme, which continuously maintains relative expression and activity, though its expression does increase with pregnancy (Dong et al., 1996; Vichai et al., 2005). COX-2 is the inducible isoform of this PG synthesising enzyme, maintaining a relatively low expression throughout gestation until the functional withdrawal of progesterone at term (Challis et al., 2000; Dong et al., 1996).

PGE_2 is one of the 2-series PG's capable of relaxing or stimulating the uterus, depending on which of its receptors it acts through. If PGE_2 acts through receptors EP_2 or EP_4 its activates the quiescent adenylyl cyclase-cAMP-PKA pathway (Challis et al., 2000, 1997). Subsequently, if PGE_2 binds to either the EP_1 or EP_3 receptor, it activates the stimulatory Ca^{2+}-Calmodulin-MLCK pathway, resulting in myometrial contraction (Challis et al., 2000, 1997). Unlike PGE_2, $PGF_{2\alpha}$ is only able to stimulate myometrial contraction via the Ca^{2+}-Calmodulin-MLCK pathway by binding to its FP receptor (Challis et al., 2000).

PG's and their receptor(s) are under tight endocrine regulation throughout gestation and parturition. Progesterone supresses PG synthesis and function by depressing EP_1, EP_3, FP, and COX-2 expression, while supporting EP_2 and EP_4 expression within the

placenta and myometrium (Challis et al., 2000). PGDH expression is maintained by progesterone during gestation, limiting the synthesis of stimulatory PG's within the chorion, but increasing the synthesis of the PG intermediary prostacyclin.

Prostacyclin contributes to uterine quiescence by activating the adenylyl cyclase-cAMP-PKA pathway (Sparey et al., 1999). As gestation progresses, cortisol induced sterile inflammation will increase the synthesis of $PGF_{2\alpha}$ within the foetal lungs, which is able to contribute to the functional withdrawal of progesterone at term through imbalance of the PRB: PRA ratio (Madsen et al., 2004). Functional withdrawal of progesterone at term will increase PGE_2 and $PGF_{2\alpha}$ synthesis, along with their receptor expression within the myometrium and placenta (Challis et al., 2000). PG release coincides with the release of matrix metalloproteinases capable of degrading collagen within the chorion, amnion, myometrium and cervix (Gomez-Lopez et al., 2013). These events contribute to the rupture of the foetal membranes, dilation of the cervix, and contraction of the uterus during term labour (Bogaerts et al., 2013).

2.6.2.6. Oxytocin

Oxytocin (OXT) is a neuropeptide synthesised within the paraventricular nuclei of the hypothalamus, being released in a pulsatile manner from the posterior pituitary gland (Arrowsmith and Wray, 2014). OXT has a range of physiological roles, but it directly

stimulates uterine contractions by promoting an influx and/or release of intracellular Ca^{2+} (Blanks and Thornton, 2003). Progesterone directly limits the synthesis of both OXT and its receptor OXTR throughout gestation to sustain relative uterine quiescence (Challis et al., 2000). Upon the functional withdrawal of progesterone at term, oestrogen increases the synthesis of OXT and the transcription of OXTR within the myometrium (Arrowsmith and Wray, 2014). Binding of OXT to its receptor results in intracellular dissociation of the G protein heterotrimeric complex, activating the Ca^{2+}-calmodulin-MLCK pathway, and stimulating contraction of the myometrium (Challis et al., 2000). Through intracellular processes, OXT is capable of contributing to its own feed-forward loop, further driving its own synthesis and contraction of the uterus during term labour.

2.6.2.7. The synergism of prostaglandins and oxytocin

PG and oxytocin are able to act upon each other's endogenous pathways to stimulate their synthesis and release from uterine tissue in a feed-forward autocrine/paracrine manner (Siemieniuch et al., 2011; Snegovskikh et al., 2006). As a result of this process, OXT will drive the synthesis of PG's, while PG's will drive the synthesis of OXT during labour (Snegovskikh et al., 2006). Despite their synergism, PG's appear to drive this relationship more. Murine knockout of OXT does not significantly prolong the duration of labour or result in any aberrant outcomes (Petraglia et al., 2010). While knockout of PGFP, COX-1, or COX-2 significantly prolongs the duration of labour, reduces OXT

sensitivity, and uterine OXTR expression (Petraglia et al., 2010; Sugimoto et al., 1997). This evidence certainly highlights the synergism of PG and OXT, and the overwhelming importance of PG's in the onset of murine parturition. It is yet to be established how this data translates to human parturition, as these hormones play an important role in labour, but human parturition is not triggered through $PGF_{2\alpha}$ induced luteolysis (Mitchell and Taggart, 2009). Despite this, PG's play a central role in the sterile inflammation induced functional withdrawal of progesterone, which is a hypothesised to be trigger of human parturition (Mesiano and Welsh, 2007).

2.6.3. Mechanical stimulus

The uterus is a robust organ that undergoes hyperplasia and hypertrophy to account for the ever-growing foetus (Shynlova et al., 2010). As gestation progresses, the uterus increases by 20 times its original volume, but eventually foetal growth will overcome uterine expansion, and place a mechanical stimulus upon it (Wray, 1993). The mechanical stimulus, better known as stretch, is crucial in activating the uterus at term (Challis et al., 2000). Upon functional withdrawal of progesterone, stretch (in conjunction with oestrogen) is able to directly increase uterine CAP expression (Challis et al., 2005). For example, stretch of a non-gravid uterine horn using plastic rods increases myometrial CX-43 expression to the same level as that of a gravid uterine horn (Ou et al., 1997). This highlights the importance of stretch

in the development of a synchronous contraction phenotype and the outcomes of labour.

2.6.4. Immunology of gestation and parturition

Research over recent decades has begun to highlight the important role of the pro-inflammatory immune response in parturition. Progesterone is capable of supressing the pro-inflammatory immune response by inactivating NF-κB (Bogaerts et al., 2013; Shynlova et al., 2013). At term, increasing activation of pro-inflammatory pathways via NF-κB results in the production of cytokines, PG's, and the invasion of leucocytes into maternal-foetal tissue, collectively known as sterile inflammation (Bogaerts et al., 2013; Golightly et al., 2011). The pro-inflammatory immune response is able to contribute to the functional withdrawal of progesterone, and the synthesis of PG's necessary to stimulate the myometrium and dilate the cervix (Bogaerts et al., 2013; Golightly et al., 2011; Shynlova et al., 2013). It is clear that the role of the immune system in the onset of parturition is important, with activation of the pro-inflammatory response potentially being one of the key stages in the shift from phase 0 to phase 2 of parturition.

2.6.5. The mechanism(s) that initiate parturition

The mechanism(s) that initiate labour remained to be fully determined. The hypothesis has evolved from a single mechanism to a multi-factorial process involving physiological, endocrine, immune, and mechanical inputs.

Despite having a more global view of the mechanisms that may be involved, the exact trigger that activates this tree of pathways remains to be determined.

It is clear progesterone supresses this array of mechanisms to maintain relative uterine quiescence (Mesiano and Welsh, 2007). As gestation progresses, CRH drives the release of cortisol and sex steroid precursors (DHEA and DHEAS), but the activity of this HPA axis is limited by CRH-BP (Golightly et al., 2011). Cortisol stimulates foetal pulmonary maturation through the activation of inflammatory pathways, by producing surfactant protein A and phospholipids (Bogaerts et al., 2013). Cell senescence has also been shown to contribute to the activation of inflammatory pathways within the foetal membranes (Polettini et al., 2015). The sex steroid precursors will circulate from maternal or foetal tissue to the placenta where they are synthesised to oestrogens (Smith, 2007). During this time, the ever-growing foetus is stretching the uterus and providing a mechanical stimulus (Wray, 1993). Circulating CRH-BP decreases at term, facilitating the feed-forward activation of the foetal HPA axis, resulting in increased cortisol and sex steroid precursor synthesis (Challis et al., 2000). Increasing release of cortisol from the foetal adrenals will drive lung maturation and the activation of pro-inflammatory pathways (Bogaerts et al., 2013). The resulting activation of pro-inflammatory pathways will contribute to increased PG synthesis within maternal and foetal tissues (Shynlova et al., 2013).

PG's are able to imbalance the progesterone nuclear receptor expression ratio, facilitating the truncated PRA isoform to supress the pro-gestational effects of the PRB isoform, functionally withdrawing progesterone (Madsen et al., 2004). Further to this, activation of the pro-inflammatory pathways will promote the synthesis and release of matrix metalloproteinases, degrading collagen within the maternal tissue and foetal membranes (Golightly et al., 2011). This in conjunction with increased apoptosis within the foetal membranes results in their rupture (Carlson et al., 2015), causing an intra-uterine flood of amniotic fluid laden in PG's and cytokines capable of stimulating the myometrium and dilating the cervix (Golightly et al., 2011).

While this is occurring, the placenta has been synthesising oestrogens, namely oestradiol and oestriol, from the sex steroid precursors released from the maternal and foetal adrenal glands (Snegovskikh et al., 2006). With the functional withdrawal of progesterone, oestrogens and stretch up-regulate uterine CAP gene transcription and uterotonin synthesis (Bogaerts et al., 2013; Challis et al., 2000). This increase in uterine CAP expression and uterotonin synthesis propagates a positive shift in the ion concentration gradient, facilitating spontaneous synchronised depolarisation of uterine myocytes (Wray and Arrowsmith, 2012). It is hypothesised that this compilation of physiological, endocrine, immune, and mechanical signals is sufficient to induce contraction of the myometrium and dilation the cervix, which

will facilitate the vaginal delivery of the foetus to the extra-uterine environment. Despite this wealth of information, we are not quite close enough to solving the puzzle that is human parturition. Currently, we are unaware of the exact mechanism(s) by which human parturition is triggered, though it is debated to be of or related to an endocrine or inflammatory origin. It is clear at this stage, parturition is a multi-factorial process requiring a number of endogenous signals to successfully occur, but further research is certainly required to fully understand parturition.

2.7. Effect of maternal obesity upon parturition

A number of clinical and epidemiological studies have highlighted the significant effect of maternal obesity upon the outcomes of labour. Maternal obesity significantly increases the risk of complications to the mother and foetus including gestational diabetes, pre-eclampsia, post-partum haemorrhaging, the development of congenital abnormalities, and even stillbirth (Bogaerts et al., 2013; Carlson et al., 2015; Centre for Maternal and Child Enquiries (CMACE), 2010; Kominiarek et al., 2011). One notable complication that can arise from maternal obesity is the development of a prolonged and dysfunctional labour ending in an emergency caesarean delivery (Bogaerts et al., 2013; Carlson et al., 2015; Poobalan et al., 2009). This prolonged and dysfunctional labour results from uterine dystocia (Carlson et al., 2015), characterised by an insufficient rate of cervical dilation and/or poor uterine contractility (Karaçam et al., 2014). Physiological study has highlighted the dose-

dependent utero-relaxant effect of increasing body mass (J Zhang et al., 2007), but the aetiology and mechanism(s) that govern maternal obesity associated uterine dystocia remain to be eludicated.

One proposed mechanism that may contribute to maternal obesity associated uterine dystocia is an incomplete functional withdrawal of progesterone, compromising uterine activation (Bogaerts et al., 2013; Carlson et al., 2015). Progesterone is the steroid hormone that promotes uterine quiescence (Mesiano and Welsh, 2007; Smith et al., 2015). At term, the withdrawal of progesterone nulls its quiescence upon the uterus, facilitating oestrogen, uterine stretch, and the pro-inflammatory immune response to activate the uterus for parturition (Smith, 2007). Incomplete uterine activation would be characterised by reduced CAP expression including CX-43, OXTR and COX-2, in conjunction with a decreased capacity to synthesise OXT and PG's (Bogaerts et al., 2013; Carlson et al., 2015). This would compromise the contractility of the uterus at term, resulting in poor labour outcomes and obstetrical intervention.

Maternal obesity is associated with dyslipidaemia, which is characterised by an elevation in circulating total cholesterol and TAG (Farias et al., 2015). Despite cholesterol having a clear biological function, excessive quantities may contribute to the poor labour outcomes of obese parturients. Increasing exogenous cholesterol concentrations impair ex vivo rat and human myometrial contractility in

a dose-dependent manner (Smith et al., 2005; Jie Zhang et al., 2007). The exact mechanisms by which cholesterol impacts upon myometrial contractility remain to be determined, but it is hypothesised that maternal obesity associated hypercholesterolemia will negatively alter the fluidity of the cell membrane, compromising integral protein expression and function (Grouleff et al., 2015; Pucadyil and Chattopadhyay, 2006). This could compromise the expression and function of GPCR's (Gimpl and Fahrenholz, 2002, 2000; Klein et al., 1995), ion channels (A Shmygol et al., 2007), and gap junctions (Elmes et al., 2011) resulting in the propagation of an aberrant contraction phenotype.

Maternal obesity associated dyslipidaemia is also accompanied by an increase in circulating SFA, MUFA, and a decrease in total n-3 PUFA (Cerf and Herrera, 2016; Cinelli et al., 2016; Grant et al., 2011), which could alter the fatty acid composition of the plasma membrane and thus its fluidity (Murphy, 1990; Wathes et al., 2007). Similar to the effect of cholesterol upon the membrane, change in the fatty acid composition of the plasma membrane could significantly impact upon the expression and function of integral proteins essential to contraction and relaxation of the uterus during labour (Murphy, 1990; Wathes et al., 2007). Despite this, further research is required to determine the validity of this hypothesis in the study of maternal obesity associated uterine dystocia, as currently no evidence is available to support this. A change in membrane esterified PUFA's concentrations could not only impact

upon membrane fluidity, it could also impact upon the synthesis of 2-series PG's (PGE_2 and $PGF_{2\alpha}$) within maternal and foetal tissues (Wathes et al., 2007). Feeding of diets high in n-6 PUFA significantly increases the synthesis of PGE_2 and $PGF_{2\alpha}$ within the endometrium and foetal allantochorion, significantly increasing the risk of pre-term labour (Cheng et al., 2005; Elmes et al., 2005). Contrastingly, feeding of diets rich in n-3 PUFA increases the risk of a prolonged and dysfunctional labour in both rats and humans (Leat and Northrop, 1981; Olsen et al., 1986), which is likely a result of EPA outcompeting AA for COX, decreasing the synthesis of uterine PGE_2 and $PGF_{2\alpha}$ (Allen and Harris, 2001; Leaver et al., 1991). PUFA's are not the only fatty acids that can alter the synthesis of 2-series PG's, as culture of ovine primary endometrial cells with the n-9 MUFA oleic acid for 24 hours attenuates the synthesis of $PGF_{2\alpha}$ (Cheng et al., 2015). The resulting change in dietary fatty acid consumption could significantly impact upon the synthesis of 2-series PG's, which stimulate contraction of the uterus and dilation of the cervix during labour (Challis et al., 2000).

Maternal obesity is characterised by the significant accumulation of subcutaneous and visceral adipose tissue prior to or above that deemed normal during gestation (Bogaerts et al., 2013). White adipose tissue is the site of energy storage, but is also an active endocrine organ secreting a range of hormones known as adipokines (Leal and Mafra, 2013). Adipokines play an essential role in appetite regulation, lipolysis, and glucose homeostasis, but also obesity induced metabolic

dysfunction (Coelho et al., 2013; Leal and Mafra, 2013). Synthesis and secretion of adipokines increases with the onset of obesity, with leptin, visfatin, and apelin displaying significant utero-relaxant effects during *ex vivo* contractility studies (AlSaif et al., 2015; Hehir and Morrison, 2012; Moynihan et al., 2006; Mumtaz et al., 2015). Despite the utero-relaxant effect of adipokines, these effects are only occurring beyond the physiological range, with some contradictory studies illustrating they have a stimulatory effect upon the uterus e.g. in the case of ghrelin (AlSaif et al., 2015). Further research is required to fully characterise the *in vitro*, *ex vivo*, and *in vivo* effects of adipokines upon term labouring uterine contractility, and the expression of these adipokines within the uterus, plasma, and fat depots of normal and obese parturients throughout gestation and parturition.

A number of hypotheses have been proposed to explain the aetiology and mechanisms of maternal obesity associated uterine dystocia, though there is limited evidence to support these proposed mechanism(s). As a result, further work is required to evaluate these and other potential mechanisms that have been proposed to compromise uterine contractility during parturition. If the mechanism(s) responsible can be identified and exploited, the risk of complications to the mother and foetus could be significantly reduced, while taking the stress off of maternity wards nationwide.

2.8. Human tissue and the use of animal models in the study of maternal obesity associated uterine dystocia

The most common approach for unravelling the mechanisms of parturition is through the analysis of human uterine tissue biopsies (Hehir and Morrison, 2012; Higgins et al., 2010; Moynihan et al., 2006; Parkington et al., 2014; J Zhang et al., 2007; Jie Zhang et al., 2007). Human uterine tissue biopsies are regularly collected from non-labouring women during an emergency or planned caesarean delivery. Once collected, biopsies can be dissected for physiological study, primary cell culture, transcriptomics, proteomics, or stored for further research. Despite the versatility of these samples, they suffer from the fundamental flaw that they are non-labouring (Mitchell and Taggart, 2009). Research by Challis and Lye has clearly established the difference between the non-labouring (phase 1) and labouring uterus (phase 2), characterising an endocrine shift, increased uterine CAP expression, and uterotonin synthesis, resulting in a synchronous contractile phenotype (Challis et al., 2000). Relative to this, the non-labouring uterus does not display such changes, and as a result it does not truly represent the labouring uterus. To overcome this significant limitation, studies have collected tissue from women in established labour, but who subsequently required an emergency caesarean delivery (Aguan et al., 2000; Sharp et al., 2016; Tattersall et al., 2008). This addresses the issue of non-labouring tissue, but it is still argued if biopsies collected during an emergency caesarean delivery truly represent a normal labour (Sharp et al., 2016). Reasons for an

emergency caesarean delivery can include uterine dystocia, pre-eclampsia, and foetal bradycardia, which cannot be classified as a normal labour (NICE, 2011). Normal labour is generally regarded as a spontaneous onset vaginal delivery at 37-42 weeks of gestation with little to no intervention (WHO, 1996). Through human tissue studies, it is also difficult to tightly control for clinical and demographic factors including age, diet, BMI, gravidity, parity, and complicating pathologies, which all have the potential to skew the data. Despite the limitations of human uterine tissue, little has been done to fully explore other options. One such option is the development of a translatable animal model to successfully investigate maternal obesity associated uterine dystocia. Animals including sheep, rodents, guinea pigs, and non-human primates have been successfully used to study parturition, with their advantages and disadvantages covered in depth within two reviews (Elovitz and Mrinalini, 2004; Mitchell and Taggart, 2009). Sheep have been a widely used model in the study of gestation and parturition, particularly pertaining to the role of the foetus in parturition (Mitchell and Taggart, 2009). They are relatively robust animals that allow for invasive surgery, but are expensive to house, commonly limiting the power of studies. Rodents offer an excellent research platform for the study of parturition, as the molecular mechanisms involved in their parturition are similar to those identified in humans (Mitchell and Taggart, 2009). Further to this, rodent studies are cost effective, tolerant to surgery, have a short gestation, and can be manipulated genetically or through their diet to induce desired pathologies (Elovitz

and Mrinalini, 2004). Guinea pigs are proposed as the ideal model to overcome the limitations of rodents, mainly as their progesterone withdrawal is functional and not a result of luteolysis at term (Mitchell and Taggart, 2009). Despite this and other advantages, the gestational period of the guinea pig is approximately 30-50 days longer than the rat, significantly reducing their cost effectiveness. Non-human primates are another animal model successfully used for the study of parturition, and offer distinct advantages as a result of their similar anatomy, physiology, and mechanisms of parturition to humans (Elovitz and Mrinalini, 2004; Mitchell and Taggart, 2009). Despite this, non-human primates can be difficult to work with, and require a great deal of financial support to house and care for them. As a result, there are a limited number of sites worldwide that support non-human primates, limiting their use as a model for many researchers. Other advantages to the use of animal models is the ability to control for genetic variation, age, diet, gravidity, parity, and the fact that tissue can be easily collected during established labour under normal conditions. Despite the inherent advantages of animal models, one has yet to be fully utilised in the study of maternal obesity associated uterine dystocia. Recently, Elmes et al. (2011) developed a model of maternal obesity for the study of its associated uterine dystocia. Chronic exposure of Wistar rat dams to a HFHC diet resulted in change in the term labouring expression of uterine CAP's including a significant decrease in uterine CX-43 and Cav-1 expression, increased plasma progesterone, and decreased $PGF_{2\alpha}$ concentrations relative to control

animals. As a result, this model illustrates potential in the study of maternal obesity associated uterine dystocia, but its efficacy and translatability has yet to be established.

2.9. Aims and hypotheses

Unravelling the aetiology and mechanism(s) governing maternal obesity associated uterine dystocia is an essential step in improving the outcome of labour for obese parturients. To achieve this, an efficacious animal model is needed to overcome the limitations of human uterine tissue biopsies.

The **overall** aim of this thesis is to evaluate the efficacy of the HFHC dietary animal model in the study of maternal obesity associated uterine dystocia. It is hypothesised that feeding of this HFHC diet will negatively impact upon uterine contractility within this animal model.

The aim of **chapter 3** was to evaluate the efficacy of the HFHC dietary animal model in the study of maternal obesity associated uterine dystocia through an *ex vivo* contractility organ bath study. It is hypothesised that chronic exposure to a HFHC diet will negatively impact upon uterine contractility *ex vivo*, potentially as a result of change in circulating progesterone and uterine CAP expression, which will be identified using ELISA and western blotting, respectively.

The findings reported within chapter 3 illustrate the significant negative effect of feeding a HFHC diet upon uterine contractility *ex vivo*. **Chapter 4** aimed to determine if this negative effect upon uterine contractility occurred *in vivo* through intra-uterine telemetry surgery. It is hypothesised that feeding of a HFHC diet will negatively impact upon uterine contractility *in vivo*, through change in intra-uterine pressure and the duration of labour.

Over the course of chapters 3 and 4, the negative impact of feeding a HFHC diet upon uterine contractility has been established. Despite this, the exact mechanism(s) responsible remain to be determined. One such mechanism could be a change in PG synthesis, resulting from a change in dietary fatty acids. **Chapter 5** aimed to investigate the effect of feeding a HFHC diet upon circulating, hepatic, and uterine fatty acids. It is hypothesised that chronic exposure to a HFHC diet will alter the fatty acid profile of these animals sufficiently to impact upon PG synthesis.

3. Modelling maternal obesity: The effect of a HFHC diet upon uterine contractility *ex vivo*

3.1. Introduction

Global female obesity rates have increased from 71 million in 1975 to 375 million in 2014 (NCD Risk Factor Collaboration, 2016). In the UK, 20% of the female population is currently obese, with this figure expected to reach 50% by the year 2050 (Butland et al., 2007). The rising rates of obesity are occurring in women of reproductive age, as 50% of women entering pregnancy are either overweight or obese (ACOG, 2013). Maternal obesity significantly increases the risk of complications occurring during gestation and labour including gestational diabetes, pre-eclampsia, post-partum haemorrhaging, and prolonged and dysfunctional labour resulting in emergency caesarean delivery (Bogaerts et al., 2013; Centre for Maternal and Child Enquiries (CMACE), 2010; Kominiarek et al., 2011; Poobalan et al., 2009). Specifically, obese parturients are at a 29% risk of emergency caesarean delivery compared to 15% in normal weight women (Poobalan et al., 2009). This increased risk of emergency caesarean delivery and prolonged dysfunctional labour results from uterine dystocia, which is the inability to produce contractions sufficient to deliver the foetus vaginally (Karaçam et al., 2014). *Ex vivo* human tissue studies have illustrated the significant utero-relaxant effect of increasing body mass (J Zhang et al., 2007). Despite the clear risks of

maternal obesity upon term labouring uterine contractility, the mechanisms behind this remain to be eluded.

During a normal labour, the uterus will produce biphasic plateau high frequency high amplitude contractions, resulting from the synchronous contraction and relaxation of the smooth muscle bundles that compose the myometrium (Anatoly Shmygol et al., 2007). To achieve this, a complex multi-factorial process must occur to shift the uterus from a relative state of quiescence to a stimulatory state (Bogaerts et al., 2013; Challis et al., 2000). A key part of this multi-factorial process is the progesterone and oestrogen ratio. Progesterone is the pro-gestational hormone that sustains the uterus in a relative state of quiescence (Smith et al., 2015). At term, the functional withdrawal of progesterone limits it quiescent effects, facilitating oestrogen to activate and prepare the uterus for labour (Mesiano and Welsh, 2007). Oestrogen activates the uterus by promoting the up-regulation of uterine CAP expression including Cav-1, CX-43, OXTR, COX-2, PG receptors (EP_1, EP_3, and FP), and Ca^{2+} ion channels (Challis et al., 2000; Turi et al., 2001). Cav-1 is the cholesterol-rich structural protein of caveolae, the omega-shaped invaginations of the cell membrane (Okamoto et al., 1998; Taggart, 2001; Wray, 2007) that support a range of ion channels (A Shmygol et al., 2007; Smith et al., 2005; Jie Zhang et al., 2007) and receptors (Gimpl and Fahrenholz, 2000; Klein et al., 1995; Turi et al., 2001) essential to contraction of the uterus. CX-43 is the 43KDa protein essential to the formation of gap junctions, which

facilitate intra-cellular electro-coupling (contraction synchronisation) and metabolite transfer (Döring et al., 2006; Sims et al., 1982). Ablation of myometrial CX-43 expression significantly prolongs the duration of labour and in some cases results in a complete failure to deliver (Döring et al., 2006). The importance of CX-43 and the formation of myometrial gap junctions cannot be over-stated for a successful labour. COX-2 is the enzyme responsible for the synthesis of bioactive PG's (PGE_2 and $PGF_{2\alpha}$) capable of stimulating uterine contractions during labour (Challis et al., 1997). The ability for PG's to stimulate uterine contractions is heightened by the increase in myometrial PG receptor expression at term (Challis et al., 2000). Oestrogen increases the synthesis of myometrial OXT and its receptor OXTR at term, which if activated promotes contractions through the Ca^{2+}-calmodulin-MLCK pathway (Arrowsmith and Wray, 2014). L-type Ca^{2+} ion channels expression increases at term, spontaneously stimulating contraction of the uterus upon membrane depolarisation (Wray, 1993). Maternal obesity has been hypothesised to sustain the pro-gestational effects of progesterone at term limiting oestrogen induced up-regulation of uterotinins and CAP's, compromising the contraction of the uterus during labour (Bogaerts et al., 2013). Though this hypothesis is widely accepted, there is little evidence available to fully support it, stipulating further investigation is required. Another mechanism that may alter uterine CAP expression is through change in cholesterol concentrations. Cholesterol concentrations gradually increase as gestation progresses (Baardman et al., 2013), and act as a raw

material for the synthesis of sex steroids (progesterone, oestrogens and cortisol) vital to gestation and parturition (Challis et al., 2000; Miller, 2013). Analysis of obese parturient serum has found their total cholesterol concentrations to be significantly greater than their normal weight counterparts (Farias et al., 2015), with increasing concentrations of exogenous cholesterol inducing dose-dependent relaxation of the rat and human myometrium *ex vivo* (Smith et al., 2005; Jie Zhang et al., 2007). Modulation of cell membrane cholesterol concentrations significantly alters membrane fluidity and integral protein expression (Bogaerts et al., 2013; Pucadyil and Chattopadhyay, 2006), but further investigation is required to understand if this directly effects uterine CAP expression.

To fully understand the pathophysiology of maternal obesity associated uterine dystocia, a standardised method is required. To overcome the limitations and variability of human tissue, Elmes et al. 2011 developed an animal model, inducing maternal obesity through chronic exposure to a formulated HFHC diet. A pilot study highlighted the significant effect of the HFHC diet upon term-labouring uterine CAP expression and plasma progesterone concentrations (Elmes et al., 2011), but the efficacy and translatability of this model is yet to be established.

3.2. Objective

The objective of this study was to evaluate the efficacy of this HFHC dietary animal model in the study of maternal obesity associated

uterine dystocia, through *ex vivo* measurement of term non-labouring (TNL) and term labouring (TL) spontaneous and stimulated uterine contractions, uterine CAP expression, circulating lipids, and plasma progesterone concentrations. It was hypothesised that chronic exposure to a HFHC diet will negatively impact upon both spontaneous and stimulated uterine contractions *ex vivo,* through change in circulating progesterone and uterine CAP expression.

3.3. Material and methods

3.3.1. Dietary animal study

All animal work was completed within the animal research facilities at the University of Nottingham, and carried out under the Animals Scientific Procedures Act (ASPA) of 1986. Forty weanling virgin female Wistar rats (*Rattus rattus*) weighing approximately 60g (Charles River, UK) were pair-housed under normal conditions (12 hour light:dark photoperiod, 21°C ± 5°C room temperature, 55% ± 5% relative humidity, food and water access *ad libitum*) and randomly assigned to be fed either a standard laboratory chow (CON) (Envigo, USA, n=20) or HFHC diet (n=20) as previously published by Elmes et al. 2011 (Table 8.1). Each rat was maintained on their respective diets for 6 weeks prior to mating with stud Wistar males (Charles River, UK). Pregnancy was confirmed upon discovery of a seminal plug, and recorded as gestational day 0. Following successful mating, pregnant females were maintained on their respective diet throughout gestation. Animals in

each dietary group were euthanised by CO_2 asphyxiation and cervical dislocation at 2 time points; term non-labour at gestational day 21 (TNL n=10) or day 22 term labour (TL n=10) following delivery of the first pup. All pups born during this study were euthanised using a 0.1ml of pentobarbital. A total number of 9 animals were not used in the trial as a result of congenital pathology or a lack of pregnancy. Blood was collected via cardiac puncture into EDTA-coated tubes (Sarstedt, Germany). Plasma was then separated using centrifugation at 13,000 rpm at 4°C for 10 minutes, and snap-frozen in liquid nitrogen. The uterus was removed from the visceral cavity, and separated from any gonadal adipose tissue. Following this, the uterus was split into its two horns, opened and cleared of the placenta, amnion, amniotic fluid and unborn pups; all unborn pups were euthanised by decapitation. One horn was washed and stored in ice-cold Krebs Henseleit buffer for the *ex vivo* contractility study. Krebs Henseleit buffer was prepared by dissolving 118mM NaCl, 4.7mM KCl, 1,2mM KH_2PO_4, 1.2mM $MgSO_4$ $7H_2O$, 25mM $NaHCO_3$, 1.25mM $CaCl_2$ $2H_2O$, and 11mM of Glucose in 1L of dH_2O. Once in solution, the pH of the Krebs Henseleit buffer was adjusted to 7.4 using 1M HCl, and gassed for 30 seconds using 95% O_2/5% CO_2.The second uterine horn was briefly washed using ice cold Krebs Henseleit buffer to remove any remnants of amniotic fluid, then snap frozen in liquid nitrogen and stored at -80°C for future work.

3.3.2. Plasma progesterone ELISA

Maternal plasma progesterone concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) (Ridgeway Science, Gloucestershire, UK) according to the manufacturer's instructions. A standard curve ranging from 0-20 ng/ml of progesterone was produced. 10µl of standard or plasma sample were incubated for 2-2.5 hours with 200µl of the progesterone enzyme label within a 96 well plate. After 3 x 2 minute wash cycles using the wash buffer, 200µl of substrate solution was added to each well and left to incubate at room temperature over a 20-30 minute period before the absorbance was read at 405nm (Tecan Grp Ltd, Switzerland). The manufacturer reports a limit of detection of 0.04 ng/ml of progesterone in plasma, with inter-assay and intra-assay coefficient of variation of 9.6% and 6.6%, respectively.

3.3.3. Total cholesterol and triacylglycerol assays

Total cholesterol and triacylglycerol in the maternal plasma were assayed through a commercial kit (Thermoscientific fisher, UK) according to the manufacturers' instructions. Standard curves ranging from 0-8 mM and 0-3 mM were produced for cholesterol and triglycerides, respectively. On a 96 well plate 250µl of cholesterol or triglyceride infinity reagent was added to 10µl of standard or plasma, and incubated for 15 mins at 37°C. The absorbance was then read at 550 nm (with a reference wave length of 655 nm) using a Sunrise absorbance plate reader (Tecan Grp Ltd, Switzerland).

3.3.4. *Ex vivo* contractility study

From each animal four 10x5mm uterine strips following the circular muscle layer of the myometrium were dissected from the ovary to the cervix of the uterine horn, and mounted within a 25ml organ bath (Letica, AD Instruments, Oxford UK) filled with physiological saline solution (PSS). PSS was prepared by dissolving 119mM NaCl, 4.7mM KCl, and 2.4mM MgSO4, 25mM $NaHCO_3$, and 1.18mM KH_2PO_4, 5.5mM Glucose, and 1.6mM $CaCl_2$ within 2L of dH_2O and gassed with 95% O_2/5% CO_2. Each strip was set to a resting tension of 20mN and the contractile activity of each strip was recorded using isometric force transducers connected to a bridge amplifier, which in turn was connected to a dedicated data acquisition system (Powerlab/8SP, AD Instruments, Oxford, UK), which was recorded and analysed by Lab Chart Software (version 6; Powerlab ASD Instruments). Myometrial strips were then left to equilibrate for 30-40 minutes. Once the uterine strips were equilibrated, a 30-minute baseline of spontaneous uterine contractility was recorded prior to the accumulative dosing of OXT (10^{-12} to 10^{-5} mol/L) or $PGF_{2\alpha}$ (10^{-10} to 10^{-3} mol/L) applied at 10-minute intervals. Viability of myometrial strips was checked at the end of each experiment by recording contractile responses to administration of 0.1M KCl. Uterine strips that did not respond to the KCl were deemed not viable and excluded from the analysis. The resultant contractile activity measured included activity integrals (area under the time-force curve), frequency of contractions, mean amplitude, slope maximum, and slope minimum.

3.3.5. Protein extraction and analysis

3.3.5.1. Tissue preparation

300mg of crushed rat uterine horn was homogenised in the presence of 2ml ice-cold extraction buffer (32.5mM Tris/HCl, 3.08mM EDTA, pH 7.5), and protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). Each sample was homogenised on ice in 30-second pulses. Homogenate for COX-2 underwent centrifugation at 13,000 g. Where as Cav-1, CX-43, *P*CX43 and OXTR were centrifuged at 3,500 g for 10 min at 4°C to isolate the supernatant. Supernatant was extracted, flash frozen using liquid nitrogen and stored at -80°C.

3.3.5.2. Protein assay

A protein assay was used to determine the total protein concentration of the supernatant. A standard curve ranging from 0-8mg/ml was produced from 1mg/ml bovine serum albumin (dissolved in 0.01M NaOH) (Sigma Aldrich, UK). 200µl of Bio-rad protein assay dye reagent (Bio-rad, USA) was incubated with 10µl of diluted supernatant (20x, 30x and 50x) within a 96 well plate; each sample was measured in duplicate. The absorbance was then read at 550 nm with a Sunrise absorbance plate reader (Tecan Grp Ltd, Switzerland).

3.3.5.3. Sample preparation

Samples were prepared for western blotting using 2x sodium dodecyl sulphate mix (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, 150 mM dithiothreitol) to total protein concentrations

2µg/µl (Cav-1), 22µg/µl (CX-43), 16µg/µl (PCX-43), 22µg/µl (OXTR) and 20µg/µl (COX-2).

3.3.5.4. Western blotting

Prepared samples were boiled for 5 minutes (samples were not boiled for the detection of COX-2) then separated using 8% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 200v for up to 80 minutes. Proteins were transferred at 200mA for 2 hours to a nitrocellulose membrane (Hybond-C extra, Amersham 4 Bioscience) within an ice cooled transfer solution (400mM Glycine, 30mM Tris base, 5% Isopropanol). The membrane was subsequently cut away from its excess and marked with indelible ink to retain the protein ladder. Successful transfer was confirmed using Ponceau-S stain. The membrane was blocked with 4% ECL Prime (GE UK Healthcare Ltd) 1xTris Buffered Saline-Tween (TBS-T) (10% TBS, 1% Tween 20) blocking solution for 1 hour at room temperature. 5 minute x 12 wash cycle using 1xTBS-T followed. The membrane was blotted overnight at 4°C with the primary antibody [(Cav-1, 22KDa, 1:1000, Cell Signalling Technology, USA), (CX-43, 43KDa, 1:5000, Cell Signalling Technology, USA), (*P*CX-43, 43KDa, 1:2000, Abcam, UK), (OXTR, 66KDa, 1:10:000, Santa-Cruz, USA), and (COX-2, 70-72KDa, 1:3000, Abcam, UK)]. A 5-minute x 12 wash cycle was carried out the following morning to reduce unspecific binding of the primary antibody. Following this wash cycle, the membrane was blotted with anti-rabbit secondary antibody IgG Horseradish peroxidase [(Cav-1 1:4000), (CX-43

1:25000), (PCX-43 1:8000), (OXTR 1:80000) and (COX-2 1:10000)] (GE Healthcare UK Ltd, UK) for 1-hour at room temperature. Again, a 5-minute x 12 wash cycle followed to reduce unspecific binding of the secondary antibody and subsequent background exposure. The membrane was incubated with ECL Prime detection reagent (GE Healthcare UK Ltd) for 5 minutes at room temperature to initiate the chemiluminescent reaction. The membrane was then exposed to photogenic film (GE Healthcare UK Ltd) under red light to capture the photons emitted from the chemiluminescent reaction. Following this the film was developed and fixed. Protein expression was quantified using a Bio-Rad Gel-doc analysis employing Quantity one analysis software (Bio-rad, USA). β-actin was used as the housekeeping protein (Sigma Aldrich, UK, 42KDa, 1-10:000) with an anti-mouse secondary antibody, [IgG horseradish peroxidase (1:40,000) (GE UK Healthcare Ltd, UK) following the same method stated previously for western blotting. Thermoscientific Restore PLUS western stripping buffer (Thermoscientific, UK) was applied to the membrane for 5 minutes at room temperature post detection of CX-43 and *P*CX-43 to prepare the membrane for blotting of the housekeeping protein.

3.3.6. Data analysis and statistics

All data was analysed using the Statistical Package for Social Science (Version 21; SPSS Inc, Chicago, IL, USA) and expressed as the mean value ± standard mean error (SEM), where $P=\leq 0.05$ was considered statistically significant. The homogeneity of the data was assessed and

if not normally distributed the data was suitably transformed to achieve a normal distribution. Normally distributed or transformed data sets were statistically assessed parametrically using independent T-test, Two-way ANOVA, or mixed-between-within ANOVA. If the data could not be suitably transformed, it was assessed non-parametrically using a Mann-Whitney U test or Kruskal–Wallis test with post hoc Mann–Whitney U test and Bonferonni analysis. All the graphs were produced using Graph Pad Prism (Graph Pad Version 6, USA). The effect of a HFHC diet upon necropsy measurements was assessed using an independent T-test and Man-Whitney U test. The effect of maternal diet and stage of labour upon plasma progesterone, total cholesterol and triglycerides were statistically assessed using a Kruskal–Wallis test with post hoc Mann– Whitney U test and Bonferonni analysis as the data could not be suitably transformed. As a result of the Bonferroni analysis the *P* value was adjusted to 0.008 to account for type I error that may be introduced through this statistical test. Two-way ANOVA was utilised to assess the effect and the interaction of maternal diet and stage of labour upon normal and percentage change in baseline spontaneous uterine integral activity, contraction frequency, amplitude, slope maximum, and slope minimum. Mixed between-within ANOVA was used to statistically assess the effect and interactions of maternal diet and stage of labour during accumulative dosing of OXT and $PGF_{2\alpha}$ upon normal and percentage change in integral activity, contraction frequency, amplitude, slope maximum, and slope minimum of uterine contractility *ex vivo*. Dose response analysis of OXT was fitted using

the standard least squares (ordinary) fit method. The effect of maternal diet on $LogEC_{50}$ values and steepness of the curve (Hill slope) for percentage change in integral activity was analysed by GraphPad Prism (Version 6; GraphPad Inc, San Diego, CA, USA) using a sigmoidal dose response (variable slope) curve to test the null hypothesis that $LogEC_{50}$ and Hill slope were the same for each data set. The effect and interaction of diet and stage of labour upon uterine CAP expression was determined using two-way ANOVA.

3.4. Results

3.4.1. Necropsy measurements

Animals sustained on a HFHC diet were significantly heavier at the end of the study (Table 3.1) relative to CON animals (*P*=0.012). Specifically, HFHC animals were 39.5g heavier at the end of the trial than CON animals. This significant difference in final weight is likely a result of a greater visceral fat mass within HFHC animals. HFHC animals had a significantly greater mass of peri-renal (13.19g ± 1.35) and gonadal (11.00g ±1.73) visceral fat than animals sustained on the CON diet (6.68g ± 0.83 and 6.07g ± 0.64), respectively. The significant difference in visceral fat mass and final weight could be attributed to the significantly greater energy intake of HFHC animals prior to (*P*=≤0.001) and during (*P*=≤0.001) gestation. Maternal diet had no significant effect (*P*=0.597) upon the litter size.

Category	Control diet (n=17) (Mean ± S.E.M)	HFHC diet (n=15) (Mean ± S.E.M)	*P* value
Final weight (g)	416.87 ± 5.77	456.37 ± 12.69	0.012
Peri-renal fat mass (g)	6.68 ± 0.83	13.19 ± 1.35	≤0.001
Gonadal fat mass (g)	6.07 ± 0.64	11.00 ± 1.73	<0.032
Energy intake prior to gestation (Kcal)	108.07 ± 1.43	140.03 ± 4.95	≤0.001
Energy intake during gestation (Kcal)	75.43 ± 1.12	89.34 ± 2.34	≤0.001
Litter size (n)	14.06 ± 0.91	13.60 ± 0.82	0.597

Table 3.1 - Summary of the effects of a CON (n=17) or HFHC (n=15) on parameters measured at necropsy.

3.4.2. Plasma progesterone concentrations

Progesterone is the pro-gestational hormone that sustains the uterus in a state of quiescence. Measurement of plasma progesterone using ELISA will determine if maternal diet or stage of labour altered the circulating concentrations of this sex steroid. At day 21 of gestation HFHC animals (129.44 ± 5.00 ng/ml) displayed significantly increased (*P*=<0.014) plasma progesterone concentrations relative to CON animals (80.26 ± 12.92 ng/ml) at this stage of gestation (Figure 3.1). With the onset of day 22 term parturition, plasma progesterone concentrations decreased within both dietary groups, though this decrease only reached significance in HFHC animals (*P*=0.003). With the onset of labour at day 22 of gestation, circulating progesterone concentrations did not significantly differ between the CON (33.33 ± 2.41 ng/ml) and HFHC (35.75 ± 2.33 ng/ml) animals.

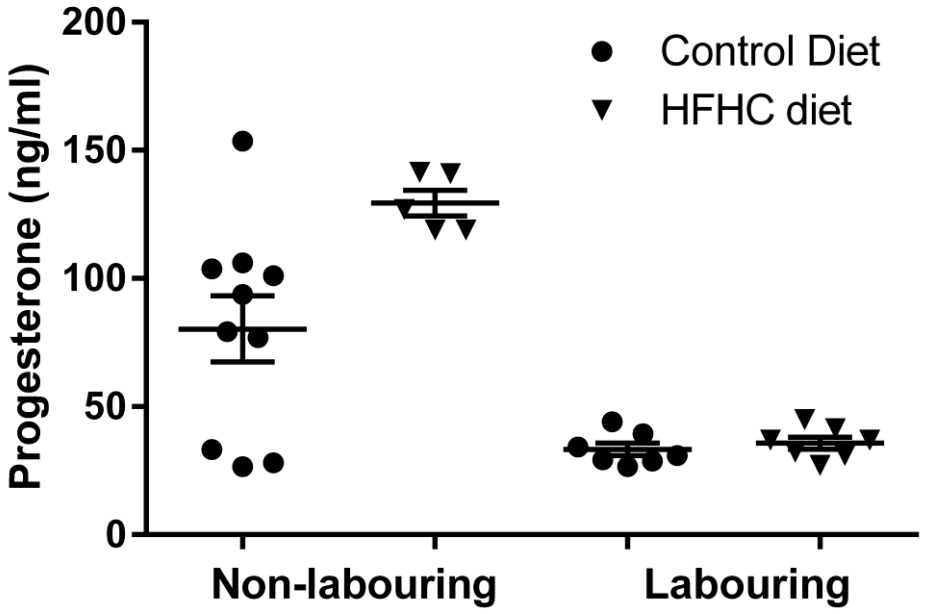


Figure 3.1 - Measurement of maternal plasma progesterone concentrations (ng/ml) using ELISA from CON (TNL=10, TL=7) and HFHC (TNL=5, TL=7) animals. Kruskal-Wallis test with post-hoc Mann-Whitney U test and Bonferonni analysis. Values are means ± SEM.

3.4.3. Maternal plasma lipid profile

It was important to establish if chronic exposure to a HFHC diet significantly altered circulating lipid concentrations including total cholesterol and TAG (Figure 3.2). Analysis of the plasma using a colourmetric assay revealed that chronic exposure to a HFHC diet significantly increased circulating total cholesterol (CON 2.63 ± 0.08mmol/L vs HFHC 9.00 ± 0.44mmol/L; *P*=<0.001) and TAG concentrations (CON 1.24 ± 0.17mmol/L vs HFHC 1.67 ± 0.14 mmol/L; *P*=0.039). HFHC animals displayed significantly increased plasma total cholesterol concentrations during TNL (CON 2.5 ± 0.1mmol/L vs HFHC 9.8 ± 0.4mmol/L; *P*=<0.001) and TL (CON 2.7 ± 0.1mmol/L vs HFHC 8.5 ± 0.6mmol/L; *P*=<0.001) relative to CON animals at the same stage of parturition. TL circulating TAG concentrations were significantly (*P*=<0.001) greater in HFHC animals (1.3 ± 0.2 mmol/L) relative to CON animals (0.56 ± 0.06 mmol/L). Plasma TAG concentrations during TNL did not significantly differ between the dietary groups (CON 1.7 ± 0.2 mmol/L vs HFHC 2.3 ± 0.06 mmol/L; *P*=0.019) because of the adjusted *P* value.

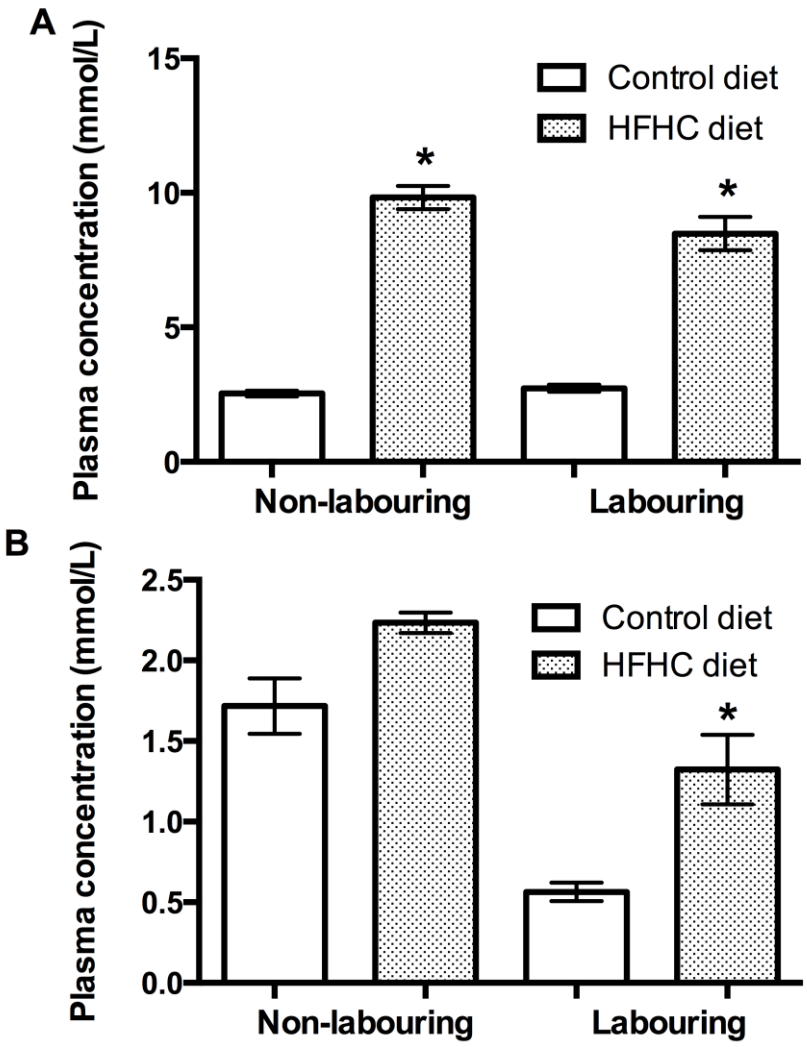


Figure 3.2 - The effect of maternal diet (CON/HFHC) and stage of labour (TNL/TL) upon plasma (**A**) total cholesterol and (**B**) triacylglycerol concentrations (mmol/L). Sample sizes CON (TNL=10, TL=7) and HFHC (TNL=5, TL=7). Kruskal-Wallis test with post-hoc Mann-Whitney U test and Bonferonni analysis. Values are means ± SEM. ***P*=≤0.008 relative to CON diet of same stage of labour.

3.4.4. *Ex vivo* contractility study

To assess the effect of maternal diet and stage of labour upon uterine contractile activity, strips of uterine tissue were dissected and mounted with an organ bath. By placing the strips in this controlled physiological environment, the effect of maternal diet and stage of labour on both spontaneous and stimulated uterine contractions could be assessed.

3.4.4.1. Vehicle control analysis

During each *ex vivo* contractility experiment a single strip of uterine tissue was not exposed to any of the experimental condition beyond the baseline recording, this is known as the vehicle control. The vehicle control strip allows the analyst to determine the effect of time upon contractile measurements recorded during the experiment, and if necessary account for the effect of time in their analysis. As the experiment progressed from the baseline recording through to the accumulative dosing of the uterine stimulants, integral activity of the vehicle control significantly decreased by 31.46% (P=<0.001), but time did not significantly interact with maternal diet or stage of labour. Time was found to have no significant effect upon the frequency of contraction (23.46%; P=0.054) nor did it significantly interact with maternal diet or stage of labour. As the experiment progressed, time had a significant (P=0.018) effect upon the mean amplitude of the vehicle control, with a 27.61% decrease by the end of the accumulative dosing period. Time was also found to significantly interact with maternal diet (P=0.035) and stage of labour (P=0.029) when measuring

mean contraction amplitude. Through analysis of the vehicle control strip it is clear that as the experiment progressed, contractile parameters were significantly decreased despite being maintained in a pristine physiological environment. Though as a result it was deemed unnecessary to account for time in the analysis.

3.4.4.2. Effect of a HFHC diet upon spontaneous uterine contractility

Upon equilibration of the uterine contractions, a 30-minute baseline was recorded with the aim of investigating the effect of maternal diet and stage of labour upon spontaneous uterine contractions. The effect of maternal diet and stage of labour upon *ex vivo* uterine contractility was assessed both through qualitative and quantitative means.

3.4.4.2.1 Qualitative measurement of spontaneous uterine contractile activity

Qualitative assessment allows the analyst to review the phenotype, rhythm, and synchronicity of the uterine contractions *ex vivo* and how maternal diet and stage of labour may affect these. Uterine tissue collected from day 21 TNL CON animals displayed a spontaneous synchronous plateau contraction phenotype, with contractions being of a short duration and fluctuating amplitude (Figure 3.3A). CON TL animals displayed a similar synchronous plateau contraction phenotype to TNL CON animals, but the amplitude did not fluctuate during the baseline recording (Figure 3.3C). Relative to the CON animals, an asynchronous spike bundle contraction phenotype was produced from

uterine tissue collected from TNL HFHC animals, which displayed a similar duration of contraction and fluctuation in amplitude (Figure 3.3B). As the HFHC animals moved from TNL to TL, the asynchronous contraction phenotype persisted, but the duration of contraction increased and amplitude variability decreased, though it still did not remain consistent (Figure 3.3D). Qualitative analysis has illustrated the significant negative effect of feeding a HFHC diet upon the uterine contraction phenotype and contraction synchronicity *ex vivo*.

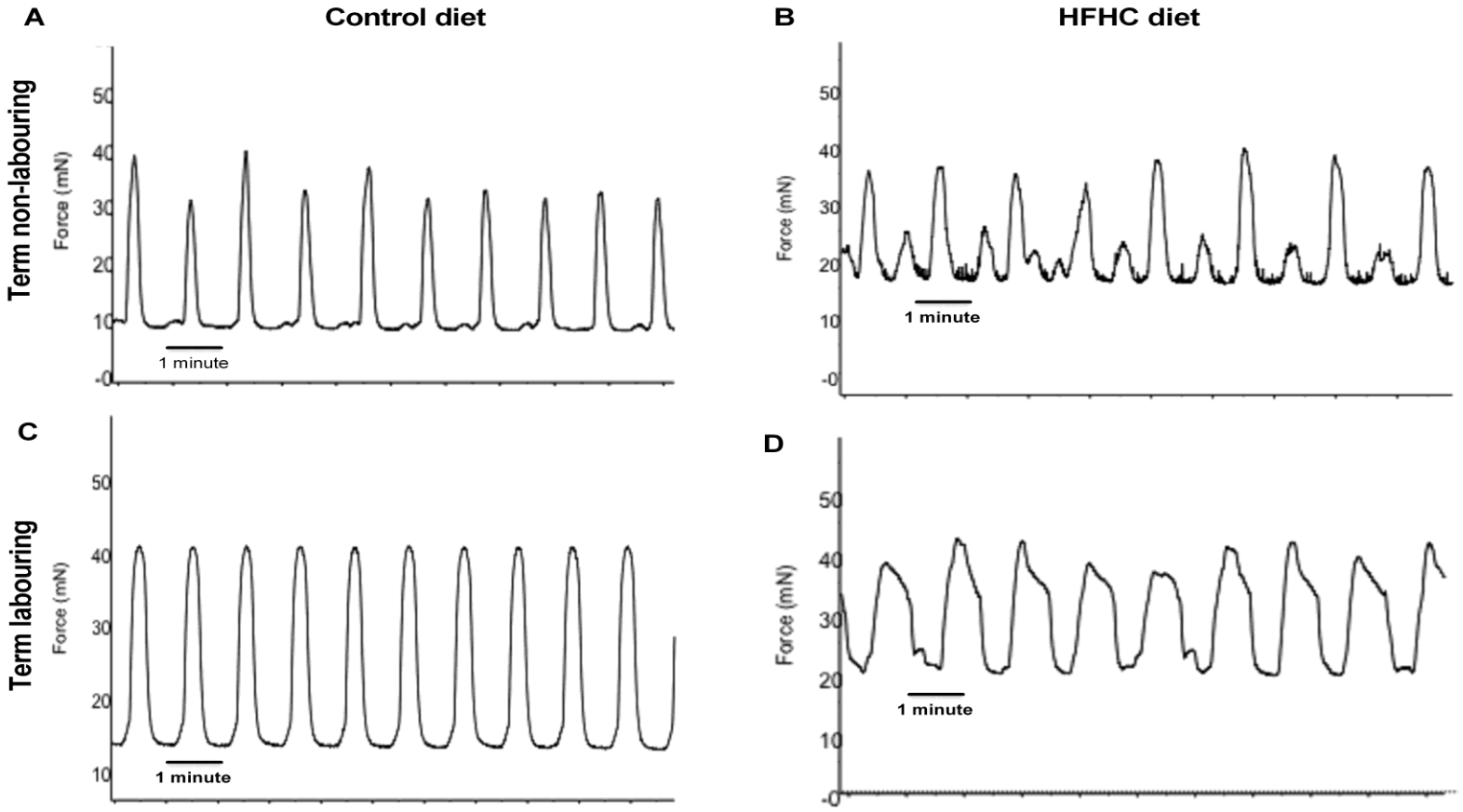


Figure 3.3 - 10 minute representative traces of *ex vivo* spontaneous baseline contractile activity from (**A**) CON TNL, (**B**) HFHC TNL, (**C**) CON TL, and (**D**) HFHC TL animals.

3.4.4.2.2 Quantitative measurement of spontaneous uterine contractile activity

Integral activity is a measure of the area under the curve as a rate of change in force per second (mN/s). Spontaneous uterine integral activity significantly (*P*=<0.001) increased with the onset of TL in CON and HFHC animals (Figure 3.4A), but maternal diet had no significant (*P*=0.098) effect upon this contractile measurement. Maternal diet (*P*=0.188) and stage of labour (*P*=0.934) were found to have no significant effect upon spontaneous uterine contraction frequency (Figure 3.4B). Contraction amplitude was not significantly affected by maternal diet (*P*=0.710), but both CON and HFHC animals displayed a significant increase with the onset of labour (*P*=0.009) (Figure 3.4C). Slope is the measurement of the rate of change in force per second during the contraction (maximum) or relaxation (minimum) phase of the event. Slope provides an insight into the duration of membrane depolarisation and its subsequent hyperpolarisation, suggesting differences in integral protein expression, though microelectrode or patch clamp analysis would be required to confirm this. During the contraction phase of the uterine event, slope max did not significantly (*P*=0.809) differ between TNL and TL animals, but maternal diet did have a significant (*P*=0.005) effect upon the rate of change during this phase of the contraction (Figure 3.4D). Maternal diet had a significant effect (*P*=<0.001) upon the rate of change during the relaxation phase of the event, but stage of labour had no significant effect (*P*=0.547) upon slope minimum (Figure 3.4E).

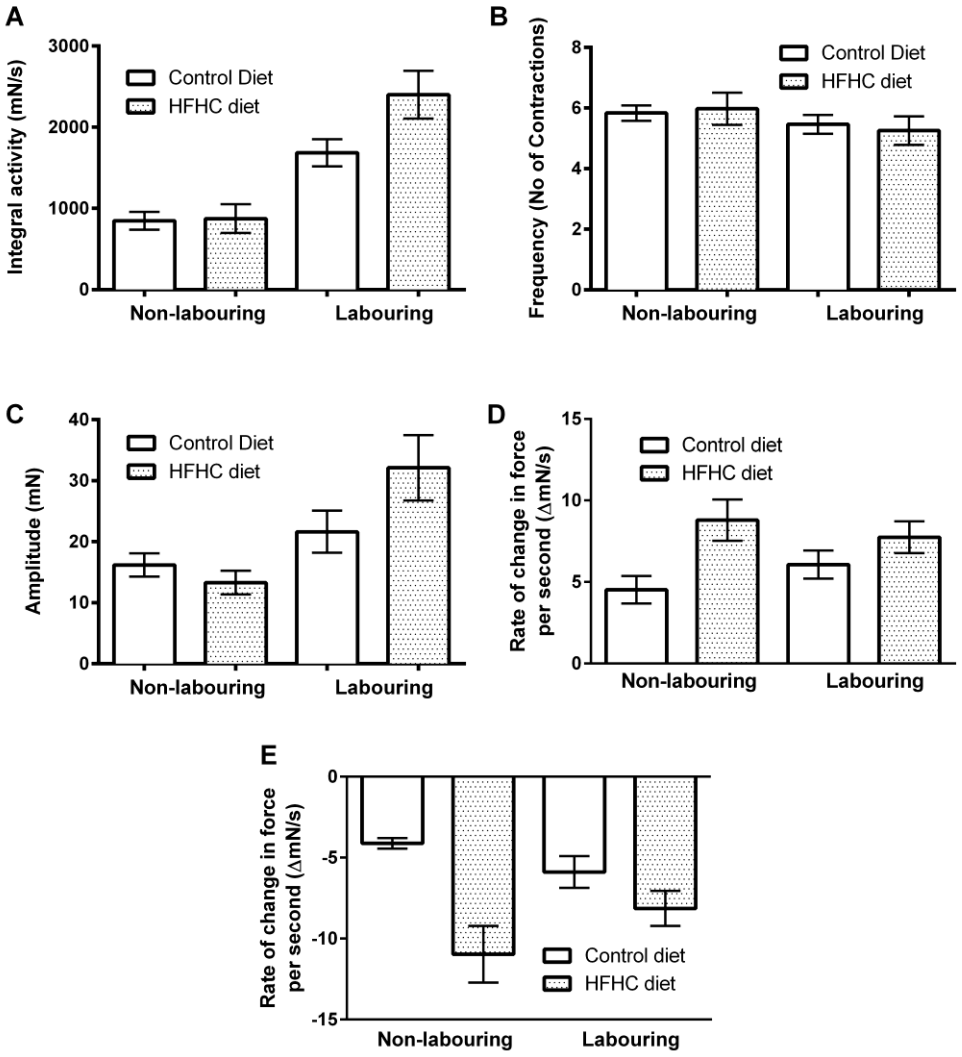


Figure 3.4 - Contractile activity measurements from uterine strips collected from TNL and TL animals fed either a CON or HFHC diet. (**A**) 30 minutes baseline integral activity, (**B**) Mean frequency of contractions during 30 minutes baseline and (**C**) Mean amplitude of uterine contractions, (**D**) Slope maximum of uterine contraction during 30 minute baseline recording, and (**E**) Slope minimum of uterine contraction during 30 minutes baseline recording. Sample sizes CON (TNL=9, TL=8) and HFHC (TNL=6, TL=8). Two-way ANOVA. Values are means ± SEM.

3.4.4.3. Effect of a HFHC diet upon uterine response to accumulative dose of oxytocin

(Synthetic) Oxytocin is utilised to positively augment the outcomes of labour in obese parturients diagnosed with uterine dystocia, but its fails to do so on a significant number of occasions. Thus it felt applicable to challenge animals sustained on a HFHC diet with accumulative doses of OXT to determine their response relative to CON animals. To achieve this, a single strip of uterine tissue from each animal was exposed to an accumulating dose of OXT from 10^{-12} - 10^{-5}M.

3.4.4.3.1 Qualitative measurement of oxytocin stimulated uterine contractile activity

Illustrated within the representative traces (Figure 3.5), uterine tissue challenged with an increasing dose of OXT had a clear effect upon the contraction phenotype *ex vivo.* As the concentration of OXT increases, the ability of the tissue to relax is impeded, leading to an increase in uterine basal muscle tone and contraction frequency. Notably, this increase in uterine basal tone and contraction frequency occurs at a lower dose (10^{-10}M) of OXT in CON than HFHC (10^{-9}M) animals. This suggests that HFHC animals may display insensitivity to OXT relative to CON animals, but further analysis will be required to determine this. Despite the delayed response to OXT, HFHC animals displayed an improvement in contraction synchronisation as the dose increased, though this benefit may be of set by the simultaneous increase in

uterine basal tone and contraction frequency. This data implies that feeding of a HFHC diet may negatively affect uterine OXT response.

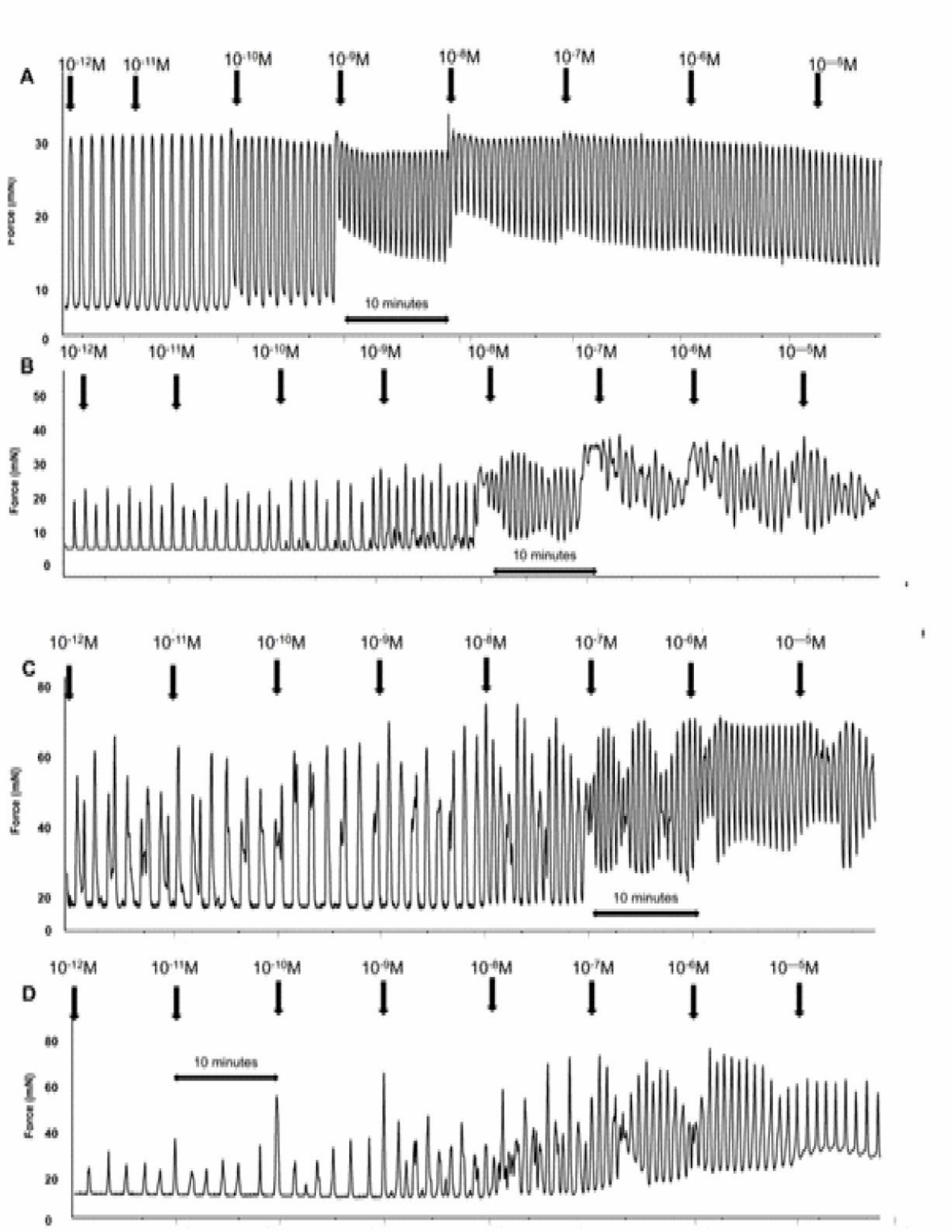


Figure 3.5- Representative *ex vivo* uterine contractility traces from accumulative dosing of oxytocin (1pM - 10µM) from (**A**) a CON TL, (**B**) CON TNL, (**C**) HFHC TL, and (**D**) a HFHC TNL animals.

3.4.4.3.2 Quantitative measurement of oxytocin stimulated uterine contractile activity

As with the spontaneous contractile activity, the same contractile parameters were measured to investigate the effect of maternal diet and stage of labour upon the uterine response to OXT *ex vivo*. Statistical analysis revealed a significant (*P*=0.004) increase in integral activity with increasing dose of OXT (Figure 3.6). Maternal diet (*P*=0.954) and stage of labour (*P*=0.121) were found to have no significant effect upon percentage change in integral activity during the OXT challenge. Despite stage of labour having no significant effect upon percentage change in integral activity, it is clear TNL animals responded to increasing concentrations of OXT more than TL animals (Figure 3.6 C and D). Further analysis split by stage of labour revealed that TNL animals displayed a significant increase (*P*=<0.001) in integral activity in response to increasing dose of OXT, while TL animals displayed no significant (*P*=0.217) change in response to OXT. Accumulative dosing of OXT resulted in a significant increase (*P*=0.011) in uterine contraction frequency *ex vivo*, though maternal diet (*P*=0.252) and stage labour (*P*=0.966) was found to have no significant effect on this measured parameter (Figure 3.7). Increasing concentrations of OXT resulted in a significant increase (*P*=0.001) in contraction amplitude, but again stage of labour (*P*=0.467) or maternal diet (*P*=0.835) had no significant effect. Despite the stage of labour having no significant effect upon contraction amplitude, TNL animals clearly displayed a physiological response to increasing dose of OXT,

relative to the more placid response of TL animals (Figure 3.8). Splitting the data by the stage of labour revealed that OXT significantly interacted with stage of labour (*P*=<0.001), with TNL (*P*=<0.001) and TL (*P*=<0.001) animals displaying a significant increase and decrease in contraction amplitude, respectively. It was important to measure the effect of increasing dose of OXT upon slope maximum and minimum, as this could illustrate if maternal diet or stage of labour were influencing membrane depolarisation or hyperpolarisation, and thus Ca^{2+} concentrations. OXT challenge had no significant (*P*=0.068) effect upon slope maximum, with maternal diet (*P*=0.126) and stage of labour (*P*=0.126) having no significant effect upon slope max during this phase of the study (Figure 3.9). Increasing dose of OXT was negligible in influencing slope minimum (*P*=0.318); as illustrated in Figure 3.10. Though OXT had no significant effect, maternal diet was found to significantly decrease slope minimum (*P*=0.02) during this phase of the study, with stage of labour's effect not quite reaching significance (*P*=0.052).

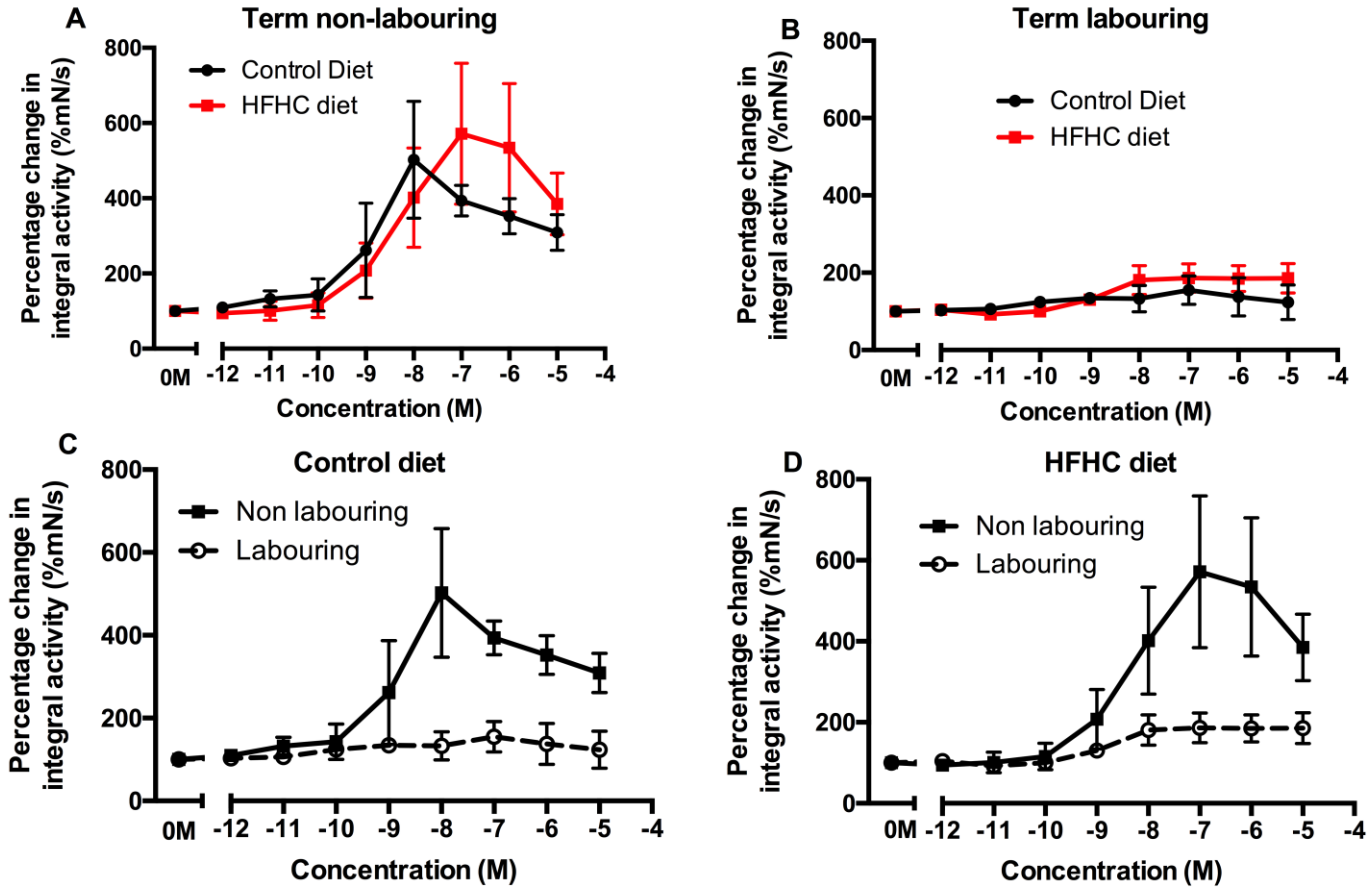


Figure 3.6 **-** Uterine percentage change in integral activity response to accumulative dosing of Oxytocin (10^{-12}M – 10^{-5}M) in (**A**) TNL, (**B**) TL, (**C**) CON, and (**D**) HFHC animals. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Mixed between within ANOVA. Values are means ± SEM.

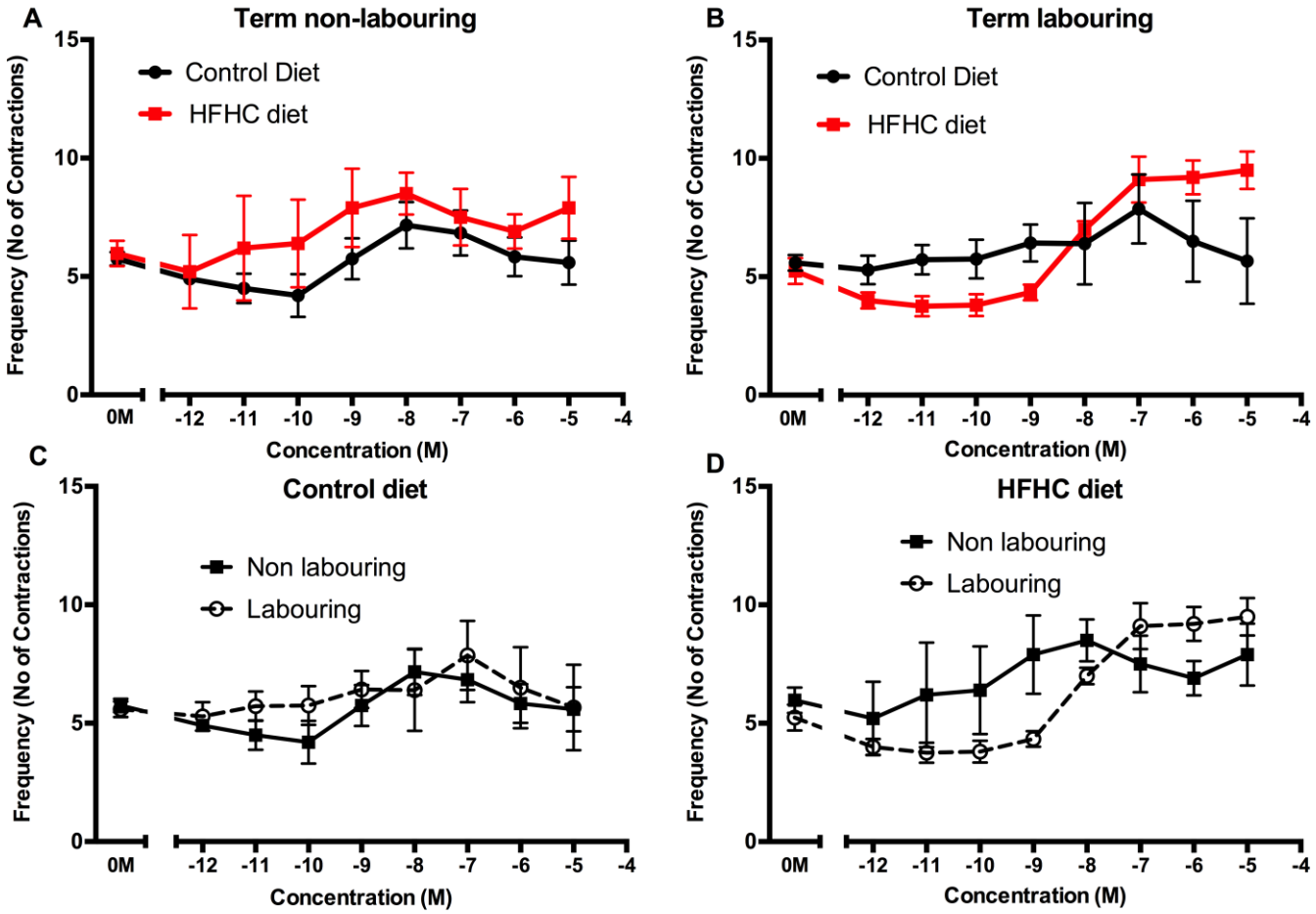


Figure 3.7 - Change in Frequency of uterine contractions resulting from accumulative dosing of Oxytocin (10-12M – 10-5M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Mixed between within ANOVA. Values are means ± SEM

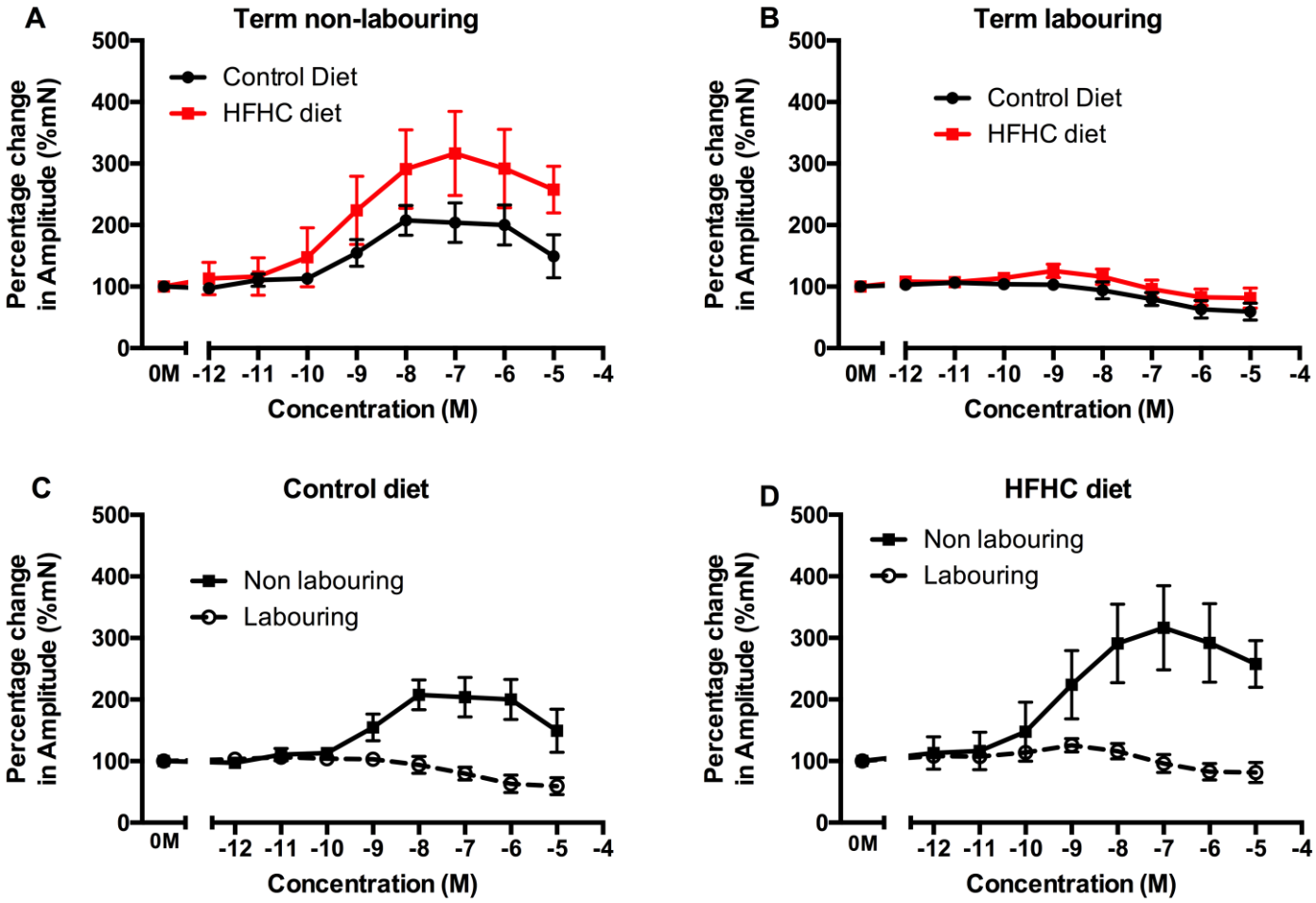


Figure 3.8 - Uterine percentage change in contraction amplitude in response to accumulative dosing of Oxytocin (10^{-12}M – 10^{-5}M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Mixed between within ANOVA. Values are means ± SEM.

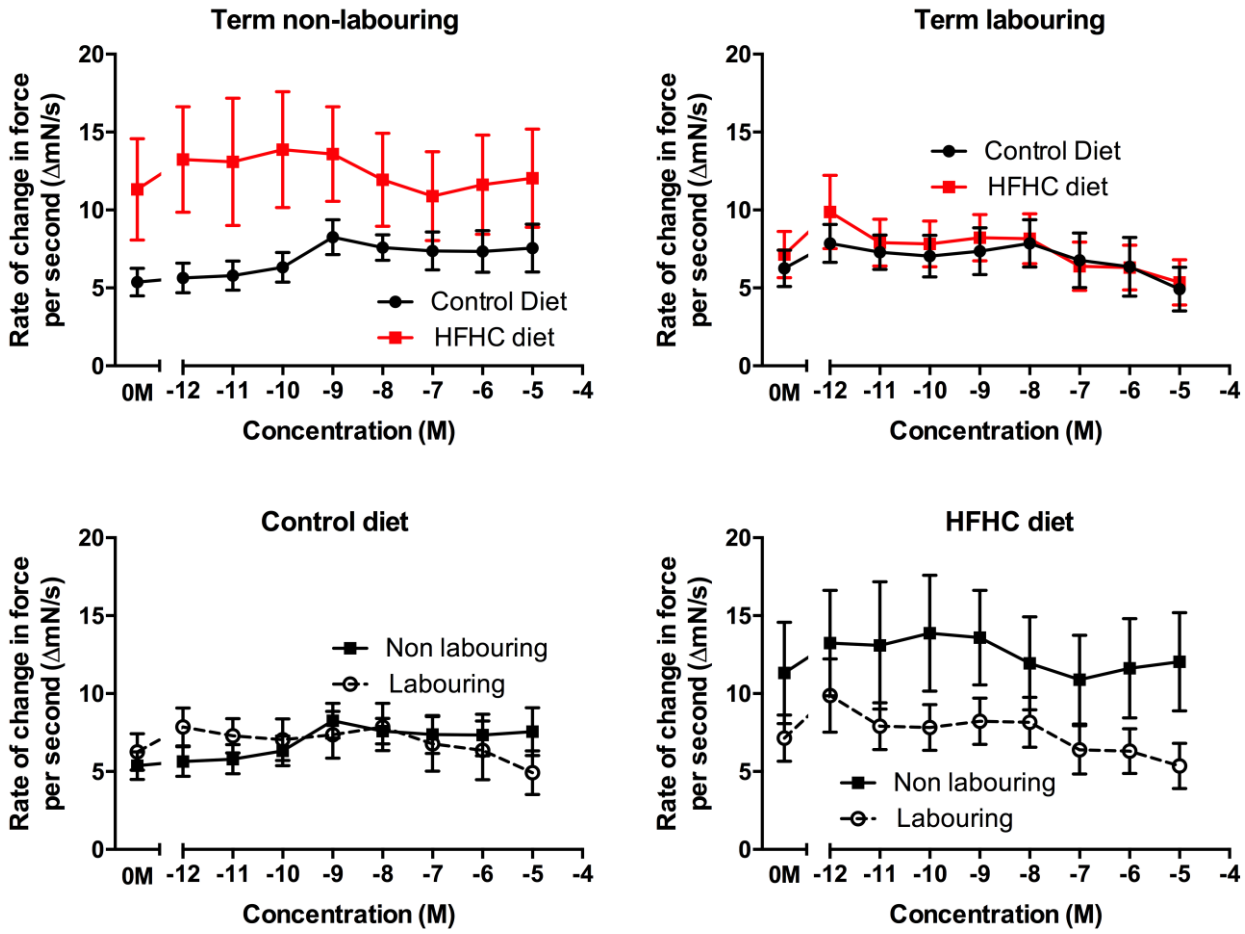


Figure 3.9 – Rate of change in force per second during the contraction phase (slope maximum) in to response accumulative dosing of Oxytocin (10^{-12}M – 10^{-5}M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals illustrating the effect of a HFHC diet upon membrane depolarisation. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Mixed between within ANOVA. Values are means ± SEM.

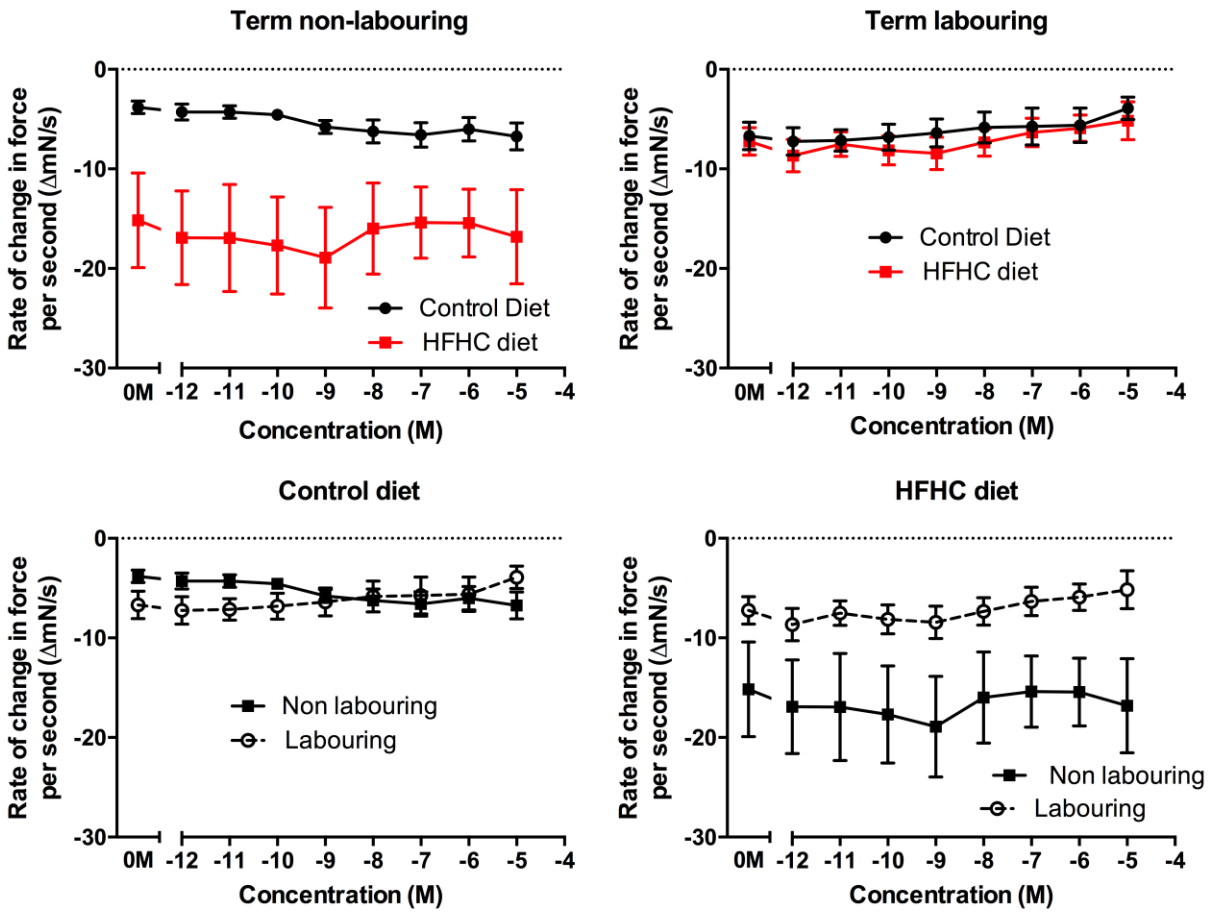


Figure 3.10 - Rate of change in force per second during the relaxation phase (slope minimum) in response to accumulative dosing of Oxytocin (10^{-12}M – 10^{-5}M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals illustrating the effect of a HFHC diet upon membrane hyperpolarisation. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Mixed between within ANOVA. Values are means ± SEM.

Dose response analysis was utilised to further investigate the potential OXT insensitivity identified during qualitative analysis of the data (Figure 3.11). At day 20 of gestation TNL, dose response analysis revealed no significant difference between the CON and HFHC animals in response to increasing concentrations of OXT. The onset of TL resulted in HFHC animals displaying a delayed response to increasing OXT concentrations, as highlighted by the significant difference in $LogEC_{50}$ (CON: 10.25M vs HFHC: 8.84M; *P*=<0.05). A significantly greater hillslope (CON: 0.90 vs HFHC: 1.55; *P*=<0.05) in HFHC may explain this delayed response to OXT, as a change in hillslope may signify increased OXTR competition or a change in ligand binding efficiency or affinity.

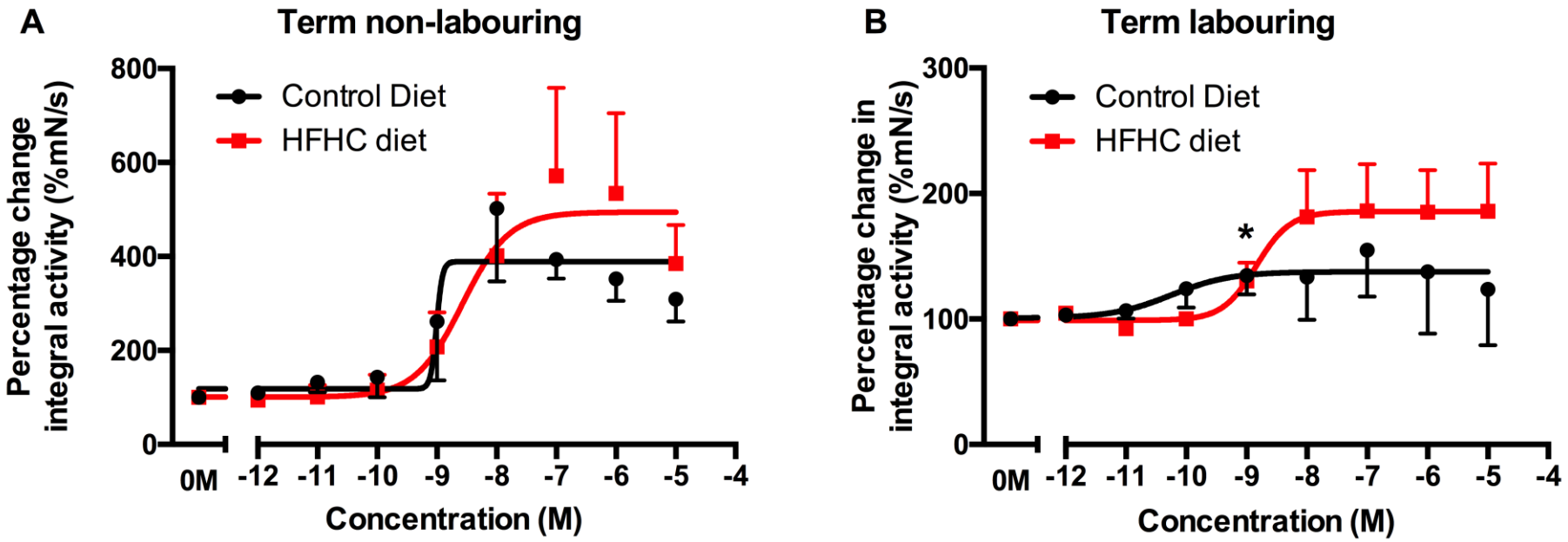


Figure 3.11- Sigmoidal agonist dose response analysis of percentage change in uterine integral contractile response *ex vivo* to increasing concentration of oxytocin (10^{-12}M – 10^{-5}M) from CON or HFHC fed rats during (**A**) TNL and (**B**) TL. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Values are means ± SEM. * defines significant difference between CON and HFHC rats at the $P \leq 0.05$ level.

3.4.4.4. Effect of a HFHC diet upon uterine response to accumulative dose of $PGF_{2\alpha}$

$PGF_{2\alpha}$ is a uterotonin that endogenously stimulates uterine contractions during parturition. $PGF_{2\alpha}$ is prescribed by clinicians to positively augment the outcomes of a prolonged and dysfunctional labour. As a result, it was important to ascertain if stage of labour or maternal diet affected uterine response to accumulating dosing of $PGF_{2\alpha}$ *ex vivo.* To determine this, a single strip of uterine tissue from each animal was exposed to an accumulating dose of $PGF_{2\alpha}$ from 10^{-10} - 10^{-3}M.

3.4.4.4.1 Qualitative measurement of $PGF_{2\alpha}$ stimulated uterine contractile activity

Maternal diet or stage of labour had no qualitative effect upon $PGF_{2\alpha}$ stimulated uterine contractions *ex vivo* (Figure 3.12). Though there was a minor increase in contraction frequency and amplitude during the 10^{-3}M dose in both dietary groups.

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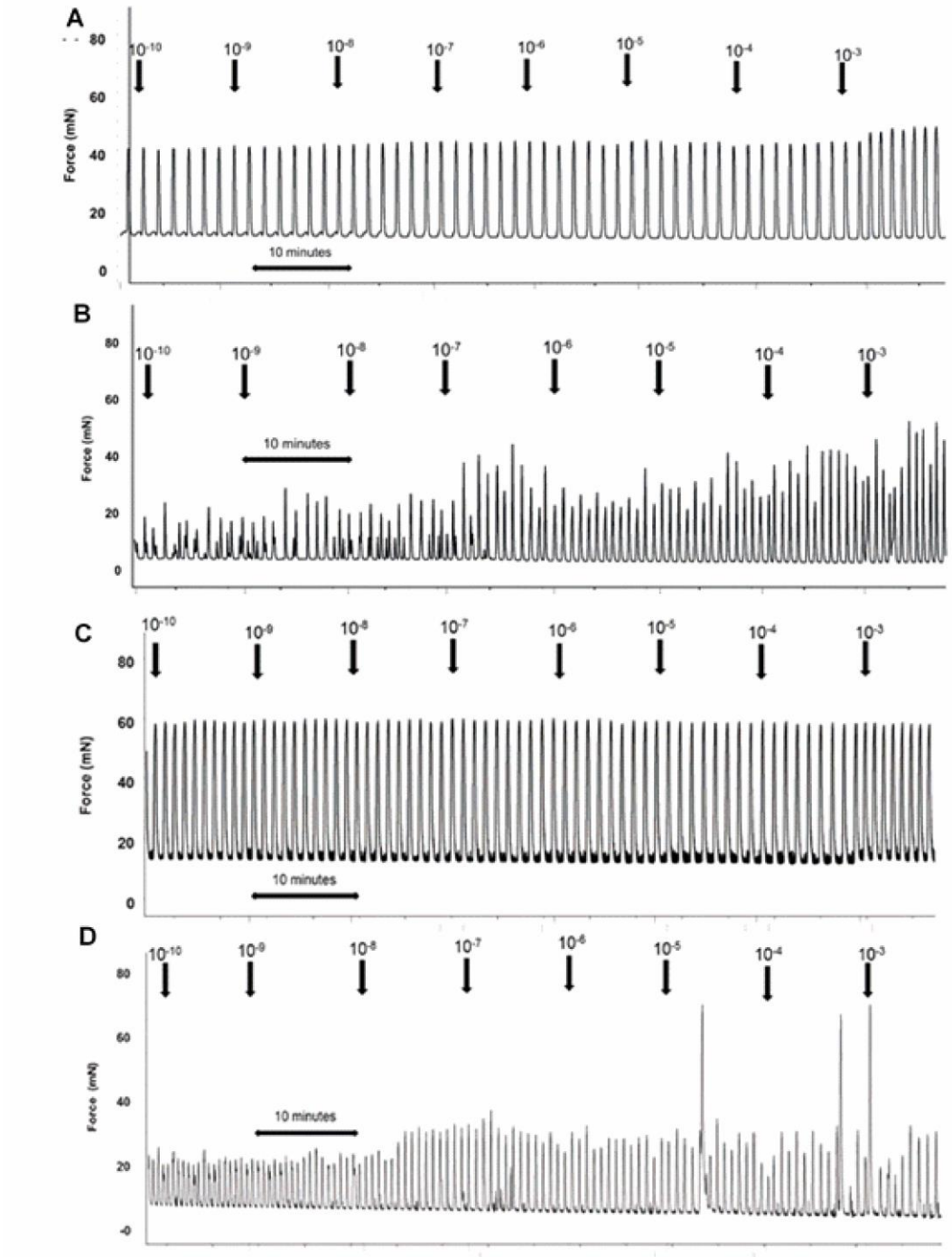


Figure 3.12 - Representative *ex vivo* uterine contractility traces from accumulative dosing of $PGF_{2\alpha}$ (10^{-14}M – 10^{-7}M) from (**A**) CON TL, (**B**) CON TNL, (**C**) HFHC TL, and (**D**) HFHC TNL animals.

3.4.4.4.2 Quantitative measurement of $PGF_{2\alpha}$ stimulated uterine contractile activity

Despite the lack of response within the traces, accumulative dosing of $PGF_{2\alpha}$ had a significant effect upon the contractile parameters measured. Increasing dose of $PGF_{2\alpha}$ lead to a significant (*P*=<0.001) increase in integral activity (Figure 3.13). The stage of labour (*P*=0.331) or maternal diet (*P*=0.069) had no significant effect upon integral activity during accumulative dosing of $PGF_{2\alpha}$. Increasing dose of $PGF_{2\alpha}$ lead to no significant change (*P*=0.142) in uterine contraction frequency, with stage of labour having no significant effect (*P*=0.876) upon this measurement. Maternal diet was found to have a significant effect upon contraction frequency during $PGF_{2\alpha}$ accumulative dosing, with HFHC animals displaying a lower contraction frequency during TNL (Figure 3.14). A significant increase (*P*=0.008) in contraction amplitude was reported during the accumulative dosing of $PGF_{2\alpha}$ (Figure 3.15). Maternal diet and stage of labour was found to have no significant effect upon contraction amplitude during the $PGF_{2\alpha}$ challenge. Slope maximum was significantly (*P*=0.017) affected by the increasing dose of $PGF_{2\alpha}$ (Figure 3.16). Maternal diet was found to have a significant effect (*P*=0.028) upon slope max, with HFHC animals displaying an increase during TL. Stage of labour had no significant (*P*=0.942) effect upon slope maximum during the accumulative dosing of $PGF_{2\alpha}$. $PGF_{2\alpha}$ was found to have no significant effect (*P*=0.088) upon slope minimum, with stage of labour having no significant (*P*=0.868) effect upon this recorded parameter (Figure 3.17). Maternal diet was

found to have a significant effect (*P*=0.004) upon slope minimum during the accumulative dosing of $PGF_{2\alpha}$. Dose response analysis could not be carried out, as the maximum dose of $PGF_{2\alpha}$ was not sufficient to reach a plateau, and allow $LogEC_{50}$ and hillslope to be calculated (Figure 3.18).

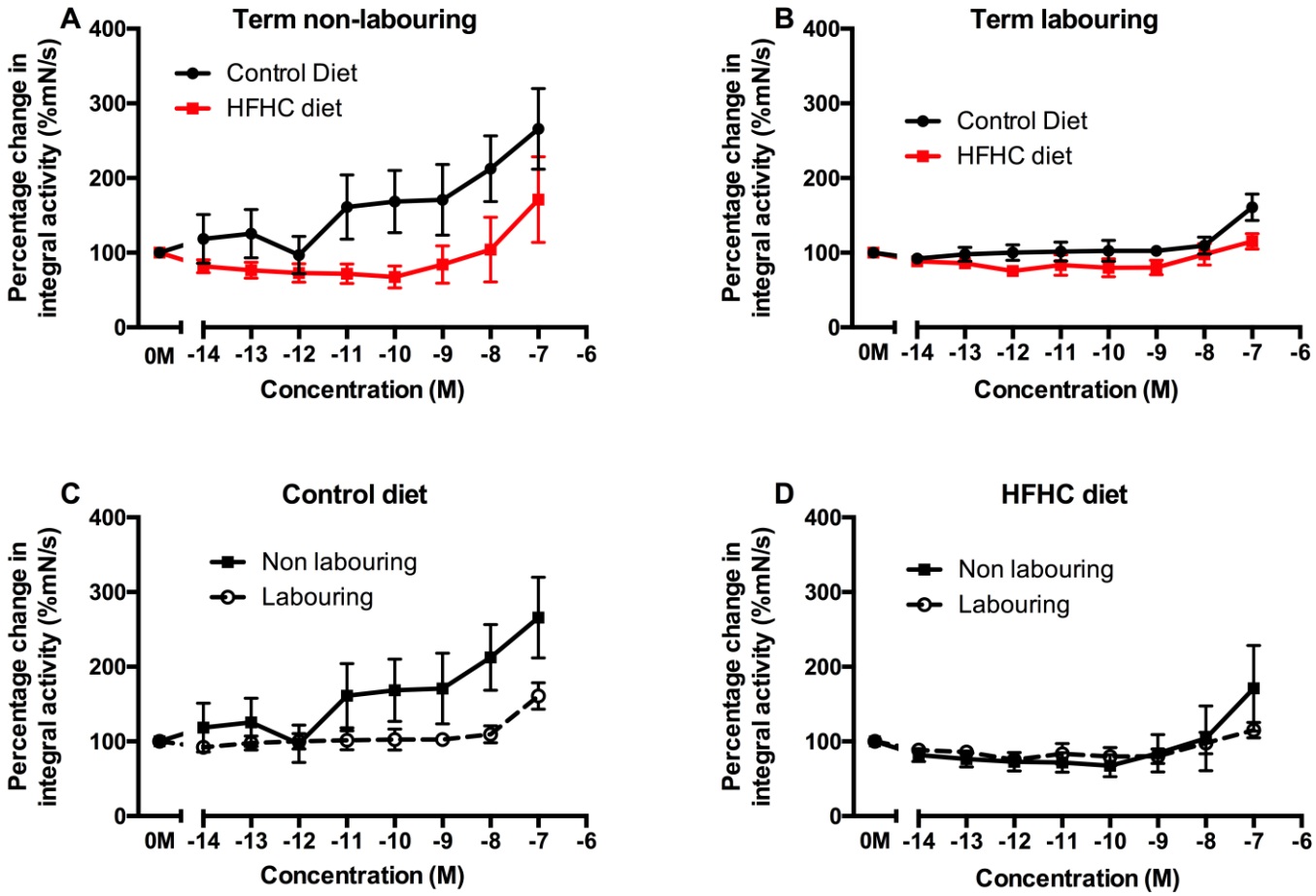


Figure 3.13 - Uterine percentage change in integral activity response to accumulative dosing of $PGF_{2\alpha}$ (10^{-14}M – 10^{-7}M) in (**A**) TNL, (**B**) TL, (**C**) CON, and (**D**) HFHC animals. Sample sizes CON (TNL=5, TL=4) and HFHC (TNL=7, TL=5). Mixed between within ANOVA. Values are means ± SEM.

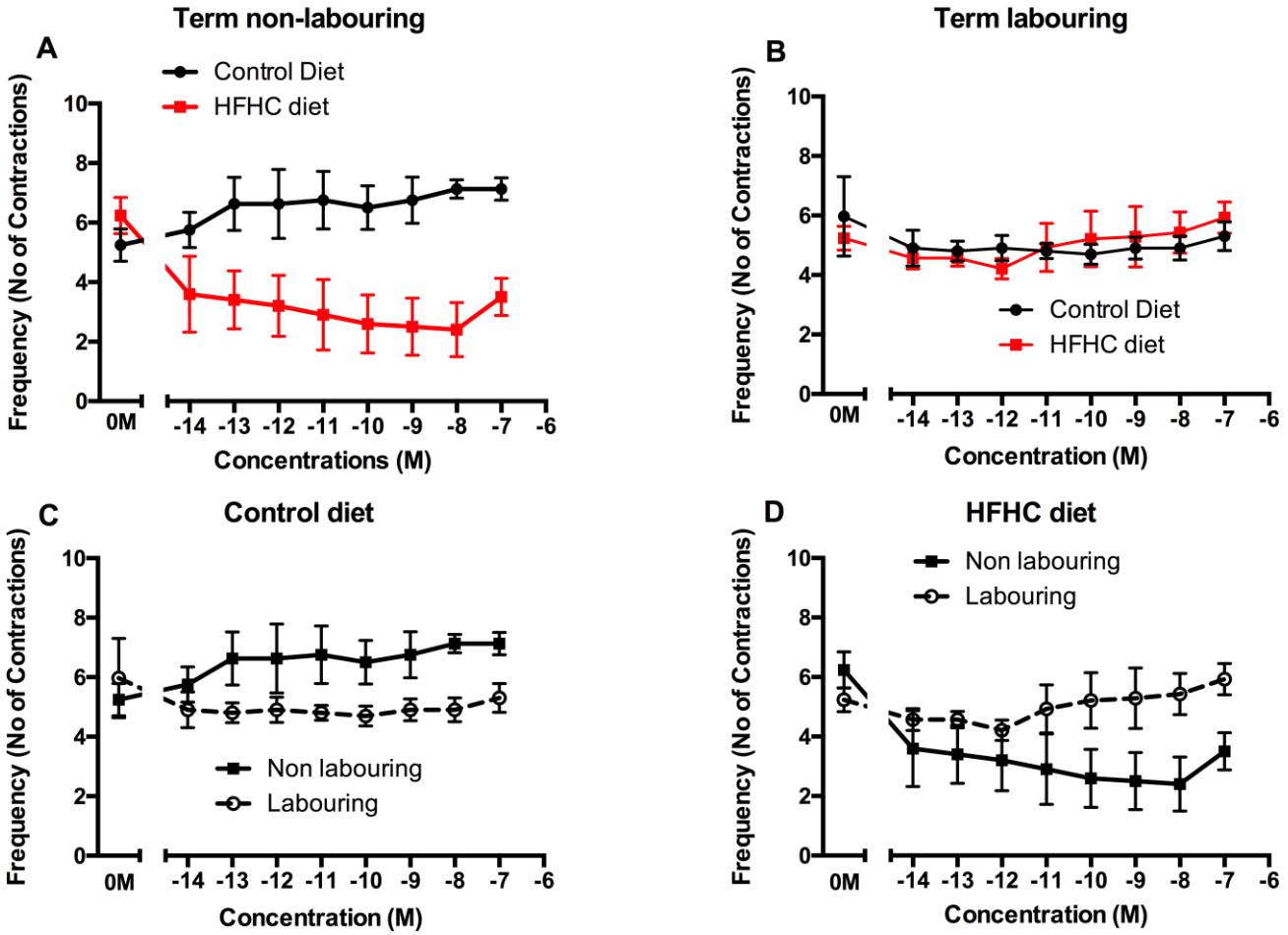


Figure 3.14 - Change in Frequency of uterine contractions resulting from accumulative dosing of $PGF_{2\alpha}$ (10-14M – 10-7M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals. Sample sizes CON (TNL=5, TL=4) and HFHC (TNL=7, TL=5). Mixed between within ANOVA. Values are means ± SEM.

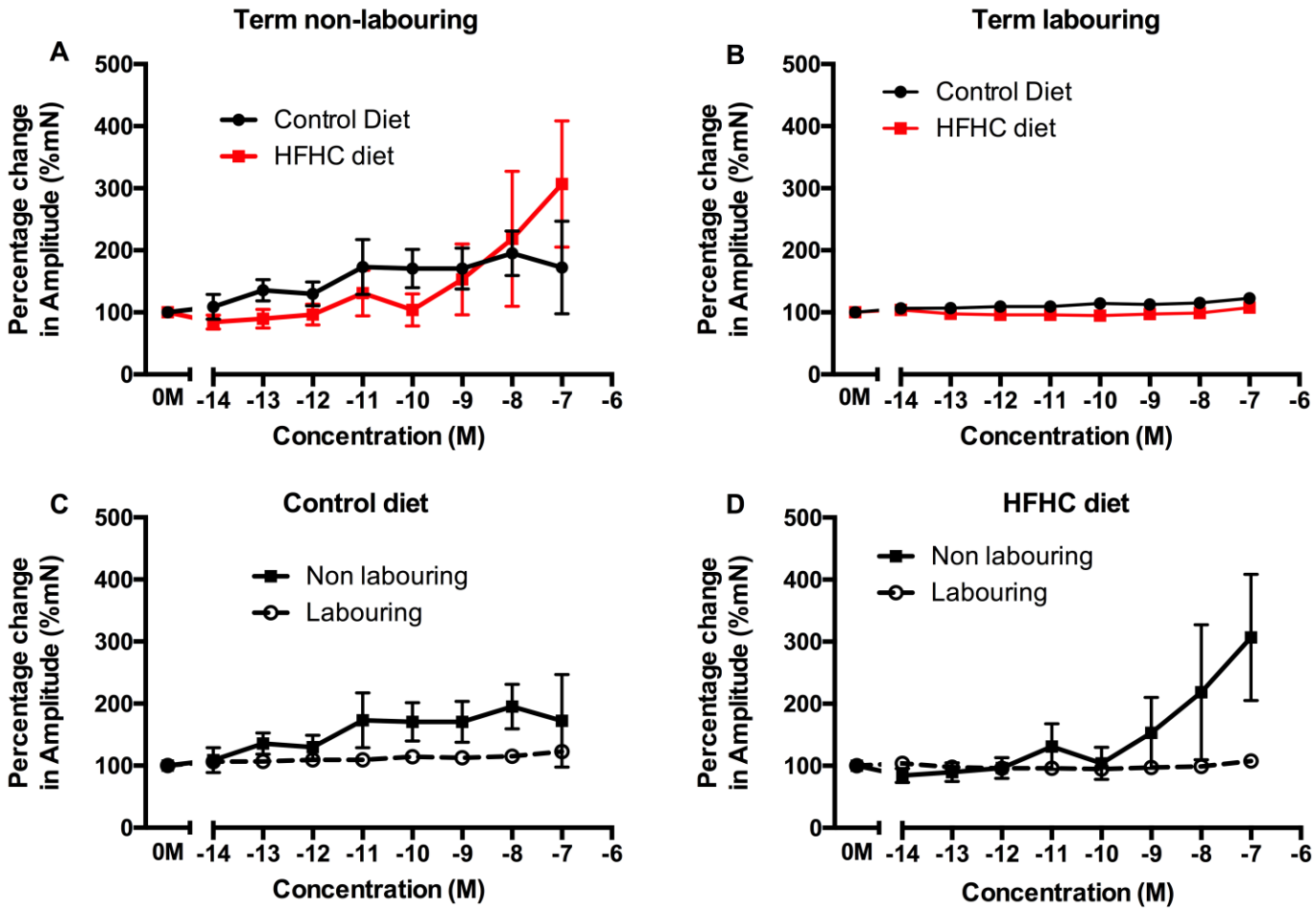


Figure 3.15 - Uterine percentage change in contraction amplitude in response to accumulative dosing of $PGF_{2\alpha}$ (10^{-14}M – 10^{-7}M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals. Sample sizes CON (TNL=5, TL=4) and HFHC (TNL=7, TL=5). Mixed between within ANOVA. Values are means ± SEM.

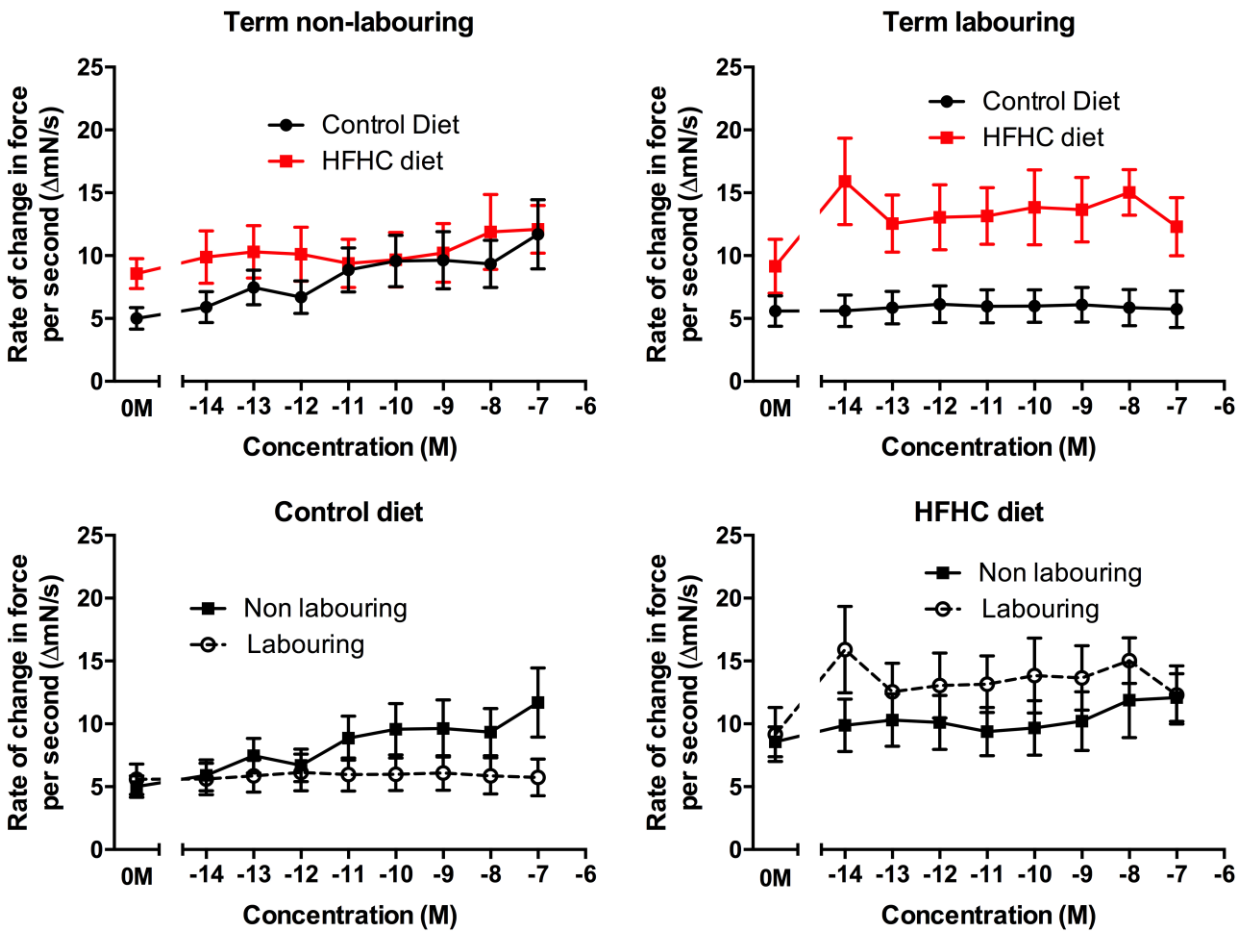


Figure 3.16 - Rate of change in force per second during the contraction phase (slope maximum) in response to accumulative dosing of $PGF_{2\alpha}$ (10^{-14}M – 10^{-7}M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals illustrating the effect of a HFHC diet upon membrane depolarisation. Sample sizes CON (TNL=5, TL=4) and HFHC (TNL=7, TL=5). Mixed between within ANOVA. Values are means ± SEM.

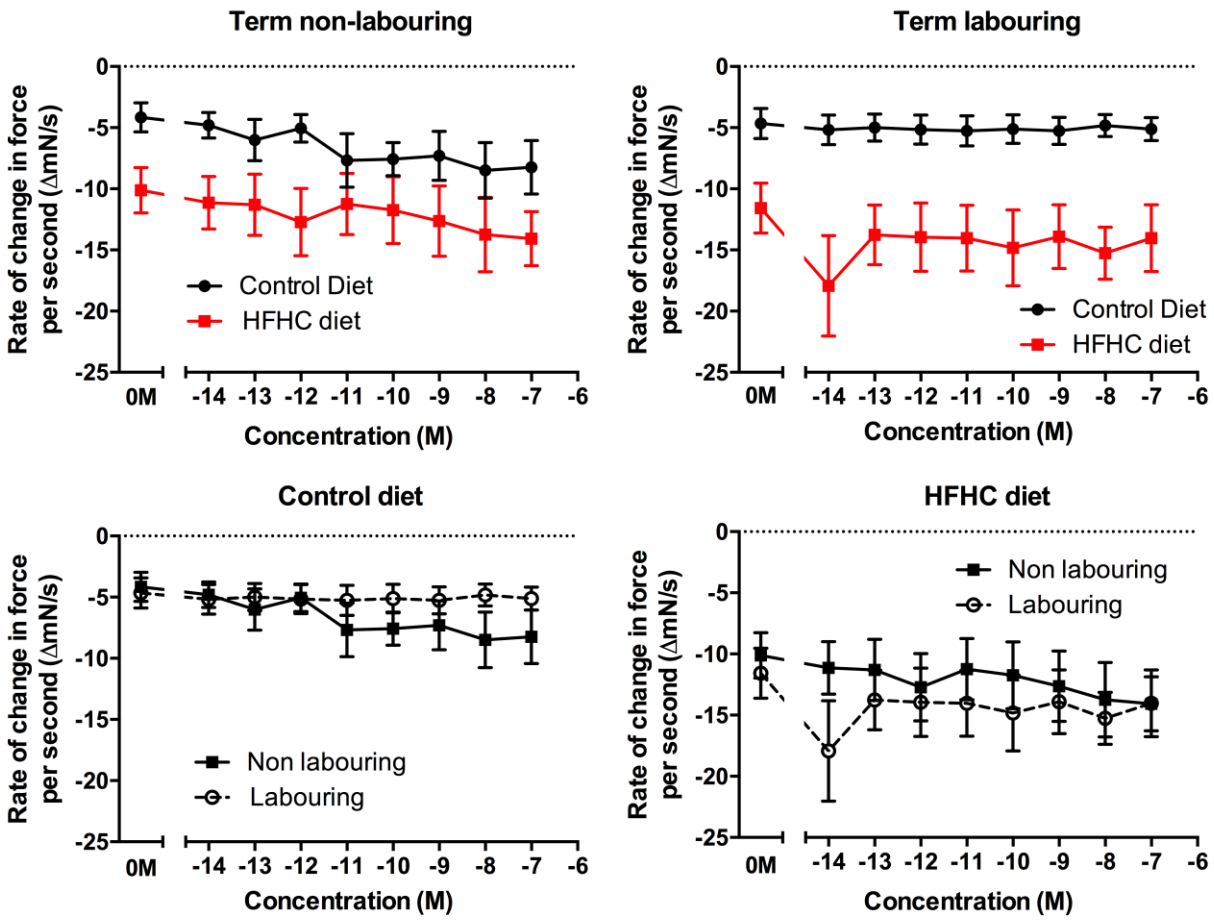


Figure 3.17 – Rate of change in force per second during the relaxation phase (slope minimum) in response to accumulative dosing of $PGF_{2\alpha}$ (10^{-14}M – 10^{-7}M) in (**A**) TNL, (**B**) TL, (**C**) CON diet, (**D**) HFHC diet animals illustrating the effect of a HFHC diet upon membrane hyperpolarisation. Sample sizes CON (TNL=5, TL=4) and HFHC (TNL=7, TL=5). Mixed between within ANOVA. Values are means ± SEM.

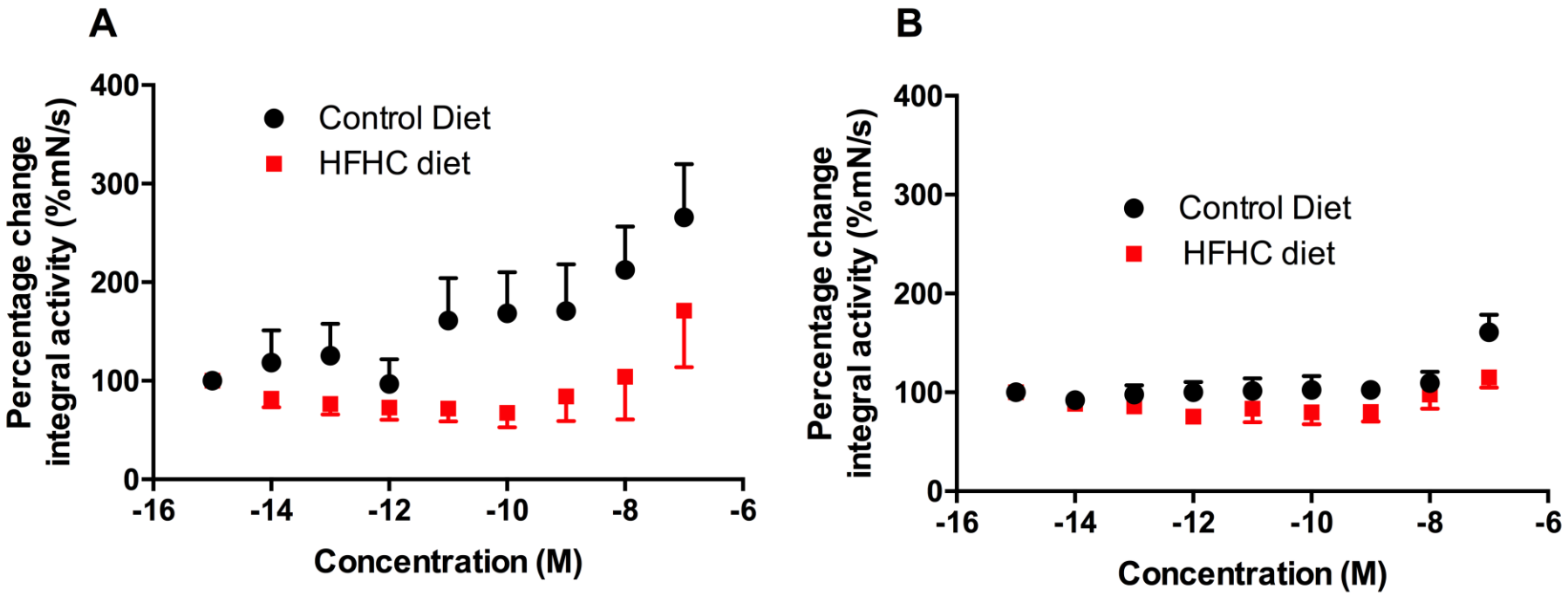


Figure 3.18 – Attempted dose response analysis of percentage change in uterine integral contractile response *ex vivo* to increasing concentration of $PGF_{2\alpha}$ (10^{-12}M – 10^{-5}M) from CON or HFHC fed rats during (**A**) TNL and (**B**) TL. Sample sizes CON (TNL=7, TL=7) and HFHC (TNL=5, TL=6). Values are means ± SEM.

3.4.5. Western blotting

Western blotting identifies proteins through the use of a targeted antibody and the measurement of their molecular weight. Analysis of uterine CAP expression was important as it may offer an initial perspective to how a HFHC diet is inducing a different contraction phenotype or delaying OXT response relative to CON animals. Feeding of a HFHC diet was found to have no significant effect upon uterine Cav-1 expression (*P*=0.793), though stage of labour was found to have a significant effect (*P*=<0.015), with HFHC animals displaying a decrease from TNL to TL (Figure 3.19A). Maternal diet (*P*=0.324) and stage of labour (*P*=0.320) were found to have no significant effect upon uterine expression of CX-43 (Figure 3.19B), but there was a significant interaction (*P*=0.024) between these two groups. Despite this lack of significance, uterine CX-43 expression decreased with the onset of labour in HFHC animals, while CON animals displayed no relative change. Transition from TNL to TL resulted in a significant change (*P*=0.031) in uterine *P*CX-43 (Figure 3.19C), with maternal diet displaying no significant effect (*P*=0.291) but significantly interacting with stage of labour (*P*=0.004). CON animals displayed a substantial decrease in uterine *P*CX-43 expression with the onset of TL, while HFHC animals displayed no relative change in expression at the same stage. Maternal diet was found to have a significant effect (*P*=0.027) upon uterine OXTR expression (Figure 3.19D), with HFHC animals displaying an increase during TL. Stage of labour was found to have no

significant effect (*P*=0.610), with CON animals displaying no relative change in uterine OXTR expression from TNL to TL. Uterine COX-2 expression significantly (*P*=0.031) changed with the onset of labour, with HFHC animals displaying an increase relative to the no change in CON uterine COX-2 expression (Figure 3.19E). Maternal diet was found to have no significant effect (*P*=0.834) upon uterine COX-2 expression.

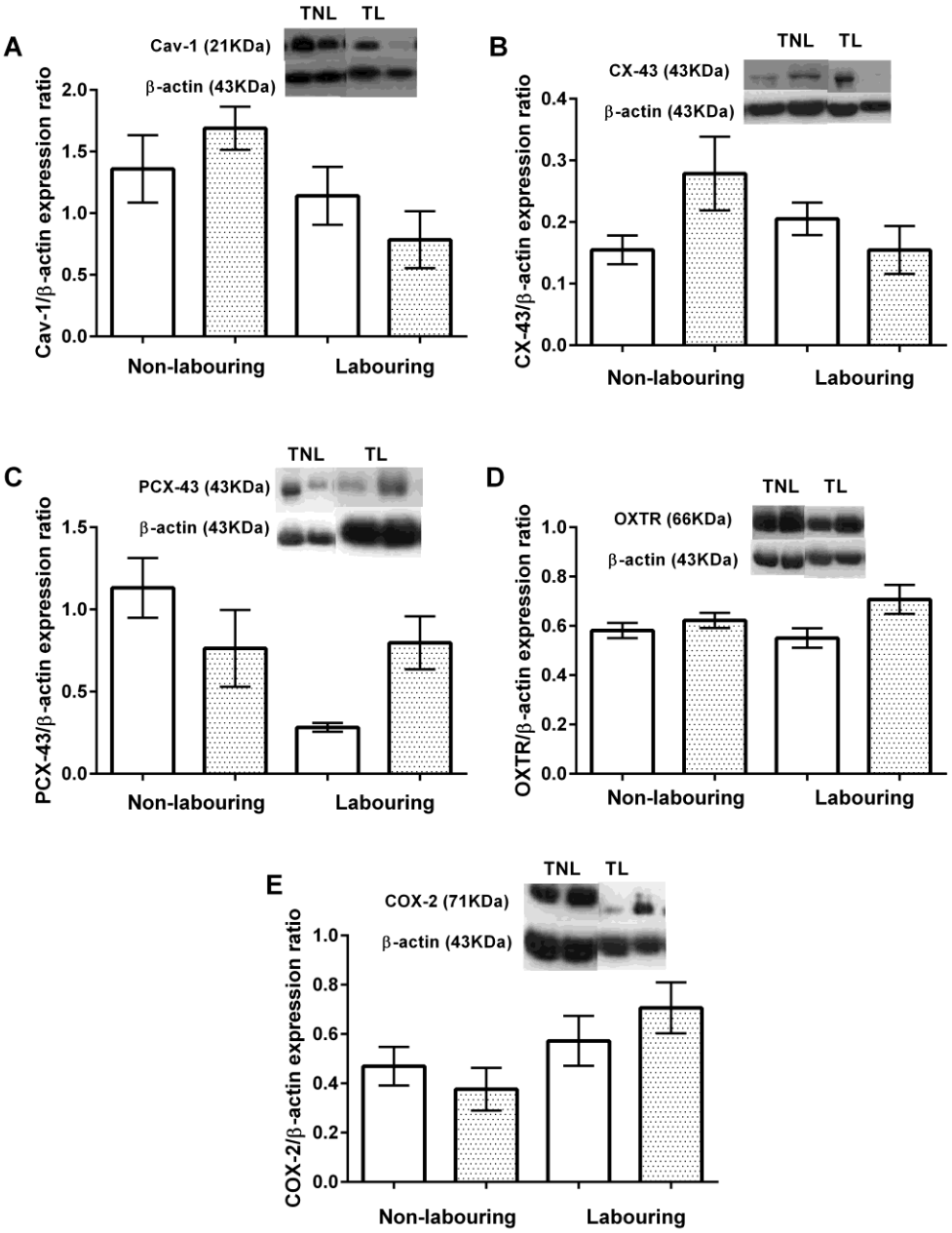


Figure 3.19 - The effect of a CON or HFHC diet upon contractile associated protein expression in the term non-labouring (TNL) or term labouring uterus. (**A**) caveolin -1 (Cav-1), (**B**) connexin-43 (CX-43), (**C**) Ser368 phosphorylated form of connexin-43 (PCX43), (**D**) oxytocin receptor (OXTR) and (**E**) cyclooxygenase-2 (COX-2). Sample sizes CON (TNL=9, TL=8) and HFHC (TNL=6, TL=8). Two way ANOVA. Values are means ± SEM.

3.5. Discussion

The objective of this study was to determine if chronic exposure to a HFHC diet significantly altered uterine CAP expression and uterine contractility *ex vivo*. This will begin to determine if this model is effective in the study of maternal obesity associated uterine dystocia.

Chronic exposure to the HFHC diet certainly induced obesity and hypercholesterolemia. Animals sustained on the HFHC diet ended this study with a significantly greater final weight (CON 416.87 ± 5.77g vs HFHC 456.37 ± 12.69g; *P*=0.012), as a result of their significantly greater visceral fat mass. Prolonged exposure to a HFHC diet significantly increased plasma total cholesterol concentrations (CON 2.63 ± 0.08mmol/L vs HFHC 9.00 ± 0.44mmol/L; *P*=<0.001) relative to CON animals, symptomatic of hypercholesterolemia.

A distinct part of this study was to establish if feeding of a HFHC diet has a significant effect upon spontaneous uterine contractile activity at TNL and TL *ex vivo*. Qualitative analysis revealed that CON animals displayed a synchronous biphasic plateau contraction phenotype, with amplitude stabilising at the onset of TL. HFHC animals displayed an asynchronous multiphasic spike bundle contraction phenotype, which mildly improved with the onset of TL. The difference in the uterine contraction phenotype between the maternal diet groups highlights a distinct lack of synchronisation in the contraction and relaxation of the smooth muscle bundles that compose the myometrium (Anatoly

Shmygol et al., 2007). A biphasic plateau contraction is a two stage single contractile event that results from synchronised contraction and relaxation of the smooth muscle bundles that compose the myometrium (Anatoly Shmygol et al., 2007). Unlike the plateau contraction phenotype, the smooth muscle bundles that compose the myometrium do not contract and relax in a synchronous manner, producing a multiphasic spike bundle uterine contraction (Anatoly Shmygol et al., 2007). The resulting multiphasic spike bundle contraction phenotype identified within HFHC animals signifies these animals may have suffered from prolonged and dysfunctional labour.

In an attempt to overcome a prolonged and dysfunctional labour, parturients can be prescribed uterotonins (predominantly OXT) to stimulate contraction of the uterus, and positively augment the outcome of labour (Arrowsmith and Wray, 2014). OXT is more likely to fail in augmenting labour in obese parturients, but if successful they are likely to require a significantly greater dose relative to normal weight parturients (Roloff et al., 2015). Similar to the study by Roloff et al. (2015), OXT failed to positively augment the asynchronous multiphasic spike bundle contraction phenotype identified within HFHC animals. Further to this, dose-response analysis also revealed that HFHC animals would need a significantly greater dose of OXT to produce the same contractile response as CON animals. Despite this, as OXT concentrations increased out of the physiological range they appeared to improve synchronisation of uterine contractions, but any benefit

could have been negated by the increase in contraction frequency, and uterine basal tone. Increased contraction frequency and uterine basal tone could restrict placental blood flow, increasing foetal distress (Budden et al., 2014).

In an attempt to elucidate the cause of the asynchronous multiphasic contractile phenotype, circulating progesterone concentration and uterine CAP expression was determined. From this study it was found that both maternal diet and stage of labour had a significant and in some cases a synergistic effect upon uterine CAP expression including CX-43, Cav-1, OXTR and COX-2. Maternal diet and stage of labour were found to have no significant effect upon uterine CX-43 expression. Though there was a distinct lack of statistical significance, CX-43 expression decreased with the onset of TL in HFHC animals, which could have a notable physiological effect. Stage of labour was found to have a significant effect upon uterine Cav-1 expression, though HFHC animals displayed a decrease with the onset of TL. Exposure to a HFHC diet was found to significantly increase uterine OXTR expression at TL. Uterine COX-2 expression significantly increased with the onset of TL in HFHC animals, unlike the CON animals that displayed no relative change in its expression.

CX-43 is a protein essential to the formation of gap junctions that directly facilitate cell-cell electro-coupling (synchronisation) and passive transfer of metabolites (Sims et al., 1982). The number of myometrial

gap junctions increase with the onset of partition to propagate synchronous uterine contractions (Wray, 1993), while a lack of gap junctions at term significantly prolongs the duration of labour (Döring et al., 2006; Tong et al., 2009). HFHC animals display a decrease in CX-43 during TL, which could significantly compromise the syncytium of the uterus, and prolong the duration of labour. In conjunction with this decrease in uterine CX-43 expression, HFHC animals displayed no relative change in *P*CX-43 expression with the onset of labour, while CON animals displayed a significant decrease in *P*CX-43 expression with the onset of TL. *P*CX-43 is the dysfunctional serine-368 phosphorylated form of CX-43 that disrupts cellular trafficking of CX-43 and the formation of intracellular gap junctions (Oshima, 2014; Su and Lau, 2014). Cholesterol enrichment of cardiomyocyte culture medium results in significant phosphorylation of CX-43, impeding gap junction assembly (Zou et al., 2014). The resulting change in uterine expression of the 'functional' CX-43 and the dysfunctional *P*CX-43 offers a potential explanation or a contributory factor to the dysfunctional HFHC contractile phenotype.

Cav-1 is the cholesterol-rich structural protein of caveoale. Caveolae are the omega-shaped invaginations of the plasma membrane (Okamoto et al., 1998) that support a range of ion channels (Brainard et al., 2009, 2005; O'Connell et al., 2004; A Shmygol et al., 2007), receptors (Gimpl and Fahrenholz, 2000; Klein et al., 1995; Turi et al., 2001) and gap junctions (Defamie and Mesnil, 2012) essential to

regulating uterine contractions during parturition. HFHC animals displayed a significant decrease in uterine Cav-1 expression with the onset of labour. Disruption of Cav-1 within murine knockout models results in impaired smooth muscle contractility (Drab et al., 2001; Je et al., 2004). This illustrates the crucial role of Cav-1 in smooth muscle contractility, with its loss potentially impacting upon uterine contractility and downstream signalling.

OXTR is localised within the caveolae of uterine myocytes, with its expression remaining low throughout gestation until the onset of labour (Fuchs et al., 1984; Gimpl and Fahrenholz, 2000). Binding of OXT to its receptor directly stimulates contraction of the uterus during labour through activation of the Ca^{2+}-calmodulin-MLCK pathway (Challis et al., 2000).

Higgins et al. 2010 has reported that increasing body mass has no effect on human uterine OXT response or OXTR expression *ex vivo*, but unlike this study HFHC diet induced maternal obesity significantly reduced OXT sensitivity and increased uterine OXTR expression during term labour *ex vivo*.

The contrasting findings of these studies are possibly a result of collecting tissue at difference stages of parturition. Research by Challis and Lye (20000) has clearly established that TNL and TL are completely distinct physiological states, with differences in uterine CAP

expression including OXTR. As a result, the tissue collected by Higgins et al. (2010) will have not completely undergone uterine activation affecting its response to OXT (Challis et al., 2000). This explains a key disparity between these two studies, and highlights the need to collect tissue during TL to thoroughly assess the effect of maternal obesity upon uterine contractility.

Through this study, maternal diet had no significant effect upon TNL OXT sensitivity or OXTR expression, which changed with the onset of TL. The use of TL uterine tissue highlights the compromising effect of maternal obesity upon OXT signalling, though further investigation is warranted to fully assess this. Unlike OXT, both CON and HFHC animals at both stages of labour displayed a lack of response to increasing concentrations of $PGF_{2\alpha}$. PG's including PGE_2 and $PGF_{2\alpha}$ are synthesised from PLA_2 membrane released AA by COX-2 (Vichai et al., 2005). Chronic exposure to a HFHC diet significantly increased HFHC uterine COX-2 expression with the onset of labour, while CON animals displayed no relative change. This increase in uterine COX-2 expression at the onset of TL may be a result of low $PGF_{2\alpha}$ concentration, which has been identified in this model previously (Elmes et al., 2011). This change in uterine CAP's identified within HFHC animal's offers evidence to why these animals are displaying an asynchronous multi-phasic contraction phenotype during TL.

Through *ex vivo* investigation it is clear that chronic exposure to a HFHC diet significantly increases visceral adiposity and circulating lipids, while adversely effecting spontaneous and stimulated uterine contractility, and uterine CAP expression. As a result, the HFHC dietary animal model could be a suitable platform to test the proposed aetiology and mechanism(s) governing maternal obesity associated uterine dystocia.

A number of hypotheses have been proposed to explain the compromising effect of maternal obesity upon TL uterine contractile activity. One such hypothesis is endocrine dysfunction characterised by an imbalance in the progesterone and oestradiol ratio at term (Bogaerts et al., 2013; Carlson et al., 2015). Specifically, the functional withdrawal of progesterone is inadequate or incomplete, allowing progesterone to sustain its pro-gestational effects to a degree. This imbalance will limit oestradiol-induced activation of the uterus, which will compromise uterine CAP expression (Bogaerts et al., 2013). Animals exposed to a HFHC diet displayed significantly greater circulating concentrations of progesterone at TNL. Increased circulating concentrations of progesterone could have been sufficient to induce the significant change in uterine CAP expression, and as a result altering the uterine contraction phenotype. Further to this progesterone will also limit the activation of the pro-inflammatory immune response (Mesiano and Welsh, 2007) and the translocation of CX-43 to the membrane (Hendrix et al., 1995), which will compromise the onset of parturition and

synchronous contraction of the uterus (Bogaerts et al., 2013). Endocrine disruption is not the only potential mechanism of maternal obesity induced uterine dystocia.

A noteworthy hypothesis is that increased cholesterol is impeding term labouring uterine contractility, resulting in prolonged and dysfunctional labour (Bogaerts et al., 2013; Carlson et al., 2015). This is a valid hypothesis as serum cholesterol concentrations are significantly raised within obese parturients (Farias et al., 2015), with exogenous cholesterol impeding both rat and human myometrial contractility in a dose-dependent manner *ex vivo* (Smith et al., 2005; Jie Zhang et al., 2007). Increased cholesterol concentrations are thought to compromise uterine contractility by modulating membrane fluidity, and thus membrane peripheral and integral protein expression (Bogaerts et al., 2013; Carlson et al., 2015; Pucadyil and Chattopadhyay, 2006). The plasma membrane of the cell separates the intra-cellular from the extra-cellular space, and supports a diverse population of peripheral and integral proteins including receptors, ion channels and gap junctions. The plasma membrane is composed of a range of lipids including phospholipids and cholesterol that result in an amphipathic lipid bilayer. A significant change in lipid concentrations within the lipid bilayer can alter its fluidity (Hac-Wydro and Wydro, 2007; Wathes et al., 2007; J Zhang et al., 2007). Increased lipid bilayer cholesterol concentrations decreases membrane fluidity compromising GPCR expression and signalling (Pucadyil and Chattopadhyay, 2006). Studies have

highlighted that methyl-β-cyclodextrin (MCD) induced modulation of uterine myocyte membrane cholesterol alters the number of high affinity OXT binding sites within the uterus (Gimpl and Fahrenholz, 2002, 2000; Klein et al., 1995). Further to this, modulation of cellular cholesterol concentrations can also significantly alter the number of caveolae located with the plasma membrane (Smith et al., 2005). Progesterone has also been shown to alter membrane fluidity (Whiting et al., 2000), which could be a contributory factor when considering this hypothesis. Change in membrane cholesterol or other lipids could directly contribute to change in myometrial expression of receptors, ion channels and gap junctions essential to contraction and relaxation of the uterus during labour (Bogaerts et al., 2013; Carlson et al., 2015; Gimpl and Fahrenholz, 2002, 2000; Klein et al., 1995; Pucadyil and Chattopadhyay, 2006).

Further to the effect of maternal diet, stage of labour was found to have a profound effect upon uterine contractile activity and CAP expression *ex vivo*. Research by Challis and Lye has established the distinct phases of parturition and the difference between these stages (Challis et al., 2000). The stages highlight a clear physiological and molecular difference between an activated uterus at term and a uterus in a stimulatory state during labour. These physiological and molecular differences include an increase in myometrial CX-43, OXTR and COX-2 expression, the synthesis of OXT and $PGF_{2\alpha}$, and activation of the pro-inflammatory immune response with the onset of term parturition

(Challis et al., 2000). The differences between the stages of parturition have been highlighted through this study by the disparity in contraction phenotype, protracted response to OXT, increased plasma progesterone, and altered uterine CAP expression, which clearly illustrate the importance of collecting tissue in labour. Thus tissue collected at term but not in labour cannot be used to make conclusions on the TL uterus, with a greater emphasis now needed on collecting TL uterine tissue, especially in the case of animal models where it is easier to obtain. Only through the evaluation of term-labouring tissue will we be able to tease out the aetiology and mechanism(s) governing maternal obesity associated uterine dystocia.

This study has evaluated and established that chronic exposure to a HFHC diet causes adiposity, hypercholesterolemia, an asynchronous contraction phenotype and a protracted response to OXT *ex vivo*, which could be a result of a decrease in uterine CX-43 or Cav-1 or an increase in OXTR, COX-2 or *P*CX-43 expression, caused by significantly increased plasma progesterone concentrations at term. Further to this, it was established that the stage of parturition has a significant effect upon the tissue. With this in mind, a greater emphasis should be placed on collecting tissue during term labour. This study suggests that this animal model may be of use to investigate the mechanisms governing maternal obesity associated uterine dystocia, as feeding of the diet compromised uterine contractility *ex vivo*. Though as with many *ex vivo* studies, this data has been collected within a

controlled physiological environment lacking the integration of numerous body systems that are imperative to parturition including the endocrine and immune system. Thus further work is required to establish if chronic exposure to a HFHC diet significantly affects uterine contractility during TL *in vivo*.

4. Modelling maternal obesity: The effect of a HFHC diet upon uterine contractility *in vivo*

4.1. Introduction

The data presented within the previous chapter clearly illustrates the significant effect of feeding a HFHC diet upon uterine contractility *ex vivo*. This was characterised by an asynchronous multiphasic spike bundle contraction phenotype and insensitivity to increasing concentrations of OXT. To continue evaluating the efficacy and translatability of the HFHC diet animal model, it would be advantages and logical to determine if chronic exposure to a HFHC diet impacts upon uterine contractility *in vivo*. Further to it being the logical next step, *in vivo* assessment of uterine contractile activity will also overcome the limitations identified within the previous *ex vivo* study. For example, the longitudinal and circular smooth muscle cross-sections that compose the myometrium are dissected and isolated from the uterus for *ex vivo* contractility study. *In vivo*, the smooth muscle cross-sections of the myometrium function collectively to deliver the foetus into the extra-uterine environment. Further to this, *ex vivo* studies place the tissue within a controlled physiological environment lacking the influence and regulation of other body systems including the endocrine and immune system, which are essential to the onset of parturition (Golightly et al., 2011; Nair et al., 2016). Through *in vivo* investigation these limitations can accounted for, which will determine if the effects of a HFHC diet upon uterine contractility *ex vivo* translate to

in vivo. A few studies have been carried out to try and overcome the limitations of *ex vivo* study through the measurement of uterine electromyographic activity and intra-uterine pressure (IUP) under terminal anaesthesia (Buhimschi et al., 1998; Moustafa et al., 1999). Though these *in vivo* studies have provided a good foundation to overcome the limitations of *ex vivo* methodology, they are limited by the fact that the animals were not in spontaneous term-labour, and that anaesthesia compromises uterine contractile activity (Baxi and Petrie, 1987; Yoo et al., 2006). The recent study by Pierce et al. (2010) overcame these limitations, by demonstrating that a blood pressure telemetry device could be implanted within the gravid uterus through a recoverable surgical procedure. This procedure facilitated the authors to continuously measure IUP during gestation, parturition and the post-partum period. By combining this surgical technique and the HFHC dietary animal model, this will account for the limitations of the *ex vivo* study and determine if chronic exposure to a HFHC diet will compromise uterine contractility *in vivo*. This will further evaluate the efficacy and translatability of this animal model in the study of maternal obesity associated uterine dystocia, and to the best of our knowledge it will be the first study to do so.

4.2. Objective

The objective of this study was to investigate the effect of feeding a HFHC diet upon uterine contractility *in vivo* using the intra-uterine telemetry surgery technique. It was hypothesised that feeding a HFHC

diet will negative impact upon uterine contractility *in vivo,* through change in in intra-uterine pressure and duration of labour.

4.3. Materials and methods

4.3.1. Dietary animal study

All animal research was carried out under the ASPA of 1986 within the animal facilities of the University of Nottingham. 12 weanling virgin female Wistar rats (*Rattus rattus*) weighing approximately 60g (Charles River, UK) were pair-housed under normal conditions (12 hour light:dark photoperiod, 21°C ± 5°C room temperature, 55% ± 5% relative humidity, food and water access *ad libitum*) and randomly assigned to be fed either a standard laboratory chow CON (Envigo, USA) or HFHC diet (Table 8.1) as previously published (Elmes et al., 2011). Each rat was maintained on their respective diet for 6 weeks prior to mating with stud Wistar males (Charles River, UK); pregnancy confirmed upon discovery of a seminal plug and recorded as gestational day 0. Following successful mating, pregnant females were maintained on their respective diet throughout gestation. Aseptic cannulation of the gravid uterus was carried out on day 9 of gestation to measure intra-uterine pressure (IUP) throughout pregnancy and labour. Upon full recovery, each animal's home cage was maintained on a designated receiver pad recording the change in intra-uterine pressure every second at a frequency of 500Hz. IUP was recorded continuously from day 14 of gestation till term delivery of the 5^{th} pup on day 22 of

gestation. Upon term delivery of the 5th pup each animal was euthanised through CO_2 asphyxiation and cervical dislocation, with blood and tissue collected post-mortem. Blood was collected via cardiac puncture into EDTA-coated tubes (Sarstedt, Germany). Plasma was separated via centrifugation at 13,000 rpm for 10 minutes at 4°C, which was subsequently snap-frozen in liquid nitrogen and stored at -80°C. The uterus was dissected out, with any remaining pups removed and euthanized, cleaned of the amnion and snap frozen in liquid nitrogen. Born pups were euthanised using a 0.1ml overdose of Pentobarbital, while pups *in utero* were euthanised by decapitation. The liver, visceral fat pads, and kidneys were also dissected out, weighed, and snap frozen using liquid nitrogen, which was stored at -80°C. Further detail on the surgical procedure, data collection and analysis are provided later in this chapter.

4.3.2. Preoperative preparation and anaesthesia

All DSI chronic use TA11PA-C40 telemetry probes (DSI, Minnesota, USA) were sterile upon first use and were re-sterilised according to manufacture instructions using Actril cold sterilant (Medivators, NJ, USA) upon re-use. At day 8 of gestation HFHC fed animals were placed on the control diet for 24 hours to prevent intra-operative regurgitation of the HFHC diet. Animals were placed in the anaesthesia chamber and pre-oxygenated for 5 minutes. Following pre-oxygenation, each animal was placed under gaseous anaesthesia (3% isoflurane 3L/Min 95%/5% O_2/CO_2) until loss of consciousness was achieved.

Once the animal was unconscious, they were removed from the chamber and placed on a nose cone with an active scavenging system supplying 2% isoflurane 1L/Min 95%/5% O_2/CO_2. To maintain the correct depth of anaesthesia, pedal reflex and breathing rate were monitored continuously throughout the procedure. To prevent and manage post-operative pain, pre-operative buprenorphine (0.0168ml/100g) and meloxicam (0.004ml/100g) were administered subcutaneously. The abdomen was prepared for the laparotomy by shaving and swabbing with chlorhexidine and Virusan (Cairn Technology, Sheffield, UK). Animals were then moved through to theatre, and placed on an inclined sterile surgical field with 2% isoflurane 1L/Min 95%/5% O_2/CO_2 supplied via an active scavenging system.

4.3.3. Surgical procedure

Aseptically, a vertical incision was made from the xyphoid process of the sternum to the bladder, exposing the muscle layer. Dead space was created between the skin and muscle using round tipped scissors to aid the closure of the skin at the end of the procedure. The muscle layer was tented and pierced near the bladder using a scapel, which was then opened along the linea alba using round-tipped scissors. Carefully manipulating the internal structures within the abdominal cavity, the gravid uterus was identified and exteriorised to count the number of embryos within each uterine horn. The uterine horn with the greatest gravidity was selected for cannulation; the other horn was

placed back into the abdominal cavity. Under a microscope, the pressure sensitive catheter was fed through an incision between the ovary and the first embryo, where it was secured in place between the 3rd and 4th embryo's using Vet Bond (3M, MN, USA). The cannulised horn was placed back within the abdominal cavity, with the transmitter anchored to the muscle layer near the linea alba, located approximately 1cm below the liver on the right-side of the animal. The muscle layer was closed using a simple continuous suture pattern. The skin was closed using a simple continuous subcuticular stitch, with an Aberdeen knot to finish. The Aberdeen knot was subsequently buried, and the area around the wound cleaned using chlorohexidine. Following cleaning, the wound was dressed with Opsite spray dressing (Opsite, London, UK).

4.3.4. Recovery

Each animal was allowed to recover from the anaesthesia on the table. Once conscious, the animal was placed into a heated (37°C) post-operative recovery cage and provided with mash (water softened chow) to provide energy and hydration. Each animal's body weight, water and food intake was monitored for four days after the operation. HFHC diet fed animals were provided both mash and the HFHC diet during the first day of recovery, and then maintained exclusively on the HFHC diet from the second day of recovery.

4.3.5. Data and tissue collection

Following a full recovery, each animal was single housed and placed upon a designated receiver pad to collect the data in a non-stressful manner. The chronic use TA11-C40 transmitter was activated post-operatively by brushing a magnet over the animal's abdomen; this was confirmed using an AM radio. The transmitted data was collected every second via telemetry over an AM frequency at 500Hz to a DSI PhysioTel receiver pad (model RPC-1). The DSI data exchange matrix would collect and process the data from the PhysioTel receiver pad, and send it to the computer for data collection, storage and real-time display via DSI Dataquest A.R.T Analysis software (version 4.33). Changes in the local atmospheric pressure were accounted for by the DSI Ambient Pressure reference monitor (model APR-1) that is connected directly to the data exchange matrix. Recording of intra-uterine pressure continued until delivery of the 5^{th} pup where the animal was euthanised using CO_2 affixation and cervical dislocation. Allowing animals to progress to term delivery of the 5^{th} pup enabled the recording of TL IUP and the collection of TL tissue. Blood was collected via cardiac puncture into EDTA coated tubes (Sarstedt, Nümbrecht, Germany), and subsequently centrifuged at 13,000rpm for 10 minutes at 4°C to isolate the plasma, which was then flash frozen and stored at -80°C. The uterus, liver, kidneys, gonadal and peri-renal fat depots were dissected out, snap frozen and stored at -80°C.

4.3.6. Data analysis and statistics

All the data was extracted using DSI Dataquest A.R.T Analysis software (version 4.33). IUP (mmHg), maximum IUP (mmHg), minimum IUP (mmHg), change in IUP (mmHg), integral activity (mmHg/s), maximum slope (ΔmmHg/s), minimum slope (ΔmmHg/s) and data frequency counts (number of counts) calculated from extracted data using DSI Dataquest A.R.T Analysis software, Microsoft Excel (Microsoft, USA), and Labchart Reader version 8 (AD instruments, New Zealand). Contraction frequency was determined by manually counting each single event with ≥5mmHg amplitude within one-hour periods from day 20 of gestation till delivery of 5th pup via graphical display using DSI Dataquest A.R.T Analysis. All data was analysed using the Statistical Package for Social Science version 21.0 (SPSS, Chicago, IL, USA) and expressed as the mean value ± SEM, with statistical significance determined by P=≤0.05. The data did not fit a Gaussian distribution and could not be suitably transformed. As a result, the data was analysed using ANOVA (mixed between-within) as it could account for deviation from a Gaussian distribution. ANOVA was used to statistically assess the effect and interaction of maternal diet (CON/HFHC) and time to delivery of 5th pup upon IUP, maximum IUP, minimum IUP, change (Max-Min) in IUP, integral activity, maximum slope, minimum slope and data frequency array counts. A data frequency array quantified the number of counts within each of the assigned IUP range bins (2, 5, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58, 62, 66, 70 mmHg) at 48, 24, 12, 6, 3 and 1 hours away from

birth of the 5th pup. All 2D graphs were produced using GraphPad Prism version 6.0 (GraphPad, San Diego, CA, USA) and all 3D graphs produced using Microsoft Excel.

4.4. Results

4.4.1. Necropsy measurements

A number of measurements were taken during necropsy (Table 4.1). Chronic exposure to a HFHC diet resulted in animals being 32.8g heavier than their CON counterparts, though this difference did not reach significance (*P*=0.105). During the course of the necropsy, the peri-renal and gonadal fat depots were each weighed and collected. HFHC animals displayed a greater peri-renal (5.3 ± 1.26g vs 2.97 ± 0.53g; *P*=0.118) and gonadal (5.1 ± 0.91g vs 2.90 ±0.74g; *P*=0.09) fat mass compared to animals sustained on the CON diet, however this did not reach statistical significance. Chronic exposure to a HFHC diet had no significant effect on the litter size (*P*=0.132) or pup sex ratio (*P*=0.804). Maternal diet in conjunction with the surgical procedure did not result in a significant number of foetal losses (*P*=0.365).

Category	Control diet (n=6) (Mean ± SEM)	HFHC diet (n=6) (Mean ± SEM)	*P* value
Final weight (g)	351.82 ± 8.27	384.62 ± 16.4	0.105
Peri-renal fat depot mass (g)	2.97 ± 0.53	5.3 ± 1.26	0.118
Gonadal fat depot mass (g)	2.90 ± 0.74	5.1 ± 0.91	0.09
Litter size (n)	11.00 ± 1.21	13.50 ± 0.92	0.132
Pup male:female sex ratio	1.22 ± 0.38	1.37 ± 0.64	0.804
Number of foetal losses	4.33 ± 1.41	2.33 ± 1.26	0.365

Table 4.1 - Summary of the effects of a CON (n=6) or HFHC (n=6) and intra-uterine telemetry surgery on parameters measured at necropsy

4.4.1.1. The effect of a HFHC diet upon term-labouring contractile activity *in vivo*

Application of IUP telemetry surgery has facilitated the continuous assessment of a HFHC diet upon uterine contractility *in vivo*, from day 20 of gestation (baseline) to term delivery of the 5th pup on day 22 of gestation. Unlike the *ex vivo* data, IUP telemetry surgery has not offered the same level of qualitative analysis. As the probes are sensitive to any oscillation in IUP, the uterine phenotype was not clearly illustrated, resulting in no conclusion being drawn. Despite this, qualitative analysis did reveal that HFHC animals generally displayed increased IUP relative to CON animals during the final hours of term labour (Figure 4.1).

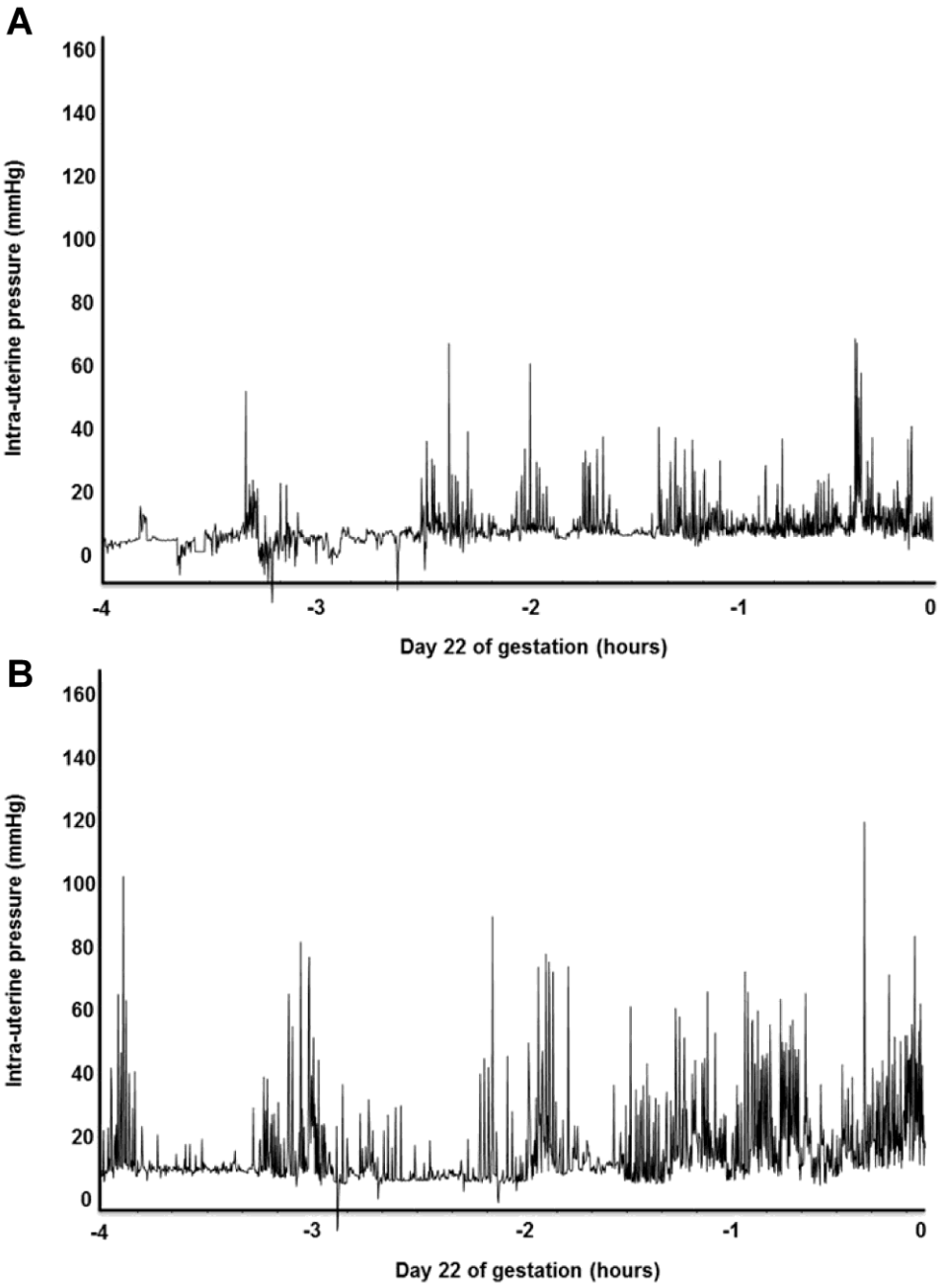


Figure 4.1- Representative traces of the change in IUP during the final 4 hours approaching term delivery of the 5th pup within (**A**) CON and (**B**) HFHC animals.

Quantitative analysis aimed to investigate the effect of maternal diet and time till delivery of the 5th pup upon uterine contractile parameters *in vivo*. IUP pressure is simply the instantaneous measurement of the pressure within the gravid uterine lumen using a pressure sensitive catheter connected to an AM telemetry transmitter.

As the animals progressed from day 20 of gestation to term delivery of the 5th pup, maternal diet was found to have a significant (*P*=0.026) effect upon IUP (Figure 4.2). As HFHC animal progress towards term delivery of the 5th pup, they displayed a significant (*P*=0.038) 1.5-fold increase in IUP. Relative to this, CON animals displayed a significant decrease (*P*=0.032) in IUP where it recovered to a 0.31-fold increase by term delivery of the 5th pup, though this did not reach significance (*P*=0.215). Contraction frequency was characterised in this study by the number of uterine events achieving ≥5mmHg amplitude within a given hour. As the animals progressed through labour, both CON (*P*=0.012) and HFHC (*P*=0.016) animals displayed a significant increase in contraction frequency (Figure 4.3). Despite both dietary groups displaying a significant increase in contraction frequency, maternal diet was still found to have a significant effect (*P*=0.013), with HFHC animals displaying a 5-fold increase relative to the CON animals 4-fold increase by term delivery of the 5th pup. Maximum IUP is the highest mean pressure sustained from day 20 of gestation to term delivery of the 5th pup (Figure 4.4A). Maximum IUP did not significantly differ between dietary groups (*P*=0.291), with time having no significant effect

upon the mean maximum IUP sustained by HFHC animals. Unlike HFHC animals, time had a significant (*P*=0.024) effect upon the mean maximum IUP sustained within the uterine lumen of CON animals, which increased as they progressed towards delivery of the 5th pup. The mean minimum pressure (also known as the baseline pressure) of the uterine lumen was also recorded during this study. Maternal diet was found to have no significant (*P*=0.104) effect upon the mean minimum IUP recorded within the gravid uterus (Figure 4.4B), with both CON (*P*=0.256) and HFHC (*P*=0.376) animals displaying no significant change over the course of this study. Maximum – minimum pressure (also referred to as the mean contraction amplitude) measured the change in mean maximum pressure relative to the mean minimum pressure achieved by each contraction event over the course of the study (Figure 4.5). Although maternal diet had a significant effect (*P*=0.004), HFHC animals did not display any significant (*P*=0.092) change in contraction amplitude from day 20 of gestation to term delivery of the 5th pup. Unlike the animals sustained on the HFHC diet, CON animals displayed a significant (*P*=0.020) increase in contraction amplitude as they progressed from day 20 of gestation to term delivery of the 5th pup. Integral activity is a measure of the area under the curve as a rate of change in pressure per second (mmHg/s). As the animals progressed towards term delivery of the 5th pup, integral activity significantly increased within both CON (*P*=0.036) and HFHC (*P*=0.05) animals, but maternal diet was found to have no significant (*P*=0.760) effect upon integral activity (Figure 4.6). Slope is a measure of the rate

of change (mmHg/s) during the contraction (maximum) and relaxation (minimum) phase of the uterine event. Chronic exposure to a CON or HFHC diet had no significant effect upon the rate of change during the maximum (P=0.357) or minimum (P=0.469) phase of the contraction (Figure 4.7). Both slope maximum (P=0.015) and minimum (P=0.033) significantly increased as CON animals progressed towards term delivery of the 5th pup. Unlike the CON animals, HFHC animals displayed no significant change in maximum (P=0.109) or minimum (P=0.076) slope from day 20 of gestation to term delivery of the 5th pup.

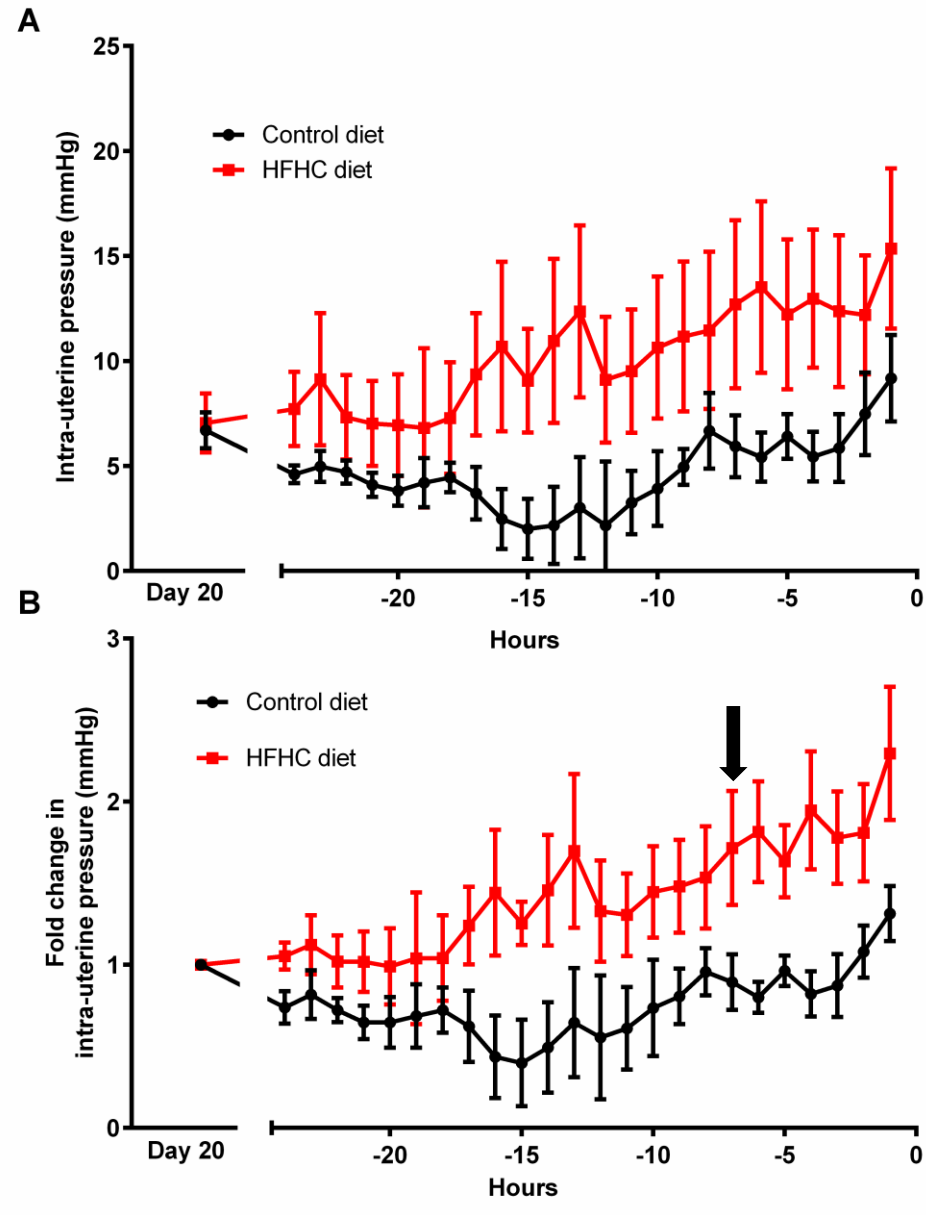


Figure 4.2 - The effect of feeding a HFHC diet upon (**A**) intra-uterine pressure and (**B**) fold-change in intra-uterine pressure; end of -1 hour marks birth of 5th pup. Sample sizes for CON (n=6) and HFHC (n=6) diet animals. Mixed between within ANOVA. Values are means ± SEM. Arrows [Black (HFHC)] identify the first significant increase above baseline.

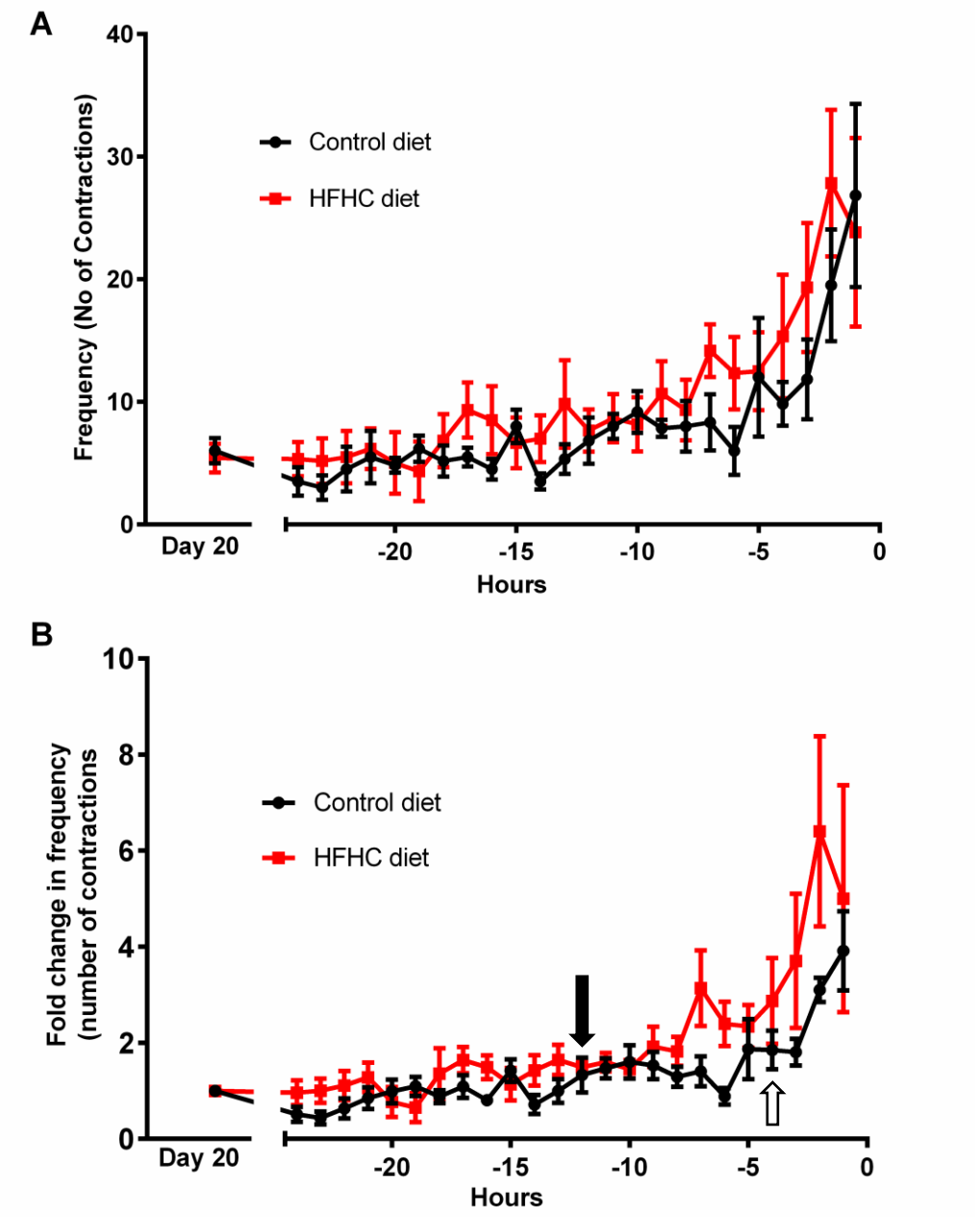


Figure 4.3 - The effect of feeding a HFHC diet upon (**A**) contraction frequency and (**B**) fold-change in contraction frequency; end of -1 hour marks birth of 5th pup. Sample sizes for CON (n=6) and HFHC (n=6) diet animals. Mixed between within ANOVA. Values are means ± SEM. Arrows [White (CON) and Black (HFHC)] identify the first significant increase above baseline.

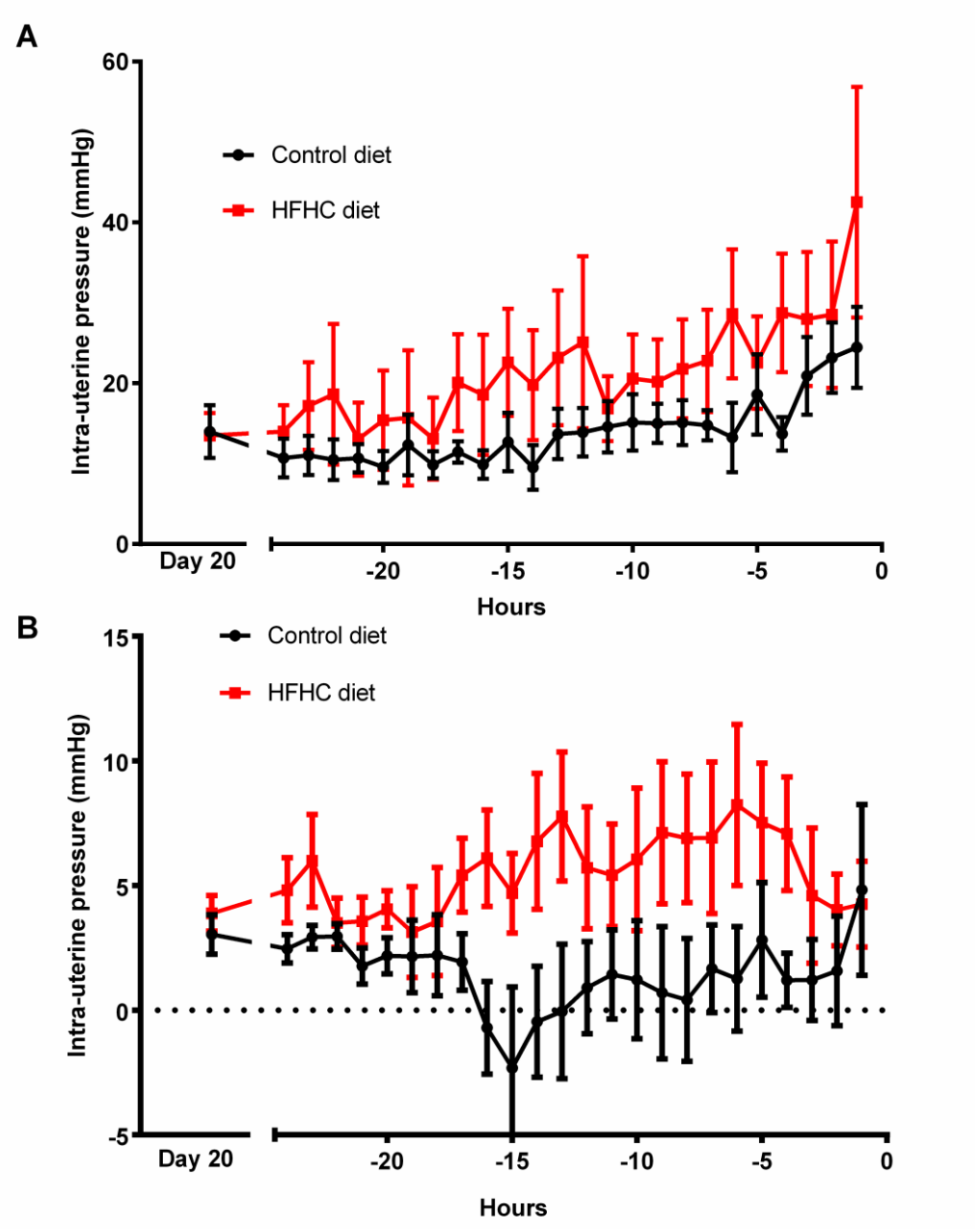


Figure 4.4 - The effect of feeding a HFHC diet upon (**A**) maximum intra-uterine pressure and (**B**) minimum intra-uterine pressure; end of -1 hour marks birth of 5th pup. Sample sizes for CON (n=6) and HFHC (n=6) diet animals. Mixed between within ANOVA. Values are means ± SEM.

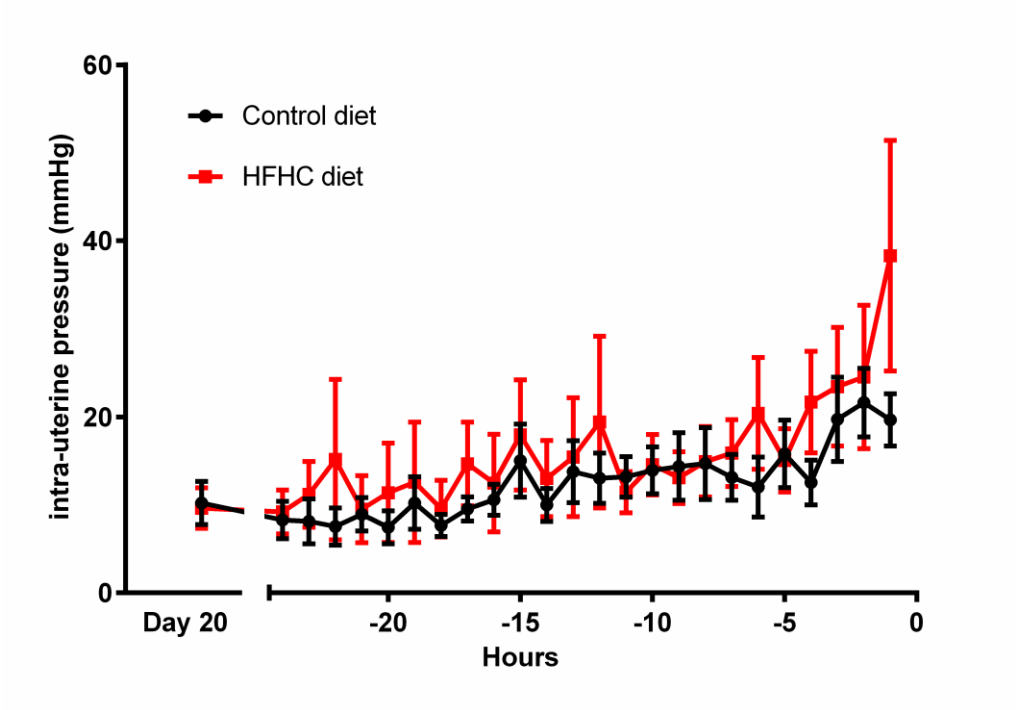


Figure 4.5 - The effect of feeding a HFHC diet upon change (maximum-minimum) in intra-uterine pressure; end of -1 hour marks birth of 5th pup. Sample sizes for CON (n=6) and HFHC (n=6) diet animals. Mixed between within ANOVA. Values are means ± SEM.

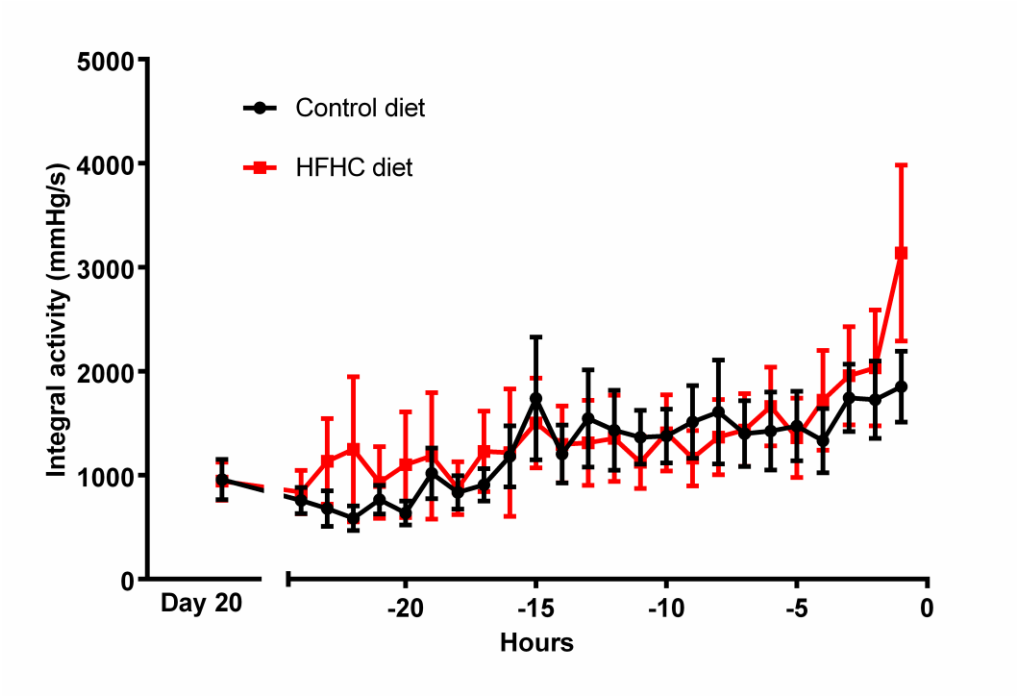


Figure 4.6 - The effect of feeding a HFHC diet upon integral activity. Sample sizes for CON (n=6) and HFHC (n=6) diet animals; end of -1 hour marks birth of 5th pup. Mixed between within ANOVA. Values are means ± SEM.

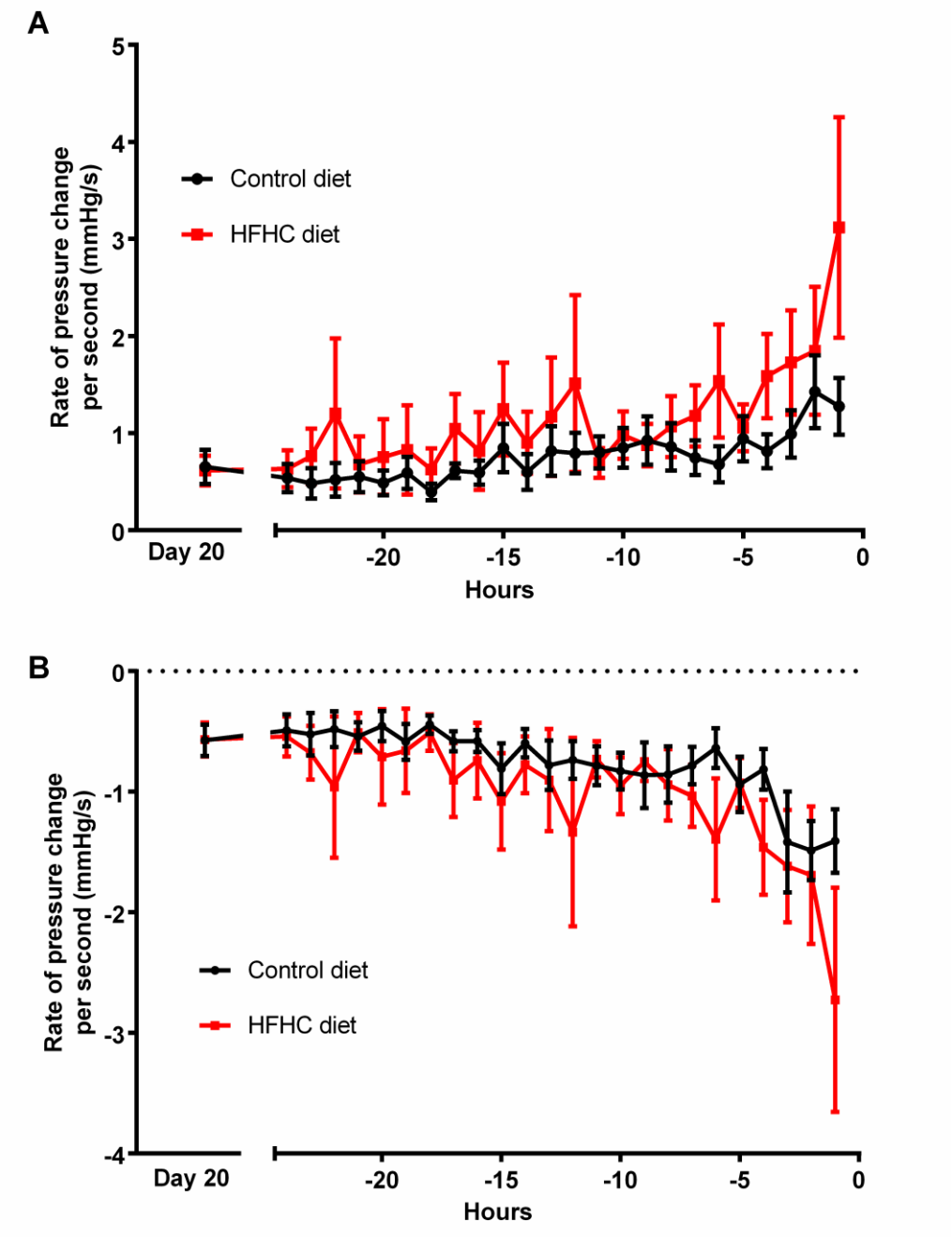
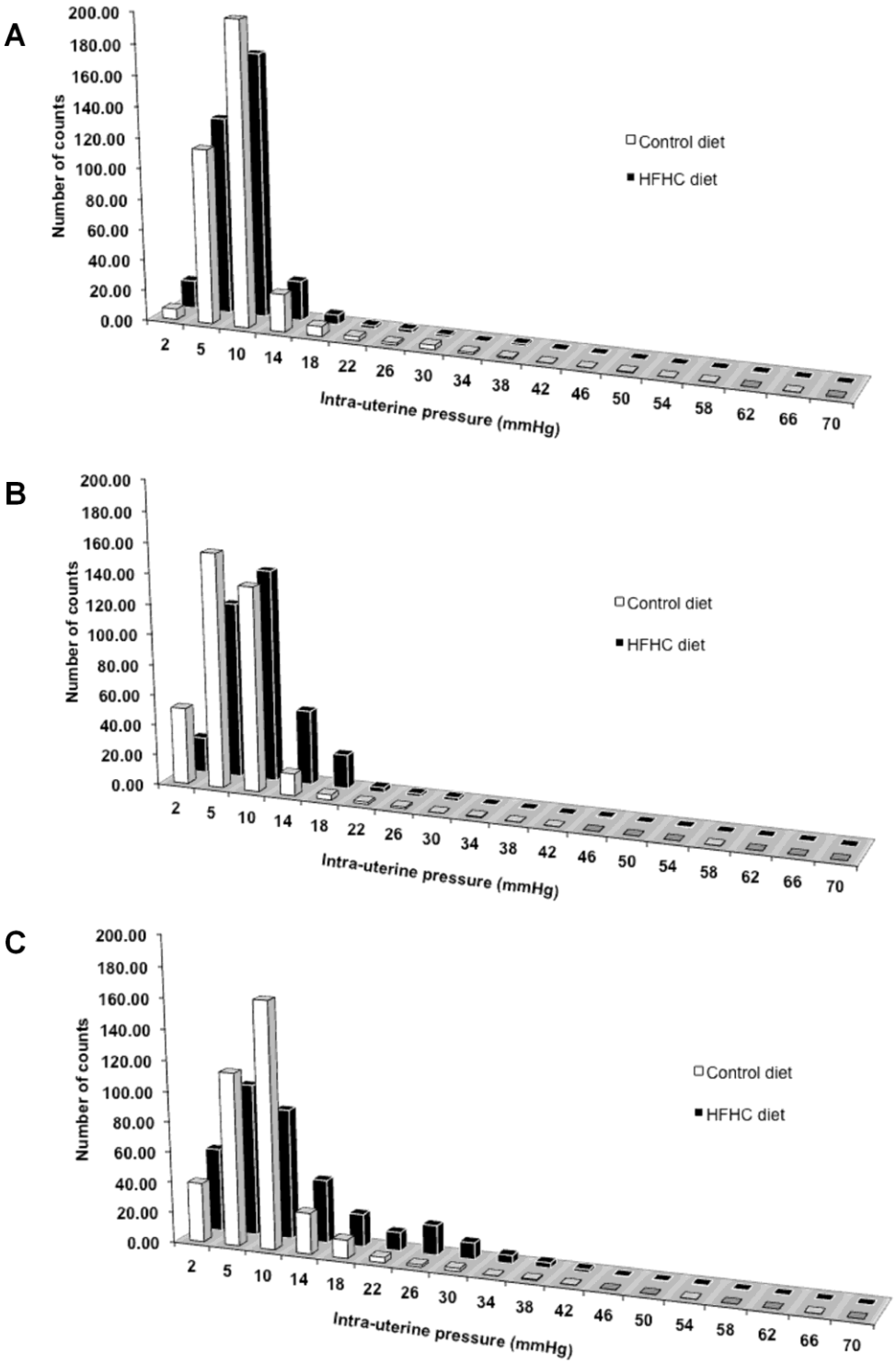


Figure 4.7 - The effect of feeding a HFHC diet upon (**A**) Maximum slope and (**B**) Minimum slope. Sample sizes for CON (n=6) and HFHC (n=6) diet animals; end of -1 hour marks birth of 5th pup. Mixed between within ANOVA. Values are means ± SEM.

4.4.2. Data frequency array count

A data frequency array was utilised to count the number of times IUP reached a specific value (2, 5, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, 58, 62, 66, 70 mmHg) at 48, 24, 12, 6, 3 and 1 hour prior to delivery of the 5th pup. It was hoped that this method of analysis would facilitate further investigation into the effect of maternal diet and time upon uterine contractility and the duration of labour *in vivo*. Statistical analysis by ANOVA (mixed between within) did not reveal a significant effect of diet or time upon the data frequency array counts. This may have been a result of the variability within the groups, consequential of the high degree of S.E.M within this data set. Despite this, qualitative analysis of the data frequency array may highlight increased contractile activity and a prolonged duration of labour in HFHC animals (Figure 4.8). At 48 hours prior to delivery of the 5th pup, maternal diet appeared to have little to no effect upon uterine contractile activity, with both dietary groups displaying a high frequency of counts below an IUP of 18mmHg. Only 24 hours later, HFHC animals began to display an increase in uterine contractile activity characterised with greater counts at 18mmHg relative to the CON animals. This trend continued, with HFHC animals displaying a rightward shift characterised by an increasing frequency of counts in high IUP bins (>18mmHg) at 12, 6, 3, and 1 hour(s) prior to delivery of the 5th pup. This shift continued within HFHC animals maxing with counts recorded at 70mmHg at 1 hour prior to delivery of the 5th pup. Unlike the HFHC animals, the frequency of

counts from the CON animals remained high within the IUP bins ≤18mmHg. Only at 1 hour prior to delivery of the 5th pup did the frequency of counts increase in high IUP pressure bins (>18mmHg), peaking at 58mmHg.


A
B
C
Number of counts
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
2 5 10 14 18 22 26 30 34 38 42 46 50 54 58 62 66 70
Intra-uterine pressure (mmHg)
Control diet
HFHC diet

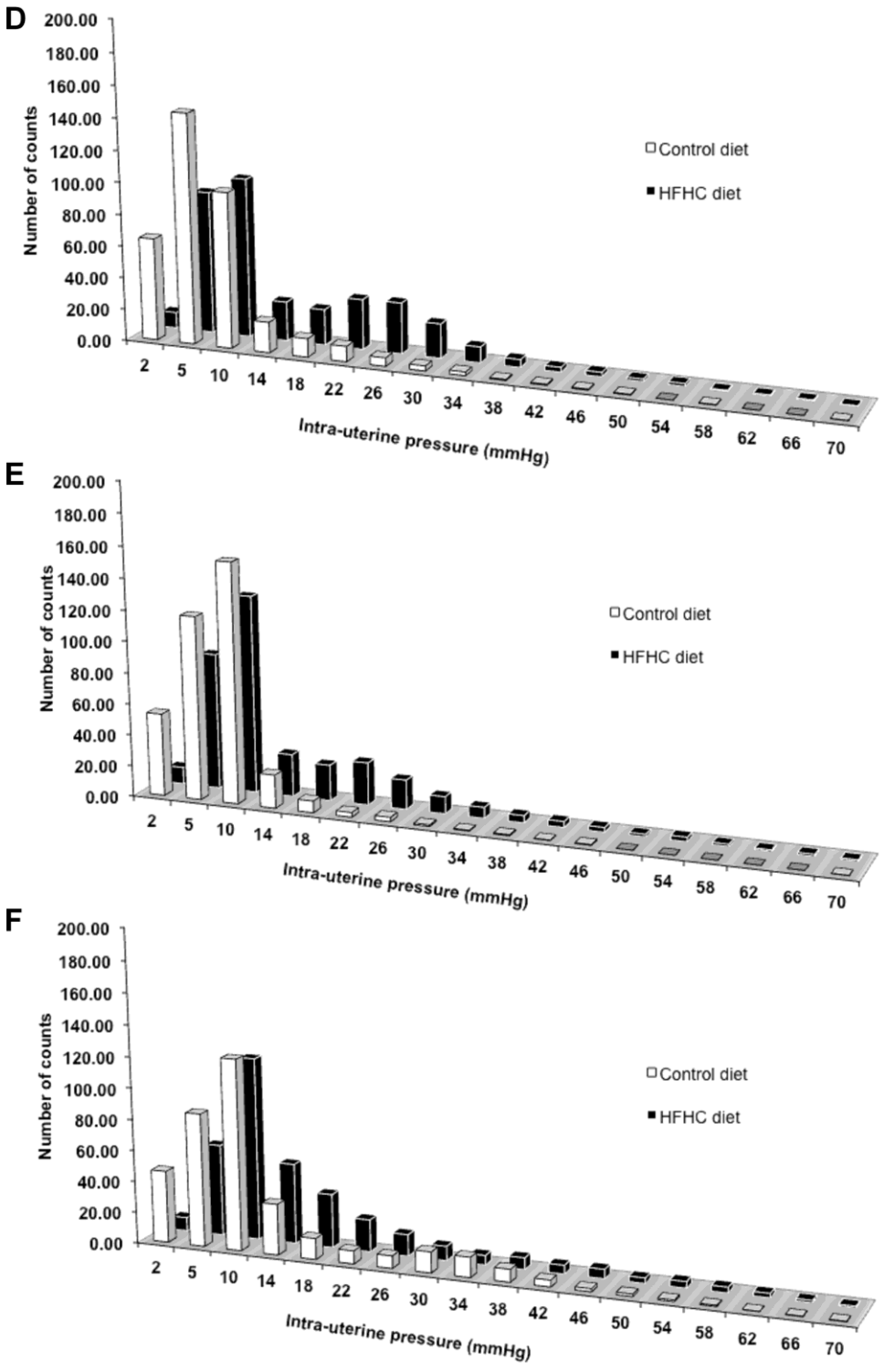


Figure 4.8 - The effect of feeding a CON or HFHC diet upon data frequency array counts at (**A**) 48 hours, (**B**) 24 hours, (**C**) 12 hours, (**D**) 6 hours, (**E**) 3 hours and (**F**) 1 hour prior to delivery of the 5th pup. Mixed between within ANOVA. Values are mean.

4.4.3. The effect of a HFHC diet upon the duration of labour

Throughout the course of this study, HFHC animals have continuously demonstrated increased uterine contractile activity from day 20 of gestation to term delivery of the 5th pup. As a result, further analysis was carried out to determine if maternal diet had a significant effect upon the duration of labour through fold-change in IUP, contraction frequency and the data frequency array analysis. To achieve this, IUP and contraction frequency were analysed by ANOVA and split by dietary groups. The duration of labour was characterised by a significant increase in IUP above that of the baseline value at day 20 of gestation. This revealed that HFHC animals displayed a significant increase (P=0.046) in IUP 8 hours prior to delivery of the 5th pup, unlike CON animals that displayed no such significant increase (Figure 4.2). Analysis of fold-change in contraction frequency revealed that HFHC animals displayed a significant (P=0.023) increase above baseline 12 hours prior to delivery of the 5th pup (Figure 4.3). In comparison, CON animals only experienced a significant (P=0.034) increase in contraction frequency above baseline 3 hours prior to delivery of the 5th pup. This suggests that HFHC animals may have laboured for 9 hours longer than the CON animals. Though the statistical analysis yielded no significant result, the data frequency array clearly illustrated a difference in the duration of labour between the dietary groups. The resulting increase in the frequency of counts to the high IUP bins (>18mmHg) within HFHC animals occurred approximately 12 hours

earlier than in CON animals (Figure 4.8). This data provides evidence that animals sustained on the HFHC diet display increased contractile activity for a longer duration relative to animals sustained on the CON diet. This increase in contractile activity and duration of labour is symptomatic of a prolonged duration of labour.

4.5. Discussion

The objective of this study was to determine if chronic exposure to a HFHC diet significantly affected uterine contractile activity *in vivo*. Through this objective it was hoped to further assess the efficacy and translatability of this animal model, while building upon the wealth of *ex vivo* data collected previously. To assess the effect of a HFHC diet *in vivo*, intra-uterine telemetry surgery developed by Pierce et al. (2010) was employed. This surgical technique allows the continuous recording of IUP by aseptically implanting a pressure sensitive probe into the gravid uterine lumen.

Previously, chronic exposure to a HFHC diet resulted in a significantly greater final weight and the accumulation of visceral adipose tissue, which did not occur during this study. This was likely a result of the 10% weight loss and depressed appetite that occurred during the post-operative recovery period. Another contributing factor to this may have been the length of the experiment. Unlike previous studies where animals were euthanised upon delivery of the 1st pup, animals were allowed to deliver to the 5th pup. This difference would have allowed the

maternal dams to utilise more of their energy stores to support parturition and deliver their pups. Though these are plausible explanations, further work is required to establish if exposure to the surgical procedure limits weight gain or the number of pups delivered effect final weight and visceral fat procurement.

This is the first known study to investigate the effect of a HFHC diet upon uterine contractility *in vivo*. Measurement of uterine contractility *in vivo* has clearly illustrated a shift from relative uterine quiescence on day 20 of gestation to a stimulatory state on day 22 of gestation. This shift in stage of parturition was characterised by a significant increase in IUP, contraction frequency, mean contraction amplitude, integral activity and a shift in the frequency of counts to high IUP bins. Though both dietary groups displayed this general shift in stage of parturition, maternal diet was found to have a significant effect upon uterine contractility *in vivo*. Unlike the CON animals, chronic exposure to a HFHC diet resulted in a significantly greater IUP, contraction frequency, and mean contraction amplitude. This increase in uterine contractile activity from day 20 of gestation till term delivery of the 5^{th} pup was coupled with a longer duration of labour. Increased uterine contractile activity and a longer duration of labour are symptomatic that HFHC animals were experiencing a prolonged and dysfunctional labour (Karaçam et al., 2014). Specifically, HFHC animals were found to be in labour for up to 9 hours longer than animals sustained on the CON diet. Unlike the HFHC animals, CON animals only displayed an increase in

uterine contractility above baseline within the final 3 hours prior to term delivery of the 5th pup. This data highlights the significant effect of the HFHC diet upon uterine contractility *in vivo*, but the efficacy and translatability of this animal model remains to be determined. Clinical and epidemiological studies have established that maternal obesity places parturients at a significantly increased risk of prolonged and dysfunctional labour (Bogaerts et al., 2013; Kominiarek et al., 2011); characterised by an inadequate rate of cervical dilation, longer duration of labour, and poor uterine contractility (Carlson et al., 2015; Karaçam et al., 2014). Specifically, Increasing body mass significantly prolongs the first stage of labour, where some obese parturients fail to progress to the 2nd stage of labour and delivery (Chin et al., 2012; Vahratian et al., 2005; J Zhang et al., 2007). However in a small of number of cases where obese parturients were allowed to progress slowly through the first stage of labour, they eventually developed a contraction profile sufficient to deliver the foetus vaginally with no obstetric intervention (Buhimschi et al., 2004). The effect of maternal obesity upon term labouring uterine contractility is translatable to what was observed in this study. HFHC animals displayed a significantly prolonged duration of labour but would in all cases eventually progress to delivery of the 5th pup. No conclusion can be made if the HFHC animals are suffering from uterine dystocia *in vivo*, but increased uterine contractility activity is not necessarily indicative of a contraction profile for a successful labour. A prolonged period of increased uterine contractile activity could end with myometrial fatigue, resulting in acidosis and increasing lactate

synthesis, impairing uterine contractility (Hanley et al., 2015; Wray, 2007). This evidence highlights the efficacy and translatability of this animal model in the study of maternal obesity associated uterine dystocia.

This model could be used to elucidate the aetiology and mechanisms that govern maternal obesity associated dystocia. Many of these hypothesised mechanisms (stated previously) could allude to the cause or at least contribute to the prolonged duration of labour identified within HFHC animal's *in vivo*, but another is the effect of dietary fatty acids on the timing and outcome of labour. Change in the n-6:n-3 PUFA ratio has been reported to effect PG production, steroidogenesis and even the timing of labour, with high n-6 diets inducing preterm labour, while diets high in n-3 PUFA's significantly prolonging labour (Cheng et al., 2005; Elmes et al., 2005; Hinckley et al., 1996; Leat and Northrop, 1981; Leaver et al., 1991; Olsen et al., 1986; Robinson et al., 2002). Further to this, culturing of cardiomyocytes with increased concentrations of n-9 MUFA oleic acid disrupts gap junction formation by promoting PKC mediated phosphorylation of CX-43 (Huang et al., 2004). This mechanism could be responsible for the decrease in CX-43 and the increase in *P*CX-43 expression within HFHC animals at the onset of TL. The dietary fatty acid intake of the HFHC animals differ from CON animals, which could be the cause or a contributory factor to the prolonged and dysfunctional labouring phenotype identified throughout the course of these *ex vivo* and *in vivo* studies.

In summary, chronic exposure to a HFHC diet led to a significant increase in uterine contractile activity and a prolonged duration of labour *in vivo*. This study has highlighted the efficacy of this animal model, but further work is required to unravel the causal mechanism(s). A potential avenue is the effect of dietary fatty acids upon parturition. Further analysis of the HFHC diet, plasma, uterus, and liver will identify if chronic exposure to a HFHC diet has resulted in a sufficient change in fatty acids capable of altering parturition

5. Modelling maternal obesity – The effect of a HFHC diet upon the maternal fatty acid profile

5.1. Introduction

Fatty acids are a component of our diet obtained through the consumption of meat, animal products, vegetables, seeds, and oils, which play a significant role in our reproductive physiology (Insel et al., 2011). Fatty acids are classified according to the number and position of C=C within their structure (Figure 2.5). SFAs lack this functional group, and can be obtained through the diet or synthesised endogenously from acetyl-CoA (Sanders, 2015; Volpe and Vagelos, 1976a). MUFAs such as oleic acid (18:1 n-9) contain a single C=C, which again can be obtained through the diet or synthesised endogenously from the SFA palmitic acid (Cook and McMaster, 2002; Lottenberg et al., 2012). PUFAs contain two or more C=C, which are further classified by the position of this functional group relative to the omega carbon (end carbon bound to a methyl group), resulting in the dominant n-6 and n-3 PUFA subclasses (Lottenberg et al., 2012). Further to this, PUFAs can be classed as non-essential or essential. Non-essential fatty acids can be synthesised endogenously or obtained through the diet. While essential fatty acids can only be obtained through the diet, as they cannot be synthesised *de novo* (Berg et al., 2011). This is a result of humans lacking the desaturase enzymes capable of inserting a C=C above carbon-9 (Wathes et al., 2007). Essential PUFAs include LA (18:2 n-6) and ALA (18:3 n-3), which can

be metabolised into AA (20:4 n-6), EPA (20:5 n-3), DPA (22:5 n-6) and DHA (22:6 n-3), respectively (Abayasekara and Wathes, 1999).

Primarily, fatty acids act as a source of storable energy, but they also play a pivotal role in female reproduction, with their dietary concentrations having a significant effect upon both oestrous and parturition (Wathes et al., 2007). Ovarian sex steroid production is essential to regulate the female menstrual cycle, which culminates in the release of the ovum from the dominant follicle (Bates and Bowling, 2013). Culture of ovarian cells with DHA decreases their capacity to synthesise progesterone and $PGF_{2\alpha}$ (Hinckley et al., 1996), which are essential to sustaining a regular cycle and luteolysis (Bates and Bowling, 2013). Further to their effects on fertility, fatty acids can also have a significant effect upon the outcomes of labour. Increased dietary LA or reduced dietary ALA intake significantly increases the risk of preterm labour in animals and humans (Elmes et al., 2005; Olsen and Secher, 2002; Reece et al., 1997). Diets rich in marine sources of n-3 PUFA have been shown to prolong the gestation of women from the Faeroe Islands by up to 0.53 weeks and significantly increase the birth weight of their offspring, compared to their Danish counterparts (Olsen et al., 1986). Animal studies have confirmed this effect, with diets rich in ALA promoting prolonged and dysfunctional labour in rats (Leat and Northrop, 1981; Waltman et al., 1977), with n-3 PUFA infusion delaying the onset of dexamethasone induced pre-term labour within the pregnant ewe by reversing the onset of labouring contractions

(Baguma-Nibasheka et al., 1999). These variations in the onset of labour result from a change in 2-series PG (PGE_2 and $PGF_{2\alpha}$) synthesis within maternal and foetal tissue (Cheng et al., 2005; Elmes et al., 2005; Kirkup et al., 2010). 2-series PG's are synthesised from PLA_2 membrane released AA by COX 1 and 2, and are able to directly stimulate contraction of the uterus and dilation of the cervix during labour (Challis et al., 2000, 1997). An increase in AA or its precursor LA, will significantly increase the synthesis of 2-series PG's within the endometrium and foetal allantochorion, resulting in the doubling of maternal and foetal circulating PGE_2 and $PGF_{2\alpha}$, contributing to the onset of pre-term labour (Cheng et al., 2005; Elmes et al., 2005). An increase in dietary n-3 PUFA EPA has been suggested to outcompete AA for position in the membrane and COX, decreasing 2-series PG and increasing 3-series PG synthesis within uterine tissue, prolonging the onset of labour (Allen and Harris, 2001; Leaver et al., 1991). Despite this suggestion, not all n-3 PUFA's will impede the synthesis of 2-series PG's, with ALA and 18:4 n-3 reported to increase the synthesis of PGE_2 within primary ovine amnion cells (Kirkup et al., 2010). PUFA's are not the only class of fatty acids capable of altering maternal PG synthesis, with the n-9 MUFA oleic acid increasing PGE_2 but stifliling $PGF_{2\alpha}$ synthesis within pregnant ewe primary endometrial cells (Cheng et al., 2015).

Maternal obesity is associated with poor labour outcomes characterised by prolonged and dysfunctional labour (Bogaerts et al., 2013; Centre

for Maternal and Child Enquiries (CMACE), 2010; Kominiarek et al., 2011). Maternal obesity induced change in 2-series PG synthesis could explain the prolonged labour demonstrated within HFHC animals, as high fat diet induced maternal obesity is associated with elevated SFA, MUFA, and reduced n-3 PUFA's (Cerf and Herrera, 2016; Cinelli et al., 2016; Grant et al., 2011). As a result, it would be important to establish if chronic exposure to a HFHC diet has a significant effect upon the maternal fatty acid profile, as this may translate to a change in PG synthesis within maternal and foetal tissue. This could explain the poor labour outcomes demonstrated by HFHC animals, and the significantly reduced plasma $PGF_{2\alpha}$ concentrations identified previously (Elmes et al., 2011).

5.2. Objective

The objective of this study was to assess if chronic exposure to a HFHC diet would significantly alter the fatty acid profiles of the maternal plasma, liver and uterus. It was hypothesised that chronic exposure to a HFHC diet will alter the fatty acid profile of the plasma, liver, and uterus compared to animals sustained on the CON diet.

5.3. Materials and methods

5.3.1. Tissue collection and preparation

The plasma, liver, and uterine tissue utilised in this study was collected previously during the IUP telemetry trial. Prior to lipid extraction, liver

and uterine tissue was crushed into a fine powder under liquid nitrogen, using a mortar and pestle.

5.3.2. Lipid extraction

300mg of crushed rat liver or uterine horn was homogenised in the presence of 2ml ice-cooled 0.9% saline in 30-second bursts. The homogenate was then vortexed in the presence of 3ml 99.9% Propan-2-ol, which was left to incubate at room temperature for 5 minutes. After the incubation period, 6ml of 98% chloroform was added to the solution, inverted, and centrifuged at 3000rpm for 10 minutes. Following centrifugation, the bottom clear organic chloroform layer was syphoned off into a fresh tube using a disposable glass pipette. This solution was dried off at 37°C using nitrogen gas. Once the solution had completely evaporated, samples were reconstituted using a 9:1 Chloroform methanol solution. Liver samples were reconstituted with 500µl, while uterine samples were only reconstituted in 100µl of the 9:1 Chloroform methanol solution. Once reconstituted, the samples could be stored at -80°C and used within 3 months.

5.3.3. Lipid analysis

The fatty acid profile of the plasma, liver, and uterus from CON and HFHC animals was analysed by Dr Ge Liu and Dr John Carragher of the Waites Lipid Analysis Service at the University of Adelaide. 20µl of plasma, reconstituted liver or uterine fatty acid extracts were pipetted onto PUFA COAT cards (Liu et al., 2014) provided by Waites Lipid

Analysis Service for stable transport. The samples were analysed for their fatty acid profile using gas chromatography flamed ionised detector (GC-FID) as described previously by Liu et al. 2014. For GC-FID analysis, all samples were trans-esterified to fatty acid methyl esters (FAMEs) using 2ml 1% sulphuric acid in anhydrous methanol. The FAMEs were then separated within the gas chromatograph using a BPX70 capillary column and detected using a flame ionised detector.

5.3.4. Data analysis and statistics

All the data was analysed using the Statistical Package for Social Science (Version 21; SPSS Inc, Chicago, IL, USA) and expressed as the mean value ± SEM, where P=≤0.05 was considered statistically significant. The homogeneity of the data was assessed and if not normally distributed the data was suitably transformed e.g. Log, square root, or inverse to achieve a normal distribution. If the data was normally distributed, it was analysed using the parametric independent student T-test. If the data was not normally distributed and could not be transformed, it was assessed using the non-parametric Mann-Whitney U test. The statistical analysis would determine if feeding of a HFHC diet had a significant effect upon the percentage of fatty acids within the plasma, liver, and uterus.

5.4. Results

5.4.1.1. Plasma fatty acid profile

The fatty acid profile of the plasma collected from animals maintained on a CON or HFHC diet for 6 weeks prior to and during gestation is illustrated within Table 5.1. As expected, chronic exposure to a HFHC diet had a significant effect upon circulating lipids, significantly increasing total MUFA and decreasing PUFA (*P*=0.004 and *P*=0.05, respectively) within the plasma, while having no effect upon total SFA levels. Despite this, HFHC animals displayed significantly increased levels of the SFA's myristic acid (CON 0.36% ± 0.01 vs HFHC 1.39% ± 0.12; *P*=0.004) within the plasma, compared to animals sustained on the CON diet. The significant difference in circulating total MUFA levels between the dietary groups could be attributed to the significantly greater quantity of oleic acid within the circulation of HFHC animals (CON 7.51% ± 0.90 vs HFHC 23.37% ± 0.96; *P*=<0.001). The significantly reduced PUFA levels within the HFHC plasma was characterised by almost half the AA (CON 18.39% ± 1.20 vs HFHC 11.57% ± 0.81; *P*=<0.001) and 3-times less DHA (CON 7.42% ± 0.62 vs HFHC 2.25% ± 0.28; *P*=<0.001) in circulation compared to CON animals, though this did not translate to a lower circulating n-6:n-3 ratio (CON 5:1 vs HFHC 12:1) within HFHC animals.

Fatty acid	Control diet (n=5) (%)	HFHC diet (n=6) (%)	*P* value
Total saturated	34.45 ± 0.53	33.83 ±0.40	0.362
Myristic acid (14:0)	0.36 ± 0.01	1.39 ± 0.12	0.004
Palmitic acid (16:0)	20.40 ± 0.75	21.01 ± 0.25	0.503
Stearic acid (18:0)	11.85 ± 0.95	9.67 ± 0.30	0.083
Total trans-unsaturated	0.04 ± 0.02	0.84 ± 0.06	0.004
Total mono-unsaturated	9.39 ± 0.96	27.10 ± 1.33	<0.001
Total n-9	7.57 ± 0.91	23.53 ± 0.97	<0.001
Oleic acid (18:1 n-9)	7.51 ± 0.90	23.37 ± 0.96	<0.001
Total n-7	1.81 ± 0.08	3.57 ± 0.41	0.004
Palmitoleic acid (16:1 n-7)	0.70 ± 0.06	2.36 ± 0.37	0.004
Vaccenic acid (18:1 n-7)	1.11 ± 0.04	1.21 ± 0.04	0.177
Total n-3 poly-unsaturated	9.77 ± 0.68	3.03 ± 0.33	0.004
Clupanodonic acid (22:5 n-3)	1.05 ± 0.12	0.31 ± 0.07	<0.001
Docosahexaenoic acid (22:6 n-3)	7.42 ± 0.62	2.25 ± 0.28	<0.001
Total n-6 poly-unsaturated	46.35 ± 0.80	35.20 ± 1.17	<0.001
Linoleic acid (18:2 n-6)	22.02 ± 1.48	17.28 ± 0.31	0.082
γ-linolenic acid (18:3 n-6)	0.59 ± 0.05	0.49 ± 0.05	0.194
Eicosadienoic acid (20:2 n-6)	0.26 ± 0.01	0.20 ± 0.02	0.126
Dihomo- γ –linolenic acid (20:3 n-6)	0.44 ± 0.05	0.48 ± 0.02	0.792
Arachidonic acid (20:4 n-6)	18.39 ± 1.20	11.57 ± 0.81	<0.001
Docosapentaenoic acid (22:5 n-6)	3.47 ± 0.54	4.45 ± 0.39	0.166

Table 5.1 – The effect of feeding a CON (n=5) or HFHC (n=6) diet upon the maternal plasma fatty acid profile. The values represent the mean percentage (%) ± SEM. Significant effect of maternal diet (*P*=≤0.05) highlighted.

5.4.1.2. Liver fatty acid profile

As the liver is an organ essential to the processing of fatty acids, it was necessary to investigate the effect a HFHC diet upon the fatty acid composition of the liver. The hepatic fatty acid profile reflects the profile of the plasma (Table 5.2), with HFHC animals displaying significantly increased total MUFA and decreased PUFA (*P*=0.004 and *P*=0.05, respectively) compared to CON animals. Unlike the plasma, HFHC animals displayed significantly (*P*=<0.001) lower levels of total SFA's within the liver, characterised by significantly decreased stearic acid (CON 17.91% ± 0.77 vs HFHC 9.61% ± 0.45; *P*=0.004) acid, but greater levels of myristic acid (CON 0.43% ± 0.02 vs HFHC 1.32% ± 0.31; *P*=0.032) within the liver. Oleic acid was again the major constituent of the total MUFA fraction, with HFHC animals displaying nearly 6-times the hepatic levels (CON 4.58% ± 0.31 vs HFHC 27.01% ± 1.63; *P*=0.004) of animals sustained on the CON diet. Continuing the trend identified within the plasma, HFHC animals again presented with a greater n-6:n-3 PUFA ratio (CON 3:1 vs HFHC 9:1), but displayed significantly lower levels of total n-6 (CON 35.60% ± 0.58 vs HFHC 30.39% ± 1.16; *P*=0.004) and n-3 (CON 13.99% ± 0.46 vs HFHC 3.30% ± 0.44; *P*=<0.001) PUFA within the liver. Despite this, HFHC animals displayed significantly greater levels of LA (CON 13.08% ± 0.83 vs HFHC 15.65% ± 0.40; *P*=0.004) within the liver, though CON animals displayed significantly greater amount of AA (CON 14.32% ±

0.64 vs HFHC 7.89% ± 0.48; *P*=<0.001) and DHA (CON 12.19% ± 0.45 vs HFHC 2.62% ± 0.37; *P*=0.004), respectively.

Fatty acid	Control diet (n=5) (%)	HFHC diet (n=6) (%)	*P* value
Total saturated	43.45 ± 0.77	34.48 ± 0.73	<0.001
Myristic acid (14:0)	0.43 ± 0.02	1.32 ± 0.31	0.032
Palmitic acid (16:0)	22.48 ± 0.83	21.96 ± 0.24	0.001
Stearic acid (18:0)	17.91 ± 0.77	9.61 ± 0.45	0.004
Total trans-unsaturated	0.02 ± 0.02	0.82 ± 0.04	0.004
Total mono-unsaturated	6.94 ± 0.32	31.02 ± 1.87	0.004
Total n-9	4.89 ± 0.24	27.29 ± 1.62	0.004
Oleic acid (18:1 n-9)	4.58 ± 0.31	27.01 ± 1.63	0.004
Total n-7	1.92 ± 0.03	3.67 ± 0.08	0.001
Palmitoleic acid (16:1 n-7)	0.65 ± 0.05	2.15 ± 0.28	0.001
Vaccenic acid (18:1 n-7)	1.27 ± 0.06	1.52 ± 0.07	0.029
Total n-3 poly-unsaturated	13.99 ± 0.46	3.30 ± 0.44	<0.001
Clupanodonic acid (22:5 n-3)	1.14 ± 0.08	0.30 ± 0.07	<0.001
Docosahexaenoic acid (22:6 n-3)	12.19 ± 0.45	2.62 ± 0.37	0.004
Total n-6 poly-unsaturated	35.60 ± 0.58	30.39 ± 1.16	0.004
Linoleic acid (18:2 n-6)	13.08 ± 0.56	15.65 ± 0.40	0.004
γ-linolenic acid (18:3 n-6)	0.56 ± 0.13	0.46 ± 0.07	0.550
Eicosadienoic acid (20:2 n-6)	0.45 ± 0.05	0.24 ± 0.11	0.003
Dihomo- γ –linolenic acid (20:3 n-6)	0.52 ± 0.05	0.40 ± 0.04	0.057
Arachidonic acid (20:4 n-6)	14.32 ± 0.64	7.89 ± 0.48	<0.001
Docosapentaenoic acid (22:5 n-6)	5.50 ± 0.60	5.01 ± 0.53	0.570

Table 5.2 - The effect of feeding a CON (n=5) or HFHC (n=6) diet upon the maternal hepatic fatty acid profile. The values represent the mean percentage (%) ± SEM. Significant effect of maternal diet (*P*=≤0.05) highlighted.

5.4.1.3. Uterine fatty acid profile

It is has been established that fatty acid supplementation can have a significant effect upon the outcomes of labour. As a result, it was important to establish if feeding of a HFHC diet altered the fatty acid profile of the uterus (Table 5.3). Surprisingly, chronic exposure to a HFHC diet had little effect upon the fatty acid profile of the uterus, with only a significant increase in DPA (CON 1.71% ± 0.30 vs HFHC 2.96% ± 0.39; *P*=0.036) and a decrease in total n-3 PUFA (CON 4.83% ± 0.66 vs HFHC 2.79% ± 0.28; *P*=0.014) reported. Similar to the plasma and the liver, HFHC animals displayed lower levels of total n-6 PUFA (CON 39.24% ± 2.82 vs HFHC 32.95 ± 1.27; *P*=0.060) characterised by reduced LA (CON 15.05% ± 4.81 vs HFHC 9.13% ± 1.27; *P*=0.247), with both the lower n-6 and n-3 PUFA still translating to a greater n-6:n-3 PUFA ratio (CON 8:1 vs HFHC 12:1) in HFHC animals.

Fatty acid	Control diet (n=5) (%)	HFHC diet (n=6) (%)	*P* value
Total saturated	40.62 ± 5.17	46.08 ± 1.69	0.306
Myristic acid (14:0)	1.31 ± 0.26	2.15 ± 0.26	0.053
Palmitic acid (16:0)	24.31 ± 1.19	23.89 ± 1.16	0.824
Stearic acid (18:0)	5.45 ± 3.12	10.83 ± 2.81	0.230
Total trans-unsaturated	0.52 ± 0.17	0.94 ± 0.13	0.089
Total mono-unsaturated	15.19 ± 2.80	17.24 ± 2.42	0.589
Total n-9	12.47 ± 2.40	14.34 ± 2.07	0.429
Oleic acid (18:1 n-9)	12.42 ± 2.34	14.16 ± 2.04	0.537
Total n-7	2.70 ± 0.53	2.82 ± 0.41	0.824
Palmitoleic acid (16:1 n-7)	1.12 ± 0.25	1.63 ± 0.26	0.354
Vaccenic acid (18:1 n-7)	1.80 ± 0.24	1.47 ± 0.10	0.195
Total n-3 poly-unsaturated	4.83 ± 0.66	2.79 ± 0.28	0.014
Clupanodonic acid (22:5 n-3)	0.91 ± 0.12	0.81 ± 0.14	0.726
Docosahexaenoic acid (22:6 n-3)	3.06 ± 0.68	1.83 ± 0.22	0.092
Total n-6 poly-unsaturated	39.24 ± 2.82	32.95 ± 1.27	0.060
Linoleic acid (18:2 n-6)	15.05 ± 4.81	9.13 ± 1.27	0.247
γ-linolenic acid (18:3 n-6)	2.18 ± 0.67	1.73 ± 0.37	0.592
Eicosadienoic acid (20:2 n-6)	0.87 ± 0.14	0.59 ± 0.10	0.113
Dihomo- γ –linolenic acid (20:3 n-6)	2.40 ± 0.61	2.04 ± 0.43	0.640
Arachidonic acid (20:4 n-6)	12.50 ± 2.97	12.24 ± 0.39	0.940
Docosapentaenoic acid (22:5 n-6)	1.71 ± 0.30	2.96 ± 0.39	0.036

Table 5.3- The effect of feeding a CON (n=5) or HFHC (n=6) diet upon the maternal uterine fatty acid profile. The values represent the mean percentage (%) ± SEM. Significant effect of maternal diet (*P*=≤0.05) highlighted.

5.5. Discussion

The objective of this study was to investigate the effect of feeding a HFHC diet upon plasma, hepatic, and uterine fatty acid profiles. This is an essential step in evaluating the HFHC diet maternal obesity model, as fatty acid supplementation has been found to have a significant effect upon female reproduction and the outcomes of labour (Wathes et al., 2007).

Chronic exposure to a HFHC diet significantly altered the fatty acid profile of the plasma, liver, and uterus. The plasma fatty acid profile of HFHC animals was characterised by significantly increased total MUFA, while exhibiting a significant decrease in total n-6 and n-3 PUFA. Further to this, the n-9 MUFA Oleic acid was significantly increased within the circulation of HFHC animals. Despite displaying reduced total n-6 and n-3 PUFA, HFHC animals displayed an increased n-6:n-3 ratio compared to CON animals in circulation. The liver exhibited a fatty acid profile similar to that of the plasma, but it appears chronic exposure to a HFHC diet had little effect upon the uterine fatty acid profile. HFHC animals only displayed a significant decrease in total n-3 PUFA and significantly increased DPA (22:5 n-6) within the uterus. Overall, the change in the fatty acid profiles of the plasma, liver, and uterus can be contributed to the composition of the HFHC diet, as other high fat diets have had similar effects upon the fatty acid profiles of rodents and their tissues (Cerf and Herrera, 2016; Grant et al., 2011).

The HFHC diet (Table 8.3) is 35% total fat, characterised by increased total SFA, trans-unsaturated, and MUFA, while being lower in total n-6 and n-3 PUFA when compared to the CON diet (Table 8.3). Despite the effect of a HFHC diet upon the fatty acid profile, it remains to be determined if this is causing or contributing to the poor labour outcomes identified within HFHC animals previously.

Change in dietary fatty acids has been shown to have a significant effect upon the outcomes of labour. Animal and human studies have illustrated the significant risk of pre-term labour when fed a diet rich in n-6 PUFA (Elmes et al., 2005; Reece et al., 1997), while diets high in n-3 PUFA significantly prolong gestation and labour (Baguma-Nibasheka et al., 1999; Leat and Northrop, 1981; Ma et al., 2000; Olsen et al., 1990, 1986; Waltman et al., 1977), resulting from a change in 2-series PG synthesis (Allen and Harris, 2001; Cheng et al., 2005; Elmes et al., 2005; Leaver et al., 1991). Diets high in n-6 PUFA's provide the substrate AA for the synthesis of 2-series PG's ($PGF_{2\alpha}$ and PGE_2), which are potent uterotonins (Challis et al., 1997). It has been suggested that the n-3 PUFA EPA displaces and outcompetes AA for COX-2, increasing the synthesis of 3-series PG's ($PGF_{3\alpha}$ and PGE_3) and decreasing the synthesis of 2-series PG's within the rat uteri (Allen and Harris, 2001; Leaver et al., 1991). As a result, a shift in the fatty acid composition of the diet can have a significant effect upon the synthesis of 2-series PG's, and as a result the outcomes of labour (Elmes et al., 2005; Leat and Northrop, 1981; Leaver et al., 1991;

Waltman et al., 1977). HFHC animals presented with prolonged and dysfunctional labour characterised by the significantly reduced plasma $PGF_{2\alpha}$ identified previously (Elmes et al., 2011), despite displaying a significant reduction in circulating and stored n-3 PUFA's. This decrease in circulating $PGF_{2\alpha}$ is likely a result of HFHC animals consuming a diet 33% lower in LA, which translates to the significantly lower circulating levels of the 2-series PG precursor AA (CON 18.39% ± 1.20 vs HFHC 11.57% ± 0.81; *P*=<0.001). Further to this, HFHC animals displayed significantly increased levels of oleic acid within circulation, which could contribute to the decrease in plasma $PGF_{2\alpha}$, as culture of pregnant ewe primary endometrial cells with oleic acid attenuates $PGF_{2\alpha}$ synthesis, even in the presence of OXT (Cheng et al., 2015). Any change in $PGF_{2\alpha}$ concentrations will significantly impact upon both PG and OXT synthesis, offering an explanation to the protracted response of HFHC animals to OXT *ex vivo*, as both PG and OXT are able to stimulate each other's synthesis through a positive feed-forward autocrine/paracrine mechanism within maternal tissue (Siemieniuch et al., 2011; Snegovskikh et al., 2006). This could have a significant effect upon contraction of the uterus during labour, as both $PGF_{2\alpha}$ and OXT are potent uterotonins (Challis et al., 2000). This dietary fatty acid induced change in PG synthesis offers an explanation to the prolonged and dysfunctional labour displayed by our HFHC animal model including the protracted response to OXT *ex vivo*, but fatty acid induced change in PG synthesis may compromise uterine contractility through alternative or additional mechanisms.

Exposure of cardiomyocytes to oleic acid in culture significantly decreases their contractility by limiting the formation of intracellular gap junctions, which is a result of the phosphorylation of CX-43 at serine 368 by PKC (Huang et al., 2004). CX-43 is the protein essential to the formation of gap junctions that act as intracellular conduits to facilitate cell-cell electro-coupling (synchronisation) and metabolite transfer (Sims et al., 1982). Phosphorylation of CX-43 compromises gap junction assembly, trafficking, and thus intracellular communication (Su and Lau, 2014). A decrease in intracellular gap junction formation has been shown to significantly reduce the synchronisation of uterine contractions, prolonging the duration of labour (Döring et al., 2006; Sims et al., 1982; Tong et al., 2009). As mentioned previously in Chapter 3, animals sustained on a HFHC diet maintained their uterine *P*CX-43 expression, while phosphorylation of CX-43 decreased in CON animals with the onset of TL. This sustained phosphorylation of CX-43 could potentially be a result of the significantly greater circulating levels of oleic acid within HFHC animals, contributing to their prolonged and dysfunctional labour. This increase in circulating oleic acid can be traced to the increased levels of oleic acid or palmitic acid within the HFHC diet, as palmitic acid can be converted to oleic acid within the liver as an effective mechanism for the storage of energy within white adipose tissue (Berry, 1997; Cook and McMaster, 2002).

As detailed within chapter 3, chronic exposure to a HFHC diet significantly increased plasma progesterone at term and altered TL uterine CAP expression including a decrease in CX-43, Cav-1, and an increase in OXTR, COX-2, and *P*CX-43. This increase in plasma progesterone at term is in agreement with other studies of maternal obesity (Crew et al., 2016; Elmes et al., 2011), which is hypothesised to compromise uterine CAP expression and the outcomes of labour (Bogaerts et al., 2013). This increase in plasma progesterone could be a result of the fatty acid composition of the diet, as bovine studies have illustrated the significant effect of dietary PUFA upon sex steroid synthesis (Mattos et al., 2000). Despite this, studies have reported conflicting results with some recording an increase in circulating progesterone upon feeding dietary n-3 or n-6 PUFA (Staples et al., 1998), while other report a decrease in plasma progesterone upon feeding of a diet rich in n-3 PUFA (Robinson et al., 2002). As a result, further work is required to determine the validity of this hypothetical mechanism and its role in maternal obesity associated uterine dystocia.

Modulation of dietary fatty acids may also compromise uterine CAP expression by altering the fluidity of the plasma membrane (Bogaerts et al., 2013). Fatty acids play a vital role in the formation of the plasma membrane lipid bilayer by reacting with glycerol and phosphate to form phospholipids essential to its structure (Berg et al., 2011). An increase in membrane n-6/n-3 PUFA or SFAs will increase or decrease the fluidity of the membrane, compromising the expression, function, and

downstream signalling of integral proteins (Murphy, 1990). For example, essential fatty acid deficiency in rats compromises the downstream signalling of the β-adrenoceptor by depressing the activity of adenylyl cyclase, with repletion of a normal membrane fatty acid profile ineffective in restoring its original activity (Murphy, 1990). Despite this information, there is currently no evidence available to the effect of dietary fatty acids upon uterine membrane fluidity and integral CAP expression. However, if the documented decrease in uterine total n-3 PUFA within HFHC animals does increase membrane fluidity sufficiently, it could compromise the expression and function of uterine CAP's including CX-43, OXTR, PG receptors, and Ca^{2+} ion channels, which are all essential to contraction of the uterus during labour (Challis et al., 2000; Gimpl and Fahrenholz, 2000; Oshima, 2014; Anatoly Shmygol et al., 2007).

It is clear that chronic exposure to a HFHC diet did significantly alters the fatty acid composition of the plasma, liver, and uterus. This is a potential mechanism of maternal obesity associated uterine dystocia. Despite this, there are some limitations to this study.

One limitation is the fact that PG's within the plasma and uterus was not measured, though this will be completed within the future work of this thesis. Although the effect of a HFHC diet upon circulating and uterine PG's has yet to be established, it is expected that chronic exposure to a HFHC diet will lower their concentrations in line with the

previous pilot study, where feeding of a HFHC diet significantly lowered circulating $PGF_{2\alpha}$ (Elmes et al., 2011). The question also remains why did exposure to a HFHC diet significantly alter the fatty acid profiles of the plasma and liver, but induce little change within the uterus. It could be that the uterus is resistant to change in dietary fatty acids like other organs e.g. the brain (Murphy, 1990). Another reason could be that by collecting tissue only from term-labouring animals, they had utilised the fatty acids within the uterus necessary for labour, and differences may only be clear prior to the onset of labour. To determine this, future samples must be collected prior to pregnancy, during gestation, and in parturition, with the analysis focusing on the fatty acid composition of the phospholipid fraction of the uterus only. This will provide a better perspective to the effect of a HFHC diet upon the circulatory, hepatic, and uterine fatty acid concentrations.

In summary, chronic exposure to a HFHC diet resulted in a significant change in both the circulating and hepatic fatty acid profiles characterised by significantly lower levels of AA and DHA, along with significantly increased levels of oleic acid. However, this difference in circulating and hepatic lipids between the dietary groups did not translate to a difference in the uterine fatty acid profiles. Despite this, the fatty acid profile of HFHC animals provides evidence that chronic exposure to a HFHC diet has the capacity to alter PG synthesis, membrane fluidity, and potentially uterine CAP expression. Despite these potential mechanisms, further work is certainly required to assess

and elucidate if these could contribute to the development of prolonged and dysfunctional labour.

6. General discussion

In 2011, Elmes et al. published a pilot study reporting on the potential of a HFHC diet animal model for the study of maternal obesity associated uterine dystocia. Chronic exposure to a HFHC diet altered uterine CAP expression, increased plasma progesterone, and decreased circulating $PGF_{2\alpha}$ concentrations (Elmes et al., 2011). These changes have the potential to impact upon term-labouring uterine contractility and the outcomes of human parturition, explaining the poor outcomes of labour associated with maternal obesity. Despite this, the overall efficacy and translatability of the HFHC diet animal model has yet to be established or the mechanism(s) elucidated.

The overall objective of this thesis was to evaluate and establish the efficacy of the HFHC diet animal model in the study of maternal obesity associated uterine dystocia. It was hypothesised that chronic exposure to a HFHC diet would compromise the uterine contractile activity of these animals compared to those sustained on the CON diet.

To assess the effect of a HFHC diet upon uterine contractility, an *ex vivo* contractility organ bath study was first carried out. Feeding of a HFHC diet 6-weeks prior to and during gestation significantly impacted upon the spontaneous contraction phenotype. CON animals displayed a biphasic plateau contraction phenotype, while HFHC animals displayed a multiphasic spike bundle contraction phenotype. The difference in the contraction phenotypes of these animals can be

attributed to the degree of synchronisation between uterine smooth muscle bundles that compose the myometrium (Shmygol et al., 2007).

The *ex vivo* contractility study also revealed that feeding of a HFHC diet had a significant impacted upon OXT response. Animals maintained on the HFHC diet demonstrated a protracted response to increasing concentrations of OXT *ex vivo*, which may have been a result of their increased circulating cholesterol. Culture of uterine myocytes with cholesterol increases the binding affinity of OXT for OXTR (Klein et al., 1995), but decreases OXT induced uterine contractility *ex vivo* (Smith et al., 2005; Jie Zhang et al., 2007). Increased circulating cholesterol could decrease the fluidity of the plasma membrane, effecting GPCR function e.g. OXTR and its downstream signalling (Pucadyil and Chattopadhyay, 2006). This could impact upon uterine contractility, but further research is required to determine this.

To continue evaluating the efficacy of the HFHC diet animal model for the study of maternal obesity associated uterine dystocia, it was deemed advantageous and logical to assess the effect of a HFHC diet upon uterine contractility *in vivo*. Intra-uterine telemetry surgery revealed that HFHC animals displayed increased uterine contractile activity for up to 9 hours longer than animals sustained on the CON diet *in vivo*. The combination of increased uterine activity for a longer duration indicated that HFHC animals exhibited a prolonged duration of labour compared to their CON counterparts.

It is clear from the evidence presented within this thesis that chronic exposure to a HFHC diet compromises uterine contractile activity within the rat, but the exact mechanism remains to be elucidated. Analysis of uterine CAP's using western blotting and fatty acid analysis using GC-FID may have revealed potential mechanism(s) by which a HFHC diet is compromising uterine contractility.

CX-43 is the CAP essential to the formation of intracellular gap junctions that increase with onset of parturition to facilitate synchronous contraction of the uterus (Sims et al., 1982; Smith, 2007; Wray, 1993). Chronic exposure to a HFHC diet resulted in a decrease in uterine CX-43 expression at the onset of term labour. This could compromise uterine contractility in the HFHC animals, as knockdown of uterine CX-43 compromises intracellular communication and contraction synchronisation, prolonging the duration of labour (Döring et al., 2006; Sims et al., 1982; Tong et al., 2009). Despite this, a number of different mechanisms could also cause or contribute to this change in uterine CX-43 expression.

It is hypothesised that maternal obesity disrupts the withdrawal of progesterone at term, allowing the hormone to limit oestrogen and stretch induced activation of the uterus (Bogaerts et al., 2013; Carlson et al., 2015). This is certainly a potential mechanism to consider, as HFHC animals displayed increased circulating progesterone at term, which could compromise the transcription and post-translational

transport of CX-43 to the membrane (Challis et al., 2000; Hendrix et al., 1995).

Progesterone is not the only endogenous substance that can impact upon the expression and function of CX-43. Enrichment of cardiomyocyte culture with cholesterol or the n-9 MUFA oleic acid increases the phosphorylation of CX-43 at serine-368, which decreases intracellular gap junction formation and intracellular communication (Huang et al., 2004; Su and Lau, 2014; Zou et al., 2014). Animals sustained on a CON diet displayed a decrease in CX-43 phosphorylation with the onset of TL, while HFHC animals displayed no relative change. This lack of change in *P*CX-43 expression may be a result of increased circulating cholesterol or oleic acid, which offers another mechanism by which feeding a HFHC diet may compromise uterine contractility.

Feeding of the HFHC diet may compromise uterine contractility in the rat by altering the synthesis of 2-series PG's. PG's (PGE_2 & $PGF_{2\alpha}$) are potent uterotonins that stimulate contraction of the uterus during labour (Challis et al., 2000), with change in their synthesis prolonging the duration of labour (Abayasekara and Wathes, 1999). Chronic exposure to a HFHC diet significantly altered circulating fatty acids causing an increase in oleic acid, while decreasing n-6 & n-3 PUFA, respectively. This change in circulating fatty acids has the capacity to limit 2-series PG synthesis within maternal reproductive tissue (Cheng et al., 2015;

Wathes et al., 2007), which could compromise uterine contractility, and explains the decrease in circulating $PGF_{2\alpha}$ identified in a previous pilot study (Elmes et al., 2011).

It is clear that a number of potential mechanism(s) may be responsible for the poor uterine contractility displayed by animals fed the HFHC diet; these mechanisms have been discussed throughout this thesis. It is also clear that this dietary animal model has displayed some clinical relevance. It was identified through the intra-uterine telemetry surgery that the HFHC animals were suffering from a prolonged duration of labour. A number of clinical studies have reported that obese parturients suffer from a prolonged first stage of labour (Chin et al., 2012; Vahratian et al., 2005; J Zhang et al., 2007), illustrating a similarity between human studies and the HFHC animal model. This similarity was also reflected during the *ex vivo* contractility organ bath study, as uterine tissue collected from HFHC animals displayed an insensitivity to OXT, requiring a significantly greater dose to reach their $logEC_{50}$. This is similar to the study by Roloff et al. (2015), who reported that obese parturients require a significantly greater dose of OXT to augment the outcomes of labour. This initial evidence highlights the potential of the HFHC diet animal model, but further research is required to establish the translatability of this animal model for the study of maternal obesity associated uterine dystocia. If its translatability can be established, this model could have a substantial

impact upon the clinical outcomes of labour for obese parturients in the future.

The overall strength of the research presented within this thesis lies in the well rounded *ex vivo* and *in vivo* characterisation of the effect of a HFHC diet upon uterine contractility. This illustrated both the effect of a HFHC diet upon the synchronisation of uterine contractions and its effect upon the duration of labour, while providing an initial insight into the potential mechanism(s) responsible. Further to this and to the author's knowledge, it is the first application of the intra-uterine telemetry surgery technique to investigate the effect of a HFHC diet upon the outcomes of labour. This thesis has provided a good foundation for future research to investigate maternal obesity associated uterine dystocia using the HFHC diet animal model.

Despite the strengths of the research documented within this thesis, there are limitations to be considered. The formulated HFHC diet did not induce obesity to the same degree as other rodent obesity models e.g. cafeteria diet or the $Apo^{-/-}$ genetic model. This raises the question to how the HFHC diet is compromising uterine contractile activity, is it through visceral fat procurement, dyslipidaemia, or another mechanism that has yet to be identified. This raises some questions to the validity of this dietary animal model for the study of maternal obesity associated uterine dystocia. Further to this, logistical problems and pathological complications may have underpowered the animal studies,

limiting their analysis and findings. Further research is required to overcome the limitations and complications identified over the course of this PhD.

In summary, the evidence reviewed and discussed throughout this thesis illustrates the potential of the HFHC diet animal model for the study of maternal obesity associated uterine dystocia. This has tested overall aim and confirmed the primary hypothesis that chronic exposure to a HFHC diet would compromise uterine contractility in the rat. Work beyond the scope of this thesis is certainly required to overcome the limitations of this thesis and to continue evaluating the HFHC diet animal model for the study of maternal obesity associated uterine dystocia.

6.1. Conclusion

In conclusion, it is clear that chronic exposure to a HFHC diet compromises uterine contractility. This has been determined by assessing the contractility of the term labouring uterus both *ex vivo* and *in vivo*, coupled with an initial investigation into the mechanisms that may be causing or contributing to the onset of maternal obesity uterine dystocia. Through this collection of evidence, the HFHC diet animal model of maternal obesity associated uterine dystocia has displayed both efficacy and translatability, and has illustrated its potential as a standardised platform to fully elucidate the pathogenesis of this disease. This research boasts the potential to improve the clinical

outcomes for obese parturients and their offspring, while having the potential to relieve the financial stress from the NHS if these mechanisms can be understood and exploited pharmacologically. As a result, this thesis has met its primary objective in evaluating the efficacy and translatability of the HFHC diet animal model for the study of maternal obesity associated uterine dystocia.

6.2. Suggestions for future research

As a result of time and financial restrictions, not every avenue of research could be fully explored within this project. As a result, suggestions for future research have been proposed.

The previously discussed mechanism proposing the insufficient (functional) withdrawal of progesterone at term shows promise, as sex steroids play a significant role in parturition. As a result, it would be advantageous to characterise the expression of all sex steroids and their relevant synthesising enzymes within both maternal and foetal tissue. This will provide further insight into the effect(s) of maternal obesity upon the endocrine milieu of parturition.

Uterine CAP expression of TNL and TL CON and HFHC animals was characterised using western blotting. This method clearly illustrated the effects of a HFHC diet and the stage of labour upon uterine CAP expression relevant to a housekeeping protein. Despite this, western blotting does not reveal the effect of a HFHC diet or the stage of labour

upon protein localisation e.g. nucleus or cytoplasm, its transport, or its co-localisation with other proteins e.g. OXTR with Cav-1 within the uterus. Further work employing immunohistochemistry and immunofluorescence will investigate the effect of maternal obesity upon the expression, function, and localisation of uterine CAP's during TL.

Determining the fatty acid profile of the plasma, liver, and uterus was carried out to provide an initial insight into the potential effect of a HFHC diet upon PG synthesis. To further this line of enquiry, it would important to establish the effect of a HFHC diet upon only the lipid composition of the plasma membrane, and the concentrations of PG's within the plasma and uterus.

Further to these suggestions, it would advantages to undertake next generation sequencing of the plasma and uterus collected from CON and HFHC animals, in an attempt to identify a novel mRNA or miRNA biomarker for increased risk of prolonged and dysfunctional labour and/or emergency caesarean delivery.

Completing these suggestions would contribute to rounding this study off, and better characterising the mechanisms that may be causing or contributing to maternal obesity associated uterine dystocia.

7. References

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8. Appendix

8.1. Diet

8.1.1. Control diet

The control diet used within all animal trials carried out at the BSU (University of Nottingham) was the Harlan Teklad Global 2018 18% protein rodent diet provided by Envigo (Indianapolis, Indiana, USA). A nutritional breakdown of the diet according to the manufacture (Envigo, 2015) is presented in Table 8.2. The fatty acid profile (Table 8.3) of the control diet was determined by Dr Ge Liu and Dr John Carragher of the Waites Lipid Analysis Service at the University of Adelaide, using gas chromatography flamed ionised detector (GC-FID).

8.1.2. HFHC diet

The HFHC diet was developed and formulated previously by Elmes et al. (2011) and illustrated in Table 8.1. Nutritional analysis was carried out by Scientific Laboratory Services (SAL) Ltd, which is presented in Table 8.2. The fatty acid profile of the HFHC diet (Table 8.3) was determined by Dr Ge Liu and Dr John Carragher of the Waites Lipid Analysis Service at the University of Adelaide, using GC-FID.

Constituents	Amount (g/kg)
Corn oil	100
Casein	200
Maize starch	218
Butter	295
Sucrose	200
Cellulose	50
Vitamin mix	5
Mineral mix	20
Methionine	10
Choline	2
Cholesterol	10

Table 8.1 – Breakdown of ingredients required to produce 1kg of HFHC diet.

Nutritional breakdown	Control diet	HFHC diet
Energy density (Kcal/g)	3.1	5.26
Total available carbohydrates (%)	44.2	30.5
Total protein (%)	18.6	18.5
Total fat (%)	6.2	35.6
Total fibre (%)	3.5	4.8
Total ash (%)	5.3	1.9

Table 8.2 – Nutritional breakdown of the control and HFHC diet.

Fatty acid	Control diet (%)	HFHC diet (%)
Total saturated	20.2	46.3
Myristic acid (14:0)	0.4	7.7
Palmitic acid (16:0)	14.8	26.8
Stearic acid (18:0)	3.8	9.9
Total trans-unsaturated	0.1	3.2
Total mono-unsaturated	20.9	29.9
Total n-9	19.4	27.7
Oleic acid (18:1 n-9)	19.0	27.6
Total n-7	1.5	2.2
Palmitoleic acid (16:1 n-7)	0.4	1.5
Vaccenic acid (18:1 n-7)	1.1	0.7
Total n-3 poly-unsaturated	6.0	0.8
Clupanodonic acid (22:5 n-3)	0.0	0.1
Docosahexaenoic acid (22:6 n-3)	0.0	0.0
Total n-6 poly-unsaturated	52.8	19.8
Linoleic acid (18:2 n-6)	52.7	19.6
γ-linolenic acid (18:3 n-6)	0.0	0.0
Eicosadienoic acid (20:2 n-6)	0.1	0.0
Dihomo- γ –linolenic acid (20:3 n-6)	0.0	0.1
Arachidonic acid (20:4 n-6)	0.0	0.1
Docosapentaenoic acid (22:5 n-6)	0	0.0
n-6:n-3 ratio	9:1	25:1

Table 8.3 –The fatty acid profiles of the control and HFHC diets