The impact of obesity on circadian rhythms in the mother, placenta and fetus in rat pregnancy

by

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Preface

The experimental work presented in this thesis was undertaken in The School of Anatomy, Physiology and Human Biology, The University of Western Australia, under the supervision of Prof. Brendan J. Waddell and Dr. Peter J. Mark. Financial assistance was provided by an Australian Postgraduate Award and UWA top-up Scholarship.

The work described is original and was performed by myself, except where the specific contributions of others are acknowledged. Chapters 5 and 6 have been published and Chapters 7-9 have been submitted for publication. All co-authors have given formal consent for each published and submitted manuscript to be presented in this thesis.

Rachael Christina Crew

May 2016
Abstract

Maternal obesity induces pregnancy complications and disturbs fetal development, but the specific mechanisms underlying these effects are unclear. Alterations in circadian rhythms are involved in maternal adaptations to pregnancy and metabolic complications associated with obesity. Accordingly, obesity-induced circadian dysfunction may drive adverse outcomes in obese pregnancy. The aim of this thesis was to characterise rhythmic function in maternal, placental and fetal tissues during obese pregnancy, with a specific focus on clock gene expression. Several other downstream physiological and metabolic processes that are implicated in the pathogenesis of obese pregnancy were also assessed.

The initial study optimised a model for maternal obesity using cafeteria (CAF) feeding in Wistar rats, through provision of calorically dense snack foods for 8 weeks prior to pregnancy, while control animals (CON) were maintained on standard rodent chow. CAF feeding was continued through pregnancy, and the impact of maternal obesity on the inflammatory status of the mother, placenta and fetus was assessed in late gestation. Despite a 63% increase in central body fat in CAF mothers, plasma inflammatory markers were not increased by obesity at day 21 of gestation. In fact, levels of IL-6, IL-12p40 and MIP2 were reduced slightly in CAF maternal plasma. Similarly, inflammatory gene expression in maternal, placental and fetal tissues remained largely unaffected by obesity, indicating that CAF-induced maternal obesity does not lead to excessive inflammation in late gestation.

Subsequent studies used the same CAF feeding model in a larger cohort of animals, where maternal, fetal and placental tissues were collected at four-hourly time-points across days 15-16 and 21-22 of gestation (term = 23 days). This allowed for rhythmic analyses across these gestational days, which span the period of maximal fetal growth. The second study analysed fetal and placental growth trajectories across each of these two gestational days, and measured the daily profiles of plasma steroid hormones and lipid markers. Fetal and placental growth restriction was evident in the CAF group across both days, however there was no change in total fetal or placental weight per litter due to slightly larger litter sizes in the CAF mothers. CON animals exhibited nocturnal declines in progesterone in maternal (39% lower) and fetal (45% lower) plasma, but these falls were absent in the CAF group. CAF mothers were also hyperlipidaemic at both days of gestation, but this effect was isolated to the dark photoperiod at day 21. Neither maternal nor fetal glucocorticoid profiles were affected by obesity.
The third study used intraperitoneal temperature loggers to assess the effect of obesity on rhythmic core body temperature (Tc) profiles. Obesity reduced the average Tc (by up to 0.29ºC) across the estrous cycle and most of pregnancy, however Tc converged between diet groups towards the end of gestation. Obesity altered the amplitude of Tc rhythms at select stages of the estrous cycle and pregnancy, but the acrophase was unaffected by diet. Photoperiod analyses revealed that obesity reduced Tc exclusively in the light period during pre-pregnancy, but only during the dark period in late gestation.

The fourth study investigated whether maternal obesity alters the rhythmic expression of clock genes and associated nuclear receptors (Ppars and Pgc1α) in maternal and fetal hepatic tissue and the placental labyrinth zone (LZ). Expression of the accessory clock gene Rev-erba was rhythmic in all tissues of CON animals, but in each case, CAF feeding reduced peak Rev-erba expression. Obesity advanced maternal hepatic clock gene and Pparδ rhythms (by approx. 1.5 h), but aside from Rev-erba, clock genes were mostly arrhythmic and unaffected by diet in placental and fetal tissues.

The final study measured the rhythmic expression of clock genes and associated nuclear receptors in maternal adipose tissue. CAF feeding suppressed the mesor and amplitude of adipose tissue clock genes (including Bmal1, Per2 and Rev-erba) across both days of gestation. The CAF diet eliminated adipose Pparγ rhythmicity, while expression of Ppara, Pparδ and Pgc1α was reduced at day 15. CAF mothers were hyperleptinaemic at both days, and in late gestation this effect was time-of-day dependent.

Collectively, these studies demonstrate that obesity disturbs maternal endocrine and Tc rhythms, as well as clock gene expression in maternal metabolic tissues. Maternal obesity had relatively little impact on circadian markers in the placenta and fetus, possibly reflecting the absence of a mature circadian system in these tissues.
Acknowledgements

Firstly I would like to acknowledge my supervisors, Brendan Waddell and Peter Mark. Brendan and Pete, thank you for sharing your invaluable time, wisdom, and guidance, but also your good humour and constant enthusiasm throughout this (mostly!) enjoyable experience. I feel incredibly lucky to have worked with both of you.

I am also thankful to the other members of our lab group, in particular Michaela Wharfe and Megan ‘MJ’ Ellyard, who have been my close friends and confidants over the years. Thank you also to Caitlin Wyrwoll and Jeremy Smith for your advice and mentorship, and a special ‘honourable mention’ to those poor souls who assisted me with midnight and 4 am tissue collections: Peter Mark, Jessica Lewis and Cassandra Yap.

I also extend my most sincere thanks to the collaborators on individual projects within this thesis; Shane Maloney and Michael Clarke, thank you for sharing your expertise with me- I learnt a great deal from both of you and have enjoyed the experience. Thank you to Trevor Mori and his team for performing lipid analyses, and to Linc Schmitt and Hugh Barrett for statistical guidance. I am also grateful to Greg Cozens, Celeste Wale and Leah Atwood for technical advice throughout my PhD.

My thanks to everyone in the School of Anatomy, Physiology & Human Biology for providing such a supportive and motivating environment to work in. I am particularly grateful to Kathy Sanders, Julie Hill, Christina Bojarski, Caitlin Wyrwoll, Jeremy Smith and Peter Mark, for allowing me the opportunity to teach in a number of undergraduate courses. I would also like to acknowledge all of the past and present ‘Room 2.36 Girls’- your friendship has made my PhD experience all the richer, thank you for the often welcome distractions and many wonderful memories.

Finally, thank you to all of my family and friends; but most importantly my parents, Joanne and Russell, who have sacrificed a great deal over the years to prioritise my education. Mum and Dad, I wouldn’t be where I am today without your love and support- thank you.
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Circadian adaptations to obese pregnancy

Thesis Format

General

This thesis is presented as eleven chapters; the first four being the conventional Introduction, Literature Review, Experimental Objectives and Materials and Methods. The experimental work is then presented as five separate chapters, comprising of two published papers (Chapters 5 and 6) and three manuscripts submitted for publication (Chapters 7-9). Each of these is an original research paper and includes an Introduction, Material and Methods, Results and Discussion section specific to that particular study. For consistency, all published manuscripts have been formatted according to this thesis rather than using specific journal guidelines. Following the experimental chapters, a General Discussion draws the findings of this thesis together and reflects on the overall significance of the work. The final chapter lists all References cited in this thesis.

Language

The majority of this thesis is written in Australian English; however for chapters which are published manuscripts, the language is kept according to specific Journal guidelines.

Presentation of data

The majority of data are presented in graphical or tabular format. Where possible, figures are placed immediately after the text in which they are referred to.

Referencing

All published work referred to in this thesis is cited using Harvard (UWA Science) formatting; that is, the author and year of publication are stated in text. When the number of authors exceeds three, the first three authors are cited, followed by et al. and the year of publication. The exception to this rule is the published experimental chapters of this thesis, which have been referred to by their Chapter name, rather than their external citation.
### List of Abbreviations

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<th>Description</th>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>11β-HSD</td>
<td>11-beta hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>11-DHC</td>
<td>11-dehydrocorticosterone</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adenocorticotropic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>bHLH-PAS</td>
<td>Basic helix-loop-helix PER ARNT SIM</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BMAL1*</td>
<td>Brain and muscle ARNT like protein 1</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT/enhancer-binding protein alpha</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CAF</td>
<td>Cafeteria</td>
</tr>
<tr>
<td>CCG</td>
<td>Clock controlled gene</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHOL</td>
<td>Cholesterol (total)</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Circadian locomotor output cycles kaput</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetres</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase (also known as prostaglandin-endoperoxide synthase; PTGS)</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CRY</td>
<td>Cryptochrome</td>
</tr>
<tr>
<td>d</td>
<td>Day(s)</td>
</tr>
<tr>
<td>d</td>
<td>Deuterated</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Dinucleotide triphosphates</td>
</tr>
<tr>
<td>DMDC</td>
<td>Dimethyl dicarbonate</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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</table>
EDTA  Ethylenediamine tetra-acetic acid
EMR   EGF module-containing mucin-like hormone receptor
FFA   Free fatty acid
$g$   Relative centrifugal force
$g$   Grams
GDM   Gestational diabetes mellitus
GLUT  Solute carrier family 2 (facilitated glucose transporter)
GR    Glucocorticoid receptor
GRO/KC Keratinocyte-derived cytokine
$h$   Hour(s)
$H_2O$ Water
$H_2O_2$ Peroxide
$HCl$ Hydrogen chloride
HDAC3 Histone deacetylase 3
HDL-C High density lipoprotein cholesterol
HF    High-fat
HPA   Hypothalamic-pituitary-adrenal
IL    Interleukin
i.m.  Intramuscular
i.p.  Intraperitoneal
IUGR  Intrauterine growth restriction
JZ    Junctional Zone
KJ    Kilojoules
kPa   Kilopascal
$L$   Litre
LC-MS/MS Liquid chromatography-tandem mass spectrometry
LDL-C Low density lipoprotein cholesterol
LEP   Leptin gene (also known as OB)
LEPR  Leptin receptor (also known as OBR)
LEPRa Short form leptin receptor (also known as OBRa)
LEPRb Long form leptin receptor (also known as OBRb)
LSD   Least significant difference
LZ    Labyrinth zone
$M$   Molar ( = moles/litre)
MCP   Monocyte chemoattractant protein
<table>
<thead>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre(s)</td>
</tr>
<tr>
<td>ML</td>
<td>Maternal liver</td>
</tr>
<tr>
<td>M-MLV-RT</td>
<td>Moloney Murine Leukemia Virus Reverse Transcriptase</td>
</tr>
<tr>
<td>mmol/L</td>
<td>Millimole(s) per litre</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl tertiary butyl ether</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>n</td>
<td>Number of samples</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram(s)</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre(s)</td>
</tr>
<tr>
<td>NPAS2</td>
<td>Neuronal PAS domain protein 2</td>
</tr>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>PER</td>
<td>Period</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram(s)</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome proliferator-activated receptor response element</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
</tbody>
</table>
Circadian adaptations to obese pregnancy

r  Correlation coefficient
r²  Coefficient of determination
REV-ERBα  Reverse erythroblastosis virus α (also known as nuclear receptor subfamily 1 group D member 1: Nr1d1)
RNA  Ribonucleic acid
RNase  Ribonuclease
RORα  Retinoic acid receptor-related orphan receptor alpha (also known as nuclear receptor subfamily 1 group F member 1: Nr1f1)
RORE  Retinoic acid receptor-related orphan receptor response element
RP  Retroperitoneal
RPM  Revolutions per minute
s.c.  Subcutaneous
SCN  Suprachiasmatic nucleus
SDHA  Succinate dehydrogenase subunit A
SDS  Sodium dodecyl sulphate
sec  Second(s)
SEM  Standard error of the mean
SNAT  System A sodium-dependent amino acid transporter
SNP  Single nucleotide polymorphism
SRY  Sex-determining region Y
Std  Standard
TAE  Tris-acetate-EDTA
Tc  Core body temperature
TG  Triglyceride (also known as triacylglycerol; TAG)
TLR  Toll-like receptor
TNF  Tumour necrosis factor
V  Volts
vol  Volume
WAT  White adipose tissue
wk  Week(s)
w/v  Weight/volume
YWHAZ  Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
ZT  Zeitgeber time
*This and all subsequent protein abbreviations are written in capitals, as per conventional nomenclature. Corresponding human genes and mRNA molecules mentioned throughout this thesis will carry the same abbreviation in italics. Corresponding rodent genes and mRNA molecules mentioned throughout the thesis carry the same abbreviations in italics, with only the first letter capitalised. These abbreviations have not been listed here.
Publications arising from this work

Published articles


Submitted manuscripts


Abstracts published in conference proceedings

1. Dietary-induced obesity suppresses expression of Rev-erbα in fetal and placental tissues; implications for circadian and metabolic development; Endocrine Society of Australia and Endocrine and Reproductive Biology Society of Western Australia Joint Annual Symposium 2015, Perth, WA, Australia.

2. Dietary-induced obesity suppresses expression of Rev-erbα in fetal and placental tissues; implications for circadian and metabolic development; Society for Reproductive Biology Annual Scientific Meeting 2015, Adelaide, SA, Australia.


4. Dietary-induced obesity suppresses clock gene expression and alters rhythmic expression profiles in maternal metabolic tissues during rat gestation; Society for Reproductive Investigation 62nd Annual Scientific Meeting 2015, San Francisco, CA, USA.
5. Dietary induced obesity suppresses clock gene expression in maternal metabolic tissues during rat gestation; Society for Reproductive Biology Annual Scientific Meeting 2014, Melbourne, VIC, Australia.

6. Dietary induced obesity suppresses clock gene expression in maternal metabolic tissues during rat gestation; Endocrine and Reproductive Biology Society of Western Australia Annual Scientific Symposium 2014, Perth, WA, Australia.

7. Impact of obesity on the maternal circadian clock during pregnancy; Society for Gynaecologic Investigation 61st Annual Scientific Meeting 2014, Florence, Italy.

8. Obesity reduces maternal core body temperature and alters the normal thermoregulatory changes of late pregnancy in the rat; Australasian Chronobiology Society 10th Annual Scientific Meeting 2013, Adelaide, SA, Australia.

9. Obesity reduces maternal core body temperature and alters the normal thermoregulatory changes of late pregnancy in the rat; The Endocrine Society of Australia Annual Scientific Meeting 2013, Sydney, NSW, Australia.

10. Impact of maternal obesity on circadian rhythms during pregnancy; The Endocrine Society of Australia (ESA) Seminar Weekend 2013, Maroochydore, QLD, Australia.

11. Placental responses to obesity: Circadian status of the placenta in obese pregnancy; Endocrine and Reproductive Biology Society of Western Australia Annual Scientific Symposium 2012, Perth, WA, Australia.

12. Fetal and Placental Growth Restriction in Obese Pregnancy is not Linked to Placental Inflammation or ER Stress; International Federation of Placental Associations Meeting 2012, Hiroshima, Japan.
Chapter 1: Introduction

Obesity during pregnancy is a major health concern in modern Western societies. Obesity rates have been increasing in women of reproductive age (Fisher et al. 2013), such that approximately 20% of women enter pregnancy obese (Guelinckx et al. 2008). The prominence of maternal obesity has significant implications for maternal and fetal health, since obese women are at an increased risk for pregnancy disorders such as preeclampsia and gestational diabetes (Marchi et al. 2015), and are more likely to experience complications such as miscarriage and neonatal death (Aune et al. 2014). Furthermore, obese mothers are more likely to give birth to infants with altered fetal growth trajectories, including either overgrowth (Sebire et al. 2001) or undergrowth (Gardosi & Francis 2009). The latter, while seemingly counter-intuitive, is likely due to an obesity-induced impairment of placental function (Higgins et al. 2011). Importantly, the consequences of maternal obesity also extend to subsequent generations, since offspring born to obese pregnancies are predisposed to develop obesity and cardiovascular disease themselves in adulthood (Thornburg 2015; Blackmore & Ozanne 2013). Moreover, later generations are likely to be affected via epigenetic processes (Aiken & Ozanne 2014).

Despite the widespread understanding that obese pregnancy elicits lasting negative health outcomes in mothers and infants, the specific biological processes behind this are still poorly understood. An important, yet relatively unexplored topic in this regard is chronobiology. Circadian rhythms have evolved in order to optimise the timing and efficiency of biological processes in effectively all living organisms, and are regulated at a molecular level via the complex interactions of clock genes in most cells of the body (Reppert & Weaver 2002). While circadian rhythms are integral to numerous biological and physiological processes, the timing of energy intake and expenditure is vital for an organism’s survival; accordingly, circadian biology is closely integrated with metabolic function (Bass & Takahashi 2010). Obesity is widely considered to be a metabolic disease, and as such, circadian rhythm disturbances are implicated in many facets of its development and pathology (Froy 2010). Indeed, circadian disruption via shift work is associated with obesity onset and metabolic dysfunction in humans (Knutsson 2003), while clock gene knockout models strongly implicate the circadian molecular system in the control of body composition and metabolic phenotype (Turek et al. 2005; Kennaway et al. 2013). Moreover, diet-induced obesity in rodents alters behavioural rhythms and clock gene expression in metabolic tissues (Kohsaka et al. 2007; Mendoza, Pévet &
Challet 2008), highlighting the reciprocal relationship between metabolic and circadian dysfunction.

Much like obesity, pregnancy is an atypical metabolic state, and recent studies in our laboratory show marked changes in several aspects of maternal circadian biology (Wharfe et al. 2016). These changes are considered important to fulfil the substantial metabolic requirements of both the mother and fetus as it grows and develops (Wharfe et al. 2016). Furthermore, maternal circadian cues, such as rhythmic glucocorticoid secretion, relay important developmental signals that promote fetal circadian development, and thereby prepare the fetus for the postnatal environment (Serón-Ferré, Valenzuela & Torres-Farfan 2007). Accordingly, circadian disruption during pregnancy has negative consequences for maternal and fetal health; for example, shift workers have higher rates of pregnancy complications (Knutsson 2003). Furthermore, studies in rats show that disruption to light cycles during pregnancy results in an adverse maternal metabolic phenotype (Varcoe et al. 2013) and programmed metabolic dysfunction in offspring (Varcoe et al. 2011).

Despite evidence that obesity and pregnancy influence the circadian system separately, it is uncertain how obesity may alter the circadian nature of maternal physiology during pregnancy. Less still is known of how the fetal or placental circadian systems may respond to the insult of maternal obesity, and what consequences this may have for offspring health outcomes. Accordingly, the overall aim of this thesis was to characterise the impact of maternal obesity on key molecular features of the circadian regulatory system in maternal, placental and fetal tissues during pregnancy. In addition to the lack of understanding regarding circadian molecular control, there is also little evidence as to whether many well-documented physiological responses to obesity, such as endocrine function (e.g. glucocorticoids and leptin) or glucose and lipid metabolism, are altered in a circadian-dependent manner during obese pregnancy. Consequently, this thesis also investigated the rhythmic biology of several key systems that may contribute to the adverse pregnancy outcomes associated with maternal obesity.

The first experimental chapter (Chapter 5) describes what was effectively a pilot study conducted to optimise a model for maternal obesity using cafeteria (CAF) feeding in Wistar rats. The aim of this study was to determine whether CAF feeding could induce an obese phenotype prior to pregnancy, and whether fetal or placental growth would be altered by the consequent maternal obesity at day 21 of gestation (term is 23 days). Since obesity is typically associated with increased inflammation, this study also measured
circulating inflammatory markers and inflammatory gene expression across maternal, placental and fetal tissues in control (CON) and CAF animals at a single daily time-point.

The second experimental chapter (Chapter 6) aimed to determine whether maternal obesity influenced the daily patterns of fetal and placental growth, and the diurnal profiles of steroid hormones and lipid biomarkers in maternal and fetal plasma. Each of these factors are considered important regulators of fetal growth, are implicated in obesity pathogenesis, and are regulated by the circadian system. The study presented in Chapter 6 involved collection of samples from CON and CAF mothers at six 4 hourly time-points over day 15-16 and day 21-22 of gestation. This large-scale model allowed for rhythmic analyses in maternal, placental and fetal tissues across two days of gestation which span the period of maximal fetal growth in the rat.

The third experimental chapter (Chapter 7) measured rhythmic core body temperature ($T_c$) profiles in a subset of CON and CAF rats during the estrous cycle and throughout pregnancy. Since $T_c$ rhythms are regulated by central circadian pacemakers and maternal $T_c$ rhythms change during rodent pregnancy, this study aimed to determine whether pregnancy-induced adaptations of $T_c$ rhythms are disrupted by an obese maternal phenotype. This comprehensive $T_c$ analysis was also considered important to ascertain whether centrally-controlled rhythmic processes are disrupted by maternal obesity.

The final two experimental chapters describe studies that characterised the impact of maternal obesity on the rhythmic gene expression of peripheral metabolic tissues in maternal, placental and fetal compartments in the same cohort of animals from Chapter 6. The fourth experimental chapter (Chapter 8) investigated the effects of obesity on the daily expression profiles of core clock genes (the molecular regulators of the circadian system) in the maternal liver, placental labyrinth zone and fetal liver. Rhythmic glucose and insulin profiles were also analysed in maternal and fetal plasma, and the expression of genes involved in glucose and lipid metabolism were measured in maternal, placental and fetal tissues. Finally, the fifth experimental chapter (Chapter 9) analysed the rhythmic expression profiles of the core clock genes and several nuclear receptors known to integrate the circadian and metabolic systems, in maternal adipose tissue. Diurnal leptin profiles were also assessed in maternal and fetal plasma as a marker of rhythmic adipose tissue function.
Collectively, the studies in this thesis provide a characterisation of the rhythmic responses to maternal obesity in several key areas related to the pathogenesis of obese pregnancy. This analysis may highlight potential areas for further investigation that could be used to improve outcomes of obese pregnancies in humans.
Chapter 2: Literature Review

2.1 Obesity during pregnancy

Obesity during pregnancy is an increasingly common condition that has detrimental effects on maternal and offspring health. Around a third of reproductive aged women in the United States are obese (Ogden et al. 2012; Flegal et al. 2010), and approximately 20% of women in Western countries enter pregnancy obese (Fisher et al. 2013; Guelinckx et al. 2008). These figures are concerning because obese women are more likely to suffer from a range of pregnancy complications, including preeclampsia, gestational diabetes mellitus, miscarriage and pre-term birth (Nohr et al. 2005; Marchi et al. 2015). Moreover, infants of obese mothers are more likely to be born either large for gestational age (Catalano et al. 2012; Sebire et al. 2001) or growth restricted (Radulescu et al. 2013; Gardosi & Francis 2009). Given the importance of the intrauterine environment in programming long-term health outcomes (Barker 1995), the postnatal phenotype is often further compromised in these offspring. Indeed, offspring born to obese mothers are prone to obesity and other adverse cardiometabolic outcomes themselves in adulthood (Drake & Reynolds 2010; Dearden & Ozanne 2015), thus perpetuating an inter-generational cycle of metabolic disease.

The mechanisms behind the pregnancy complications and adverse programming outcomes associated with maternal obesity remain poorly understood. It is likely that excessive maternal adiposity in combination with altered nutritional cues disrupt the maternal hormonal and inflammatory milieu (Jansson et al. 2008; Pantham, Aye & Powell 2015). This in turn may compromise placental function by way of altered vascular formation, nutrient transport, or oxygen supply, and thereby result in disturbed fetal development (Higgins et al. 2011). Intriguingly, animal models demonstrate that offspring of obese mothers often exhibit similar phenotypes to those of malnourished mothers (Cunha et al. 2015; Ford & Long 2011), suggesting that maternal over- and undernutrition alter similar developmental pathways to elicit adverse programming outcomes.

2.1.1 Rodent models of obese pregnancy

Rodents are a popular tool for modelling maternal obesity, likely due to their ease of conception, relatively short gestation period and well documented reproductive biology (Suckow, Weisbroth & Franklin 2005). Maternal obesity is predominantly achieved by
dietary manipulation, however the diet composition and length of exposure is often inconsistent among studies, resulting in considerable variation in maternal and offspring phenotypes. Conventionally, the most popular approach to induce maternal obesity has been via the provision of semi-pure, high-fat (HF) diets. This dietary intervention is useful since a consistent macronutrient composition minimises experimental variation, and dietary fats are a major contributing factor to obesity development in humans (Bray, Paeratakul & Popkin 2004). This HF diet approach does have limitations, however, since no standard HF formula exists and there are often marked differences in the fat content and composition of diets, making comparison between studies difficult. Furthermore, rodents (particularly rats) often exhibit autoregulation of caloric intake when provided with HF diets (Taylor et al. 2003; Khan et al. 2005; Howie et al. 2009; Mark et al. 2011), such that HF diet consumption is reduced compared to the control diet, and significant weight gain is not always achieved. Indeed, the initial approach used in our laboratory resulted in autoregulation of food intake in HF-fed pregnant rats, and was thus not considered an appropriate model for maternal obesity (Fig 2.1).

Cafeteria (CAF) diets provide an alternative approach to overcome the autoregulatory response to HF feeding. The CAF model essentially mimics human obesity development, in that animals are given traditional Western style snack foods, that is, calorically dense food items with excessive fat and sugar content, usually in addition to standard rodent chow. The increased palatability and novelty of food items appears to play a major role in determining food intake in rodents; indeed, CAF-fed rats exhibit hyperphagia and subsequent weight gain more readily than their HF-fed counterparts (Sampey et al. 2011). This CAF-induced hyperphagia may be partly due to central leptin resistance and increased neuropeptide Y (NPY) sensitivity induced by HF, high-sugar combination diets, compared to HF or high-sugar alone (van den Heuvel et al. 2014). Moreover, providing a choice of food items appears to stimulate more prolonged hyperphagia in rats consuming HF, high-sugar diets (la Fleur et al. 2014), thereby leading to subsequent obesity development. CAF feeding can be disadvantageous, however, since it is difficult to accurately calculate the nutritional composition of the diet. Furthermore, because animals may exhibit individual preferences for certain food items, this could increase variability in outcomes. As such, the desirability of CAF over HF feeding may depend on experimental objectives.
Figure 2.1 Caloric autoregulation in pregnant rats maintained on a high-fat (HF) diet. Female Wistar rats fed a HF diet throughout gestation (19 KJ/g; 43% energy as fat; \( n=10 \)) consumed less food (in grams) than control animals maintained on a standard semi-pure diet (Std; 15 KJ/g; 12% energy as fat; \( n=9 \)). This resulted in a marginal increase in total daily caloric intake in the HF animals over the first three days of gestation only, but no significant difference in body weight between diet groups. \( \dagger P<0.01 \) overall diet effect (repeated measured ANOVA); \( * P<0.05 \) compared to Std (t-test following Diet x Gestational Day interaction in repeated measures ANOVA). Data are the mean ± SEM and are adapted from (Crew 2010).
2.1.2 Heterogeneous maternal and fetal responses to obesity

Obesity is a heterogeneous condition, in that its aetiology and symptoms exhibit substantial variability among individuals. This heterogeneity also extends to pregnancy; for example, it is recognised that obese women are at an increased risk for having either small (Gardosi & Francis 2009; Radulescu et al. 2013) or large (Marchi et al. 2015) for gestational age babies. Accordingly, maternal, placental and fetal physiological responses to obesity can vary substantially between individuals, populations or different animal models. For example, studies investigating the mammalian target of rapamycin (mTOR) signalling pathway, which regulates placental amino acid transport, have demonstrated that obesity is associated with increased placental mTOR signalling in conjunction with fetal overgrowth in humans (Jansson et al. 2013) and mice (Rosario, Powell & Jansson 2016). Conversely, other rodent models have reported unchanged fetal growth with reductions to placental mTOR signalling (Lager et al. 2014), or restricted fetal growth and no change to placental mTOR (Mark et al. 2011) in HF-fed mothers. These diverse findings indicate that the amino acid transfer capacity of the placenta during obese pregnancy may be dependent on individual population or model characteristics, and this likely extends to other areas of maternal, placental and fetal physiology.

In light of these differential responses to obesity, it is perhaps unsurprising that rodent models of obese pregnancy yield variable results in fetal growth phenotypes; these include fetal overgrowth (Bouanane et al. 2009; Jones et al. 2009; Rebholz et al. 2011; Rosario et al. 2015), fetal growth restriction (Bayol, Simbi & Stickland 2005; Akyol, Langley-Evans & McMullen 2009; Howie et al. 2009; Mark et al. 2011; Hayes et al. 2012) and unaltered fetal growth (Khan et al. 2005; Akyol, Langley-Evans & McMullen 2009; Elmes et al. 2011; Ong & Muhlhausler 2011). Factors that determine which growth trajectory a fetus undergoes are unknown, and could be partially due to subtle differences in individual experimental design. Notably, all of these models are likely to be relevant to some extent, since this range of fetal growth outcomes is also evident amongst obese human populations. Furthermore, several studies have demonstrated that placental dysfunction (Lager et al. 2014) and adverse programming outcomes in offspring (Varcoe et al. 2011; Samuelsson et al. 2008) can occur after gestational insults despite no significant alterations to fetal growth. As such, fetal weight is not an entirely reliable marker for dysfunctional development, and studies that show no overt changes to fetal growth trajectories after developmental insults remain valuable.
2.2 Circadian rhythms

Circadian rhythms are endogenous timing processes within biological organisms that have evolved in response to the light-dark cycle. The circadian system regulates daily oscillations in a range of physiological and behavioural functions, such that they occur at the optimal time within a 24 h period (Mohawk, Green & Takahashi 2012). Indeed, nearly all cell types exhibit some form of autonomous circadian function (Nagoshi et al. 2004; Yoo et al. 2004; Balsalobre, Damiola & Schibler 1998). Consequently, most aspects of physiology, including metabolism, endocrine activity and core body temperature, exhibit circadian rhythmicity at the cellular, organ, or whole body level (Mohawk, Green & Takahashi 2012; Reppert & Weaver 2002).

The circadian system is organized in a hierarchical manner; the master regulator of circadian function is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN, or ‘central clock’, is linked to photoreceptive retinal ganglion cells via the retinohypothalamic tract, and thus responds directly to external light cues (Schmidt, Chen & Hattar 2011; Quintero, Kuhlman & McMahon 2003). The SCN contains approximately 20,000 neurons which function as individual circadian oscillators. Collectively, these neurons coordinate the physiological rhythms of ‘peripheral clocks’ in other organs and tissues, such that their function is aligned to the photoperiod (Welsh, Takahashi & Kay 2010; Liu et al. 1997). Peripheral clocks exist in most organs, such as the liver, kidney and heart, to regulate localised circadian function. Since they cannot respond directly to the light-dark cycle, peripheral oscillators rely on centrally-controlled rhythmic cues, such as feeding patterns, glucocorticoid and melatonin secretion or body temperature fluctuations, to align with the external environment (Reppert & Weaver 2002; Schibler, Ripperger & Brown 2003). These cues are termed zeitgebers (or ‘time givers’) and are essential for overall synchronisation of the circadian system to the 24 h period. True circadian oscillations persist in the absence of external stimuli, but most rhythms will gradually alter their period if not entrained to the 24 h period by zeitgebers, a phenomenon known as ‘free running’ rhythms (Aschoff 1984).
2.2.1 The molecular clock

In mammals, circadian rhythms are controlled at a molecular level by clock genes, which include circadian locomotor output cycles kaput (Clock), brain and muscle Arnt like protein-1 (Bmal1), Period (Per) -1, -2 and -3 and Cryptochrome (Cry) -1 and -2. Clock and Bmal1 are basic helix-loop-helix PER ARNT SIM (bHLH-PAS) transcription factors, which regulate their own expression through a sequence of positive and negative transcriptional-translational feedback loops (Reppert & Weaver 2002).

The positive arm of this oscillatory loop occurs when CLOCK and BMAL1 proteins form a heterodimer which translocates to the nucleus and induces transcription of Per and Cry via binding to their E-box (5’-CACGTG-3’) and E-box-like promoter sequences, or by a range of other control elements, including CREB, HSF1, SRF, GR (Schibler et al. 2015). Once levels of PER and CRY proteins have accumulated sufficiently in the cytoplasm, they dimerize and the resultant PER:CRY complexes translocate to the nucleus and inhibit CLOCK:BMAL1 transcriptional activity, thereby acting as a negative regulator of Per and Cry transcription (Figure 2.2). Accessory arms add stability to this system, wherein the CLOCK:BMAL1 dimer also induces the transcription of two nuclear receptors, namely reverse erythroblastosis virus α (Rev-erbα) and retinoic acid receptor-related orphan receptor α (Rora). Rev-erbα inhibits Bmal1 transcription via binding to ROR response elements (RORE) on its promotor (Preitner et al. 2002), whereas Rora aids the positive arm of the system by competing with Rev-erbα for RORE binding and thus stimulating Bmal1 transcription (Sato et al. 2004) (Figure 2.2). Rev-erbα also represses Clock transcription (Crumbley & Burris 2011; Preitner et al. 2002), whereas Rora does not appear to directly influence Clock (Crumbley & Burris 2011). Clock also has a functional paralog, Npas2, which can bind to Bmal1 and function in the same capacity (Landgraf et al. 2016; DeBruyne, Weaver & Reppert 2007).

The timing of these core clock feedback loops is tightly regulated by numerous post-translational modifications that control the stability of clock proteins and the efficiency of their dimerization, cytosolic accumulation, and nuclear translocation (Lee et al. 2001). For example, casein kinase Iε phosphorylates PER proteins to enhance their degradation (Eide et al. 2005), and CRY protein stability is modulated by E3 ubiquitin ligase complexes (Yoo et al. 2013). As such, the period of this clock gene expression cycle is approximately 24 h, with peak levels of CLOCK and BMAL1 accumulating in antiphase to those of PER and CRY (Figure 2.2).
The molecular clock exerts control over specific biological functions by regulating the expression of many clock-controlled genes (namely through E-box motifs on their promoters), which themselves are transcription factors for downstream metabolic and physiological pathways (Bozek et al. 2009). As such, it is estimated that between 5 and 20% of the transcriptome exhibits circadian rhythmicity, depending on the tissue type in question (Froy 2010).
Figure 2.2 Core clock machinery. Schematic representation of the positive and negative transcriptional and translational feedback loops of the molecular clock. This molecular feedback system is present within individual cellular oscillators in most organs and tissues of the body, and acts to drive rhythmic physiological functions via clock controlled genes.

← Stimulation  ↓ Inhibition
2.2.2 Central signals entrain peripheral clocks

The SCN entrains peripheral clocks via numerous signalling mechanisms, among which are the rhythmic control of central endocrine outputs and the regulation of core body temperature \((T_c)\) oscillations (Mohawk, Green & Takahashi 2012). Of the central endocrine signals, variations in glucocorticoid and melatonin secretion are particularly important for rhythmic entrainment, and are discussed below.

2.2.2.1 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a hierarchical endocrine feedback system that controls the adrenal synthesis of glucocorticoids, a group of steroid hormones critical in the regulation of the stress response and important metabolic functions (Stratakis & Chrousos 1995). Briefly, corticotrophin releasing hormone (CRH) is released by the paraventricular nucleus (PVN) of the hypothalamus in response to stress signals and other circadian stimuli from the SCN (Kolbe, Dumbell & Oster 2015; Stratakis & Chrousos 1995). CRH reaches the pituitary via the portal vessels, where it stimulates the production of adenocorticotropic hormone (ACTH), which induces glucocorticoid production in the adrenal cortex. Circulating glucocorticoids (primarily cortisol in humans and corticosterone in rodents) elicit their effects via interactions with the glucocorticoid receptor (GR) in target tissues, and promote overall HPA axis homeostasis by inducing negative feedback at the PVN or pituitary level, thus inhibiting the original CRH and/or ACTH production (Chung, Son & Kim 2011).

Glucocorticoid secretion occurs in a circadian manner, with peak levels evident immediately prior to the active phase (i.e. early morning for humans, early evening for rodents) (Kalsbeek et al. 2012; Chung, Son & Kim 2011). This circadian regulation is tightly controlled by autonomous links between the SCN, PVN and adrenal cortex (Buijs et al. 1999; Ishida et al. 2005), and by clock gene expression in the adrenal cortex (Oster et al. 2006). As such, glucocorticoid rhythms are robust indicators of circadian function within an organism (Kalsbeek et al. 2012). Regulation of the glucocorticoid rhythm is crucial, since glucocorticoids are potent entrainers of peripheral oscillators. Synthetic glucocorticoids induce phase changes to clock gene expression in cultured fibroblasts, and \textit{in vivo} in tissues such as the liver, kidney and heart (Balsalobre et al. 2000; Pezük et al. 2012). This regulation of clock gene transcription is facilitated by the presence of glucocorticoid-response elements (GREs) in clock gene promoter regions (Reddy et al.
2007). Interestingly, the SCN does not express glucocorticoid receptors, so these feedback effects do not extend to the central clock (Rosenfeld et al. 1988).

### 2.2.2.2 Melatonin

Melatonin is a nocturnally secreted hormone, synthesised by the conversion of serotonin in the pineal gland (Kennaway & Wright 2002). Pineal melatonin synthesis is driven by neuronal outputs from the SCN and is inhibited by light; as such, melatonin exhibits a distinct circadian secretion pattern, with peak levels occurring nocturnally in both diurnal and nocturnal species (Pevet & Challet 2011). This nocturnal melatonin secretion is an important temporal cue for peripheral tissues expressing the melatonin receptor. Loss of melatonin rhythmicity by pinealectomy disrupts peripheral clock gene rhythms in the liver, duodenum and adipose tissue (Houdek et al. 2016; de Farias et al. 2015). Melatonin also entrains clock gene rhythmicity in the adult (Torres-Farfan et al. 2006b) and fetal (Torres-Farfan et al. 2006a) adrenal gland, suggesting that it may also influence peripheral clocks indirectly via regulation of glucocorticoid synthesis. Interestingly, while pinealectomised rats exhibit suppressed hepatic and duodenal clock gene expression after one year, they retain rhythmicity in feeding and locomotor behaviour (Houdek et al. 2016). This reinforces the concept that multiple circadian signals integrate to collectively regulate rhythmic function. As such, individual signals may exhibit a degree of redundancy when the rest of the circadian signalling system remains intact. In this context, it is noteworthy that C57BL6 mice (and most other laboratory mouse strains) are deficient in melatonin (Ebihara et al. 1986), yet retain behavioural and physiological rhythmicity (Stehle, von Gall & Korf 2003).

Since the SCN expresses melatonin receptors, melatonin also exerts direct feedback to the central clock, making it a potential drug target for re-entrainment of the circadian system following chronodisruption (Pevet & Challet 2011). Moreover, melatonin functions in numerous other important capacities; it regulates the sleep-wake cycle (Cajochen, Kräuchi & Wirz-Justice 2003), acts as an antioxidant (Reiter 2000) and anti-inflammatory agent (Bruck et al. 2004), and regulates core body temperature (Cagnacci, Elliott & Yen 1992). Thus, disrupted melatonin signalling can lead to a range of metabolic complications (Mühlbauer et al. 2009; de Farias et al. 2015; Peschke 2008).

### 2.2.2.3 Core body temperature (Tc) rhythms

The SCN regulates daily rhythms in core body temperature (Tc) via interaction with hypothalamic thermoregulatory centres (Saper, Scammell & Lu 2005) such that Tc is
lowest during the inactive period (i.e. during the light phase for rodents and nocturnally for humans) (Refinetti & Menaker 1992). These circadian Tc oscillations adjust peripheral clock function accordingly. For example, minor Tc fluctuations rapidly entrain circadian oscillations in fibroblast, liver, lung and pituitary function (Saini et al. 2012; Brown et al. 2002; Kornmann et al. 2007; Buhr, Yoo & Takahashi 2010). Moreover, recent evidence indicates that the SCN communicates with the arcuate nucleus to govern Tc rhythmicity (Guzmán-Ruiz et al. 2015). This implies that circadian temperature regulation is linked to metabolism, which supports findings that altered energy intake moderates Tc rhythmicity in rodent and sheep models (Mendoza, Pévet & Challet 2008; Maloney et al. 2013; Goh, Mark & Maloney 2016).

2.3 Circadian control of metabolism

Circadian rhythms govern a range of biological processes, but are particularly vital for metabolic function. The intricate relationship between the circadian and metabolic systems optimises the timing of metabolic processes, which is crucial for successful energy homeostasis. This connection occurs at a central level, since SCN projections extend to the arcuate nucleus and thus allow direct interaction between the central clock and hypothalamic centres controlling food intake and energy expenditure (Yi et al. 2006). The relationship is also maintained at the peripheral level, since metabolic tissues (such as the liver and adipose tissue) express clock genes with robust rhythmic profiles, and thus display highly rhythmic functions (Zanquetta et al. 2010; Ferrell & Chiang 2015). The circadian function of these peripheral metabolic tissues is regulated by neurohumoral signals from the SCN and other central outputs controlled by the SCN, including core body temperature oscillations, temporal feeding patterns, and rhythmic endocrine functions (Schibler, Ripperger & Brown 2003).

Importantly, the relationship between the circadian and metabolic systems is highly reciprocal in nature. The circadian clock exerts fundamental control over a range of metabolic pathways, but metabolic signals can also directly moderate rhythmic output (Bass & Takahashi 2010; Froy 2010). Indeed, temporal food intake patterns rapidly entrain rhythmicity in peripheral metabolic tissues (Vollmers et al. 2009; Damiola et al. 2000), and specific nutritional components can alter central and peripheral clock gene expression (Froy 2007) (Figure 2.3). Moreover, while metabolic hormones such as insulin, leptin and adiponectin all exhibit strong circadian secretion patterns (Barnea et al. 2015; Kalsbeek et al. 2001; Sadacca et al. 2010), these hormones also act on the SCN
to signal feeding times and adjust central circadian output accordingly (Challet 2015). As such, metabolic disruptions, including those caused by obesity or altered feeding regimes, lead to various forms of circadian dysfunction (Froy 2010). Conversely, circadian disturbances (for example, via shift work or excessive light exposure at night) are associated with metabolic disease and obesity development (Knutsson 2003; Fonken & Nelson 2014).

2.3.1 Nuclear receptors link the circadian clock to metabolism

From a molecular standpoint, the intricate relationship between the circadian and metabolic systems is largely facilitated by nuclear receptors. These are a family of transcription factors whose ligands are small molecules such as steroid hormones, fatty acids and other dietary metabolites. As such, nuclear receptors function as sensory molecules to align metabolic tissue function with external stimuli (Chawla et al. 2001). Importantly, nuclear receptors are also closely linked to the circadian system, and therefore communicate metabolic input to the circadian clock. Indeed, 20 of 41 nuclear receptors expressed in the mouse liver, and 19 of 42 expressed in white adipose tissue exhibit rhythmic expression profiles (Yang et al. 2006).

2.3.1.1 Rev-erbα and Rorα

The nuclear receptors Rev-erbα and Rorα form an integral part of the clock machinery; as discussed in Section 2.2.1, their transcription is stimulated by the CLOCK:BMAL1 heterodimer, and they act in turn to modify Bmal1 transcription by binding to ROR response elements (RORE) on its promotor region (Preitner et al. 2002; Sato et al. 2004) (Figure 2.2 & 2.3). As well as these direct roles in the molecular clock network, Rev-erbα and Rorα have widespread metabolic effects, including regulation of lipid metabolism in the liver and adipose tissue. As such, they are considered key factors linking the circadian clock to metabolism (Duez & Staels 2008; Solt, Kojetin & Burris 2011). The functions of Rev-erbα include promotion of adipocyte differentiation in white (Chawla & Lazar 1993; Fontaine et al. 2003) and brown (Nam et al. 2015) adipose tissue, regulation of core body temperature rhythmicity via brown adipose tissue-regulated pathways (Gerhart-Hines et al. 2013), stimulation of the NF-κB inflammatory signalling pathway (Migita, Morser & Kawai 2004), and control of pancreatic β cell function (Vieira, Merino & Quesada 2015). Rorα often elicits opposing actions to those of Rev-erbα, repressing adipocyte differentiation (Ohoka et al. 2009) and inflammation (Delerive et al. 2001).
Interestingly, a recent report suggests that while Rev-erbα elicits control over clock genes via Rorα-competitive binding to RORE, it controls downstream metabolic pathways by a separate molecular mechanism involving the HDAC3 corepressor (Zhang et al. 2015). This indicates that Rev-erbα may regulate core clock gene rhythms independently of its tissue-specific modulation of metabolic functions.

2.3.1.2 Peroxisome proliferator-activated receptors

The peroxisome proliferator-activated receptors (PPARs) and their co-regulator, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1α) are another group of nuclear receptors closely aligned with the circadian clock. The PPAR isoforms are important regulators of metabolic function; Ppara functions mostly in the liver to stimulate hepatic fatty acid oxidation, while Pparγ is prominently expressed in adipose tissue where it regulates adipogenesis and lipid storage (Smith 2002). Pparδ is more ubiquitously expressed, but plays a particularly important role in stimulating hepatic glucose metabolism (Bojic & Huff 2013). Importantly, the three PPAR isoforms (Ppara, Pparδ and Pparγ) also exhibit rhythmic expression patterns (Yang et al. 2006); PPARα contains E-boxes and E-box like motifs, which enables CLOCK and BMAL1 to stimulate its rhythmic transcription (Oishi, Shirai & Ishida 2005). Similarly, PPARγ contains an E-box motif that enables transcriptional control by SREBP-1 and SREBP-2 (Fajas et al. 1999). In addition to E-box-dependent transcriptional control, the rhythmic expression of all PPAR isoforms is likely induced via hormonal mechanisms and transcriptional regulation by other clock controlled genes (Chen & Yang 2014).

In addition to being expressed in a circadian manner and controlling many critical metabolic functions, the PPARs and Pgc1α also exert functional control over the circadian clock itself by binding to peroxisome proliferator-activated receptor response elements (PPREs) on the Bmal1 promoter (Figure 2.3). Accordingly, Ppara, Pparγ and Pgc1α can directly stimulate Bmal1 expression (Canaple et al. 2006; Liu et al. 2007; Wang et al. 2008), while Ppara and Pparγ also regulate Rev-erbα transcription (Fontaine et al. 2003; Gervois et al. 1999). The role of Pparδ in the control of the molecular clock is less certain, although a recent report in zebrafish by Kopp et al. (2016) found no E-box response elements (i.e. the binding site for CLOCK:BMAL), but three Rev-erbα binding sites on the Pparδ promoter, suggesting that Rev-erbα is central in moderating Pparδ transcription (Kopp et al. 2016). Indeed, another study noted a positive correlation
between *PPARδ* and *BMAL1* expression in leukocyte mRNA transcripts of pregnant women (Pappa et al. 2013), and further evidence suggests that *Pparδ* is essential for circadian variation in hepatic lipogenesis (Liu et al. 2013). This indicates that *Pparδ* has direct functional links to circadian molecular regulation.
Figure 2.3 Circadian control of metabolism. Schematic representation of the interactions between the central clock and peripheral metabolic oscillators in the liver and adipose tissue. Central signals regulated by the SCN and external nutritional cues influence clock gene expression in metabolic tissues via the action of nuclear receptors. Clock genes then further influence the expression of these nuclear receptors and other clock controlled genes (CCG’s), resulting in rhythmic function in metabolic tissues. This rhythmic metabolic output feeds back to the central clock, ensuring a reciprocal relationship between the circadian and metabolic systems.
**2.3.2 Adipose tissue as a rhythmic organ**

Adipose tissue is an essential metabolic organ which regulates energy homeostasis via several mechanisms, including its direct roles in lipid metabolism. Adipose tissue function is linked to the circadian system, such that between 7 and 21% of the transcriptome in murine white adipose tissue exhibits circadian rhythmicity (Ptitsyn *et al.* 2006), while 25% of genes in human subcutaneous adipose tissue biopsies exhibit time of day variation (Loboda *et al.* 2009). Clock genes are rhythmically expressed in adipose tissue (Zvonic *et al.* 2006) and are essential factors in governing adipocyte function; indeed, adipocyte differentiation is regulated by Bmal1 (Shimba *et al.* 2005), while global loss of Bmal1 in mice results in increased adiposity, arrhythmic adipose tissue expression of clock and metabolic genes, and disruption to circulating adipokine profiles (Kennaway *et al.* 2013). Additionally, Pparγ transcription, which is vital for regulation of adipogenesis and adipose tissue lipid metabolism, exhibits circadian oscillations (Yang *et al.* 2006), and regulates clock gene expression. For example, PPARγ activation induces Bmal1 (Wang *et al.* 2008) and Rev-erbα transcription (Fontaine *et al.* 2003), which stimulates adipocyte differentiation, while administration of the synthetic REV-ERBα ligand SR6452 promotes adipogenesis in a similar manner to the PPARγ ligand, rosiglitazone (Kumar *et al.* 2010). In contrast to the stimulatory effects of REV-ERBα, RORα inhibits PPARγ binding and represses adipogenesis (Ohoka *et al.* 2009).

This circadian regulation of adipose tissue function allows for daily oscillations in lipid metabolism; lipidomics analysis reveals that approximately 13% of circulating lipid metabolites exhibit rhythmic daily profiles in healthy men (Chua *et al.* 2013). Studies in mice demonstrate that mobilisation of lipids from white adipose tissue (WAT) occurs in a circadian manner, and that this process is impaired after global loss of Clock and Bmal1. These clock gene deficient mice exhibit accumulation of triglycerides in WAT and disrupted circulating free fatty acid and glycerol rhythms (Shostak, Meyer-Kovac & Oster 2013). In another example of the bi-directional relationship between the circadian and metabolic systems, circulating lipids can also elicit functional control over the circadian clock by acting as ligands for PPARs (Section 2.3.1.2). Consequently, hyperlipidaemia may contribute to disrupted circadian rhythms in obese individuals (Adamovich, Aviram & Asher 2015).

Adipose tissue also functions in an endocrine capacity, regulating metabolic hormone and inflammatory cytokine secretion (Kershaw & Flier 2004). Consequently, appetite-
regulating adipokines such as leptin and adiponectin exhibit periodic oscillations in their secretion patterns (Gavrila et al. 2003; Zvoníc et al. 2006), and these oscillatory profiles are altered in clock gene mutants (Kennaway et al. 2012; Kennaway et al. 2013). Accordingly, the adipose tissue clock may influence rhythmic feeding behaviour by moderating the activity of hypothalamic appetite regulatory centres. Consistent with this idea, mice with an adipocyte-specific loss of Bmal1 exhibit increased daytime food intake and corresponding obesity development (Paschos et al. 2012).

The expression of the glucocorticoid receptor (GR) is also rhythmic in rat adipose tissue, with peak transcription levels occurring around the onset of the active phase (i.e. the dark photoperiod for rats) (Sukumaran et al. 2010). This expression profile corresponds to the circulating corticosterone peak in rats (Almon et al. 2008; Atkinson & Waddell 1997), and the early morning (i.e. start of the active period) cortisol peak in humans (Chung, Son & Kim 2011), indicating a possible role for the adipose tissue clock in the regulation of circadian glucocorticoid function.

2.3.2.1 Leptin

Leptin is a peptide hormone produced by adipocytes which communicates with hypothalamic neurons to suppress food intake and stimulate energy expenditure (Jéquier 2002). A product of the Lep gene, leptin elicits its appetite-regulatory effects by binding to receptors predominantly located the arcuate nucleus of the hypothalamus; this regulates the production of neuropeptides involved in appetite control and energy balance. As such, leptin moderates energy homeostasis by communicating between peripheral energy stores and the CNS (Jéquier 2002).

Leptin levels are proportional to the amount of adipose tissue within an organism (Maffei et al. 1995) and also exhibit postprandial increases, possibly due to insulin-mediated stimulation of leptin production, although this mechanism remains controversial (Tsai et al. 2012). Because obese individuals have elevated leptin levels but do not show suppressed appetite, obesity is considered a state of leptin resistance (Sáinz et al. 2015; Jéquier 2002). The leptin deficient ob/ob and leptin receptor deficient db/db mouse models provide useful tools for studying the effects of leptin in intact biological systems; these mice exhibit hyperphagia, obesity, and other symptoms associated with the metabolic syndrome (Coleman 1978).
2.3.2.2 Circadian functions of leptin

Leptin exhibits rhythmic daily secretion patterns, peaking nocturnally in humans (Saad et al. 1998; Heptulla et al. 2001; Yildiz et al. 2004) and rodents (Kalsbeek et al. 2001; Cano et al. 2009; Ando et al. 2005). Interestingly, leptin rhythmicity is maintained under altered feeding regimes, but is lost by ablation of the SCN (Kalsbeek et al. 2001). Furthermore, a recent study by Kettner et al. (2015) demonstrated that the CLOCK:BMAL1 heterodimer controls C/EBPα-mediated transcription of leptin in adipose tissue, such that it follows a similar transcription pattern to that of Per and Cry mRNA. Moreover, circadian disruption via simulated jet-lag disrupts rhythmic leptin production independently of feeding cues or physical activity (Kettner et al. 2015). Taken together, this evidence suggests that central and peripheral clocks interact to govern rhythmic leptin secretion independently of feeding patterns.

Leptin also displays circadian-regulating properties, with leptin receptors expressed in the SCN of both humans (Couce et al. 1997) and rats (Guan et al. 1997). Leptin treatment induces a phase advance in neuronal activity of cultured rat SCN tissue (Prosser & Bergeron 2003) and modulates SCN clock gene expression in response to irregular light exposure in female C57BL/6 mice (Mendoza et al. 2011). Another recent study demonstrated that leptin administration in ob/ob mice normalised their response to light changes, but suggests that leptin may moderate circadian rhythms by indirect stimulus to the SCN via the medial hypothalamus (Grosbellet et al. 2015). Given its bidirectional relationship to the circadian system and its control over energy homeostasis, leptin is likely a major factor connecting the circadian clock to metabolic function, and could therefore be involved in the circadian disruption associated with obesity and metabolic disease.

2.3.3 Rhythmic hepatic function: implications for glucose homeostasis

The liver is also a crucial metabolic organ that functions in close alignment with the circadian clock. The liver displays robust clock gene expression profiles, which regulate clock-controlled genes that influence numerous metabolic processes (Oishi et al. 2003). As such, approximately 9% of the hepatic transcriptome (Vollmers et al. 2012; Akhtar et al. 2002) and up to 20% of the hepatic proteome (Reddy et al. 2006) exhibit rhythmic variation in the mouse. Accordingly, the hepatic regulation of glucose, lipid, and bile acid metabolism occurs in a circadian-dependent manner (Ferrell & Chiang 2015).
The liver is a central regulator of glucose homeostasis; it takes up excess circulating glucose and stores it as glycogen in periods of hyperglycaemia. In periods of hypoglycaemia, the liver produces glucose via gluconeogenesis, or mobilisation of existing glycogen stores by glycogenolysis. As such, the circadian regulation of hepatic glucose metabolism is particularly critical for overall energy balance across daily feeding-fasting periods, and enables the maintenance of adequate blood glucose levels over the inactive period when food consumption is less likely. Consequently, hepatic glycogen content, gluconeogenesis and glycogenolysis are moderated according to the circadian cycle (Kalsbeek, la Fleur & Fliers 2014). The hepatic clock works in conjunction with autonomous stimulation from the SCN (Cailotto et al. 2005; la Fleur et al. 2001) and rhythmic pancreatic secretion of insulin and glucagon (Sadacca et al. 2010; Vieira, Merino & Quesada 2015) to achieve overall glucose homeostasis across the day. Rhythmic feeding patterns also entrain hepatic function, although some aspects of hepatic rhythmicity are still maintained even in the absence of food intake cues (Vollmers et al. 2009). Indeed, circulating glucose and insulin levels exhibit periodic oscillations that exist independently of feeding rhythms in humans and rodents, with glucose levels maximal just before the onset of the active period (Kalsbeek & Strubbe 1998; Van Cauter, Polonsky & Scheen 1997), while postprandial glucose tolerance is higher in the morning and declines over the day in humans (Saad et al. 2012).

A functional hepatic clock is required for overall glucose homeostasis, since mice with a liver-specific loss of Bmal1 exhibit hypoglycaemia during the fasting phase of the daily cycle, and abolished hepatic rhythmicity of genes regulating glucose metabolism, such as glucose transporter 2 (Glut2) (Lamia, Storch & Weitz 2008). Moreover, various aspects of the metabolic syndrome, such as diabetes and obesity, seem to disrupt the hepatic clock. Several studies have reported altered clock gene expression in the liver of HF-fed rodents (Kohsaka et al. 2007; Hsieh et al. 2009) (see Section 2.4.2.2 for further details), and this effect potentially extends to humans, since obese individuals do not exhibit the typical decrease in glucose tolerance across the day that is present in healthy controls (Lee et al. 1992). Furthermore, obese subjects actually experienced an increase in glucose tolerance from morning to evening (Van Cauter et al. 1994), suggestive of an inversion of diurnal glucose tolerance with obesity, which could be indicative of arrhythmic hepatic or pancreatic clock function.

Although the hepatic clock maintains a small amount of endogenous rhythmicity in the absence of rhythmic feeding (Vollmers et al. 2009), it is highly reliant on feeding cues
Circadian adaptations to obese pregnancy

for entrainment. Hepatic clock gene expression is altered within an hour of food consumption, whereas other peripheral oscillators do not exhibit such a rapid entrainment (Oike et al. 2011). Thus, when consumption of standard chow is restricted to the inactive (light) photoperiod in rodents, the phase of hepatic clock gene expression rapidly shifts by 10-12 h, whereas SCN rhythms remain largely entrained to the light-dark cycle (Stokkan et al. 2001; Hara et al. 2001; Damiola et al. 2000). This central-peripheral clock misalignment induces various aspects of metabolic dysfunction, such as obesity, diabetes and hyperlipidaemia (Mukherji et al. 2015). Interestingly, a recent study found that PPARα and glucagon are key regulators of this hepatic clock gene response to time-restricted feeding (Mukherji, Kobiita & Chambon 2015). Specifically, Mukerji and colleagues reported that restriction of feeding to the inactive (light) phase in mice led to hypoinsulinaemia and subsequent increased free fatty acid (FFA) levels during the active (dark) phase. Because FFAs act as ligands for hepatic PPARα, the resultant increase in PPARα activation altered Rev-erba transcription. This shift in Rev-erba rhythmicity then suppressed Bmal1 transcription via RORE binding, and led to phase shifts in hepatic clock gene expression (Mukherji, Kobiita & Chambon 2015). Glucagon levels were also increased by time-restricted feeding, which led to cAMP response element binding protein (CREB)-mediated activation of hepatic Per1 and Per2 expression (Mukherji, Kobiita & Chambon 2015). A subsequent study by the same group revealed that the phase-inversion between central and peripheral clocks in time-restricted feeding models occurs because, unlike the liver, the SCN does not express either glucagon receptors or PPARα (Mukherji et al. 2015) and thus is unresponsive to these phase shifts.

2.4 Circadian rhythms and obesity

2.4.1 Circadian dysfunction leads to obesity development: evidence from humans and animal models

Obesity is widely considered to be a metabolic disease, consistent with it being a key component of the metabolic syndrome. Excessive visceral adiposity disrupts the overall metabolic phenotype by inducing hyperglycaemia, insulin resistance, systemic and tissue-specific inflammation, and altered circulating adipokine profiles, amongst other complications (Despres & Lemieux 2006). Given the fundamental associations between circadian rhythms and metabolic function (Section 2.3), many of the pathological symptoms of obesity could be facilitated by chronodisruption. Accordingly, shift workers have higher rates of obesity, cardiovascular disease and other metabolic complications
Circadian adaptations to obese pregnancy

(Knutsson 2003), while reduced sleep quantity is associated with increased BMI (Dashti et al. 2015; Cappuccio et al. 2008), suggesting that circadian disturbances facilitate obesity development. Moreover, various clock gene polymorphisms have been linked to metabolic disease states in humans; for example, single nucleotide polymorphisms (SNPs) that reduce transcription from the BMAL1 locus have been associated with hypertension and type II diabetes (Woon et al. 2007), while distinct DNA methylation patterns in CpG sites of CLOCK, BMAL1 and PER2 are associated with obesity and metabolic syndrome incidence, suggesting that epigenetic mechanisms regulate the circadian control of the metabolic phenotype (Milagro et al. 2012).

The influence of circadian disruption on key metabolic pathways is also clearly evident in clock gene knockout models, which display markedly altered metabolic phenotypes. For example, mice with a global loss of Clock expression exhibit altered daily feeding patterns, obesity, and hormonal imbalances consistent with the metabolic syndrome, including hyperglycaemia and hyperleptinaemia (Turek et al. 2005). Bmal1 global knockout mice have decreased body weight (Shimba et al. 2011; Kennaway et al. 2013) but increased adiposity (Kennaway et al. 2013) relative to wild type controls, in conjunction with elevated plasma triglycerides and free fatty acids (Rudic et al. 2004; Kennaway et al. 2013; Shimba et al. 2011). Furthermore, global loss of Per2 results in attenuated diurnal feeding rhythms and subsequent obesity development (Yang et al. 2009). These results provide compelling evidence for the importance of a functional circadian system in the maintenance of body composition and metabolic homeostasis.

Interestingly, while global loss of Clock results in an obese phenotype (Turek et al. 2005), ClockΔ19 + MEL mice, which exhibit a loss of peripheral rhythmic function but retain intact central SCN and pineal rhythmicity (Kennaway et al. 2003; Kennaway et al. 2006), do not become obese. This is despite exhibiting decreased glucose tolerance and abolished rhythmicity in genes that regulate hepatic glucose metabolism (Kennaway et al. 2007). The ClockΔ19 + MEL model was developed from the melatonin-proficient CBA mouse strain, because the standard C57BL6J background strain (like most other laboratory mice) lacks melatonin (Ebihara et al. 1986), and thus any circadian disruptions present may be attributed to the absence of melatonin signalling rather than disrupted clock gene expression per se (Kennaway et al. 2003). ClockΔ19 + MEL mutants produce endogenous melatonin but maintain a peripheral loss of circadian function, and actually have decreased levels of circulating free fatty acids, increased adiponectin and enhanced insulin sensitivity relative to wild type controls (Kennaway et al. 2007). Moreover, they
respond better to HF feeding, exhibiting lower glucose, leptin and free fatty acid levels, and show no change in weight gain relative to wild type animals maintained on the same HF diet (Kennaway et al. 2012). This highlights the complex control that peripheral and central clocks exert over metabolic phenotype, and reinforces the importance of genetic background in clock gene manipulation studies.

Aside from gene manipulation studies, environmental modifications further implicate chronodisruption in the regulation of metabolic disturbances. For example, rodents and sheep exposed to altered light cycles, designed to mimic shift work or jet lag, exhibit disrupted expression of clock and metabolic genes in tissues such as the hypothalamus, liver and skeletal muscle, in conjunction with metabolic aberrations such as obesity and altered glycaemic control (Herrero et al. 2015; Varcoe et al. 2014; Fonken & Nelson 2014). Interestingly, the provision of a HF diet in combination with manipulations to the external photoperiod exacerbates metabolic disturbances in rodents (Qian et al. 2015; Coomans et al. 2013). This suggests that excessive caloric intake in combination with modern lifestyle factors such as shift work, travel between time zones, or excessive exposure to light at night, likely contribute to the high incidence of obesity and the metabolic syndrome in modern Western populations.

### 2.4.2 Disruption of circadian rhythms by obesity

As discussed above, chronodisruption alters metabolic phenotype and leads to obesity development, however the bi-directional relationship between the circadian and metabolic systems (Section 2.3) means that obesity and its associated metabolic complications may also alter circadian function.

#### 2.4.2.1 Clock gene disruptions in obese humans

Several studies show that adipose tissue clock gene expression is disrupted in obese humans, but given the intrinsic difficulty in obtaining serial tissue samples, results are often conflicting. One study reported that PER2 expression in omental adipose tissue was inversely correlated to waist circumference, while BMAL1, PER2 and CRY1 expression in subcutaneous adipose tissue was inversely correlated to cholesterol and low density lipoprotein (LDL) levels in obese individuals (Gomez-Abellan et al. 2007). Similarly, a more recent study noted that weight loss in overweight humans increased the expression of PER2 in subcutaneous adipose tissue (Pivovarova et al. 2016). A limitation of these studies was that clock gene expression was measured only at a single time-point. Otway et al. (2011) performed serial subcutaneous adipose tissue samples and found no
difference in clock gene expression between lean and obese individuals, while another study noted a positive correlation between BMI and rhythmic clock gene expression in visceral adipose tissue explants from obese women (Vieira et al. 2014). The inconsistencies amongst these studies may be due to the limited nature of single time-point analyses, the in vitro approach by Viera et al. (2014), and differences in clock gene rhythmicity between subcutaneous and visceral adipose tissue (Gómez-Santos et al. 2009). Differential clock gene responses to obesity between these adipose tissue subtypes would not be surprising, given that increased visceral adipose tissue is strongly associated with metabolic disease, while elevated subcutaneous adipose tissue conveys relatively little metabolic risk (Després et al. 2008).

2.4.2.2 Diet-induced obesity models

Rodent models of diet-induced obesity are particularly valuable for characterising the circadian responses to obesity, since they allow for rhythmic sampling of key metabolic tissues and comprehensive analysis of behavioural rhythms in controlled environments. Diet-induced obesity may alter central circadian output, since HF-fed mice experience slower re-entrainment of rhythmic functions after light cycle shifts, including delayed recovery of core body temperature and physical activity rhythms (Mendoza, Pévet & Challet 2008). This could be facilitated by SCN adaptations, since this study also noted reduced c-FOS induction in the SCN of HF-fed mice after nocturnal light pulses (Mendoza, Pévet & Challet 2008). Although diet-induced and genetic obesity both alter clock gene expression in the caudal brainstem (Kaneko et al. 2009), hypothalamic clock gene expression is not affected in HF-fed mice, despite altered feeding and locomotor behaviour in these animals (Kohsaka et al. 2007).

HF feeding also alters peripheral tissue rhythmicity; several studies have reported attenuation of rhythmic clock gene expression in the liver and adipose tissue of HF-fed mice (Kohsaka et al. 2007; Prasai et al. 2013; Eckel-Mahan et al. 2013). Conversely, subtle increases in hepatic and adipose clock gene expression were induced by HF feeding in female mice, although no account was taken of estrous cycle stage in this study (Yanagihara et al. 2006). Other studies which reported increased hepatic and adipose tissue clock gene expression in HF-fed animals only conducted analysis at one (Miranda et al. 2013) or two (Hsieh et al. 2009) daily time-points. As such, they provide no information on rhythmic (i.e. phase or amplitude) changes in clock gene profiles. A comprehensive study by Eckel-Mahan and colleagues (2013) indicates that such rhythmic changes are prominent with diet-induced obesity; of 2,828 rhythmic hepatic transcripts
analysed in mice fed a HF diet for 10 weeks, they observed that 27% of transcripts were rhythmic in both control and obese groups, and among these, 62% exhibited reduced amplitude in the HF group. Moreover, around two thirds of transcript rhythms were phase-shifted by HF feeding, with the majority (79%) exhibiting a phase-advance; this included a minor attenuation and phase advance in hepatic Bmal1 and Per2 expression (Eckel-Mahan et al. 2013).

2.4.2.3 Nutritional components entrain rhythmic function: Implications for diet-induced obesity models?

Specific nutritional components such as glucose, amino acids, sodium, ethanol, and caffeine, can induce changes to clock gene expression and other rhythmic functions in central and peripheral clocks (Froy 2007). This is relevant for diet-induced obesity models, since glucose administration (Iwanaga et al. 2005) and high fat diet consumption (Eckel-Mahan et al. 2013) can impact rhythmic function without associated adiposity. Indeed, provision of a daily palatable meal of chocolate alters locomotor activity rhythms and PER1 and c-FOS signalling in the rat SCN without associated obesity (Mendoza, Angeles-Castellanos & Escobar 2005a). This effect may be moderated by links between the SCN and reward centres in the brain via the PVN (Mendoza, Angeles-Castellanos & Escobar 2005b; Mendoza, Angeles-Castellanos & Escobar 2005a). Accordingly, it can be difficult to determine whether rhythmic disruptions observed in diet-induced obesity models are the result of adiposity-related parameters or nutritional cues from the obesogenic diet. HF feeding was shown to induce a 4-h phase advance in hepatic PER2: LUCIFERASE bioluminescence, in conjunction with altered feeding and locomotor rhythmicity (Branecky, Niswender & Pendergast 2015). Interestingly, switching animals to a standard diet restored normal feeding rhythms within two days and amended the liver clock phase-advance within a week, whereas locomotor rhythmicity was still disrupted two weeks after reintroduction of the normal diet (Branecky, Niswender & Pendergast 2015). These findings suggest that certain aspects of obesity-induced circadian dysfunction occur via relatively immediate dietary cues, whereas others are likely mediated via obesity per se.

2.4.3 The timing of food intake alters metabolic phenotype

There is evidence amongst rodent models that the timing of food intake, rather than nutritional composition per se, also alters the metabolic phenotype. Restriction of food intake to the light period (i.e., an atypical eating period for nocturnal rodents) alters
Corticosterone rhythmicity and induces phase shifts in rhythmic hepatic and adipose tissue gene expression (Zvonic et al. 2006; Bray et al. 2013). These changes have profound downstream effects on metabolic function, such that animals fed a standard diet exclusively during atypical hours develop obesity, diabetes and dyslipidaemia, whereas animals with comparable caloric intake during typical feeding times do not develop these adverse phenotypes (Mukherji et al. 2015).

The influence of feeding time on metabolic and circadian parameters also has implications for obesity studies. Thus, mice fed a HF diet exclusively during the daylight hours exhibit increased weight gain relative to dark or ad libitum fed counterparts (Arble et al. 2009; Yasumoto et al. 2016). Similarly, mice fed a HF diet at the end of the active period exhibit increased adiposity and a range of metabolic complications compared to mice fed at the start of the active period (Bray et al. 2010). Further evidence suggests that restricting HF diet access to the standard nocturnal eating period in rodents reduces weight gain and improves metabolic outcomes (Arble et al. 2009; Hatori et al. 2012; Haraguchi et al. 2014). Nocturnally-restricted feeding in mice also prevents the obesity onset and disruptions to hepatic gene expression caused by exposure to altered light cycles (Oike et al. 2015). This indicates that modification of temporal feeding patterns, rather than alteration of dietary composition per se, could be a potential treatment for obesity and/or circadian disruption. Interestingly, several studies have noted that weight changes driven by atypical feeding patterns occur despite no significant difference in energy intake or physical activity between different food exposure groups (Arble et al. 2009; Hatori et al. 2012; Oike et al. 2015), while others have found that atypical daytime feeding patterns induce hyperphagia and reduce activity levels, thus facilitating obesity development (Yasumoto et al. 2016; Bray et al. 2013).

From a mechanistic perspective, disrupted temporal feeding patterns may elicit adverse metabolic effects due to a misalignment between food intake cues and the endogenous rhythms of peripheral metabolic clocks. Indeed, digestive organs function in a circadian manner (Mazzoccoli et al. 2012; Konturek, Brzozowski & Konturek 2011), and therefore may be less primed to respond to food intake effectively at certain times of the circadian cycle. Consequently, eating at atypical times may result in compromised digestive function and subsequent metabolic aberrations. Recent evidence suggests that the microbiome may also be involved in the timing of digestive efficiency. The bacterial composition of the intestinal microbiome exhibits circadian rhythmicity in mice and humans, and this is modulated by the central circadian clock in combination with
temporal food intake patterns (Thaiss et al. 2014). Interestingly, a recent study by Leone et al. (2015) demonstrates that gut microbial rhythmicity is disrupted by HF feeding in mice, and that specific metabolites (e.g. butyrate) of these gut microbiota directly alter hepatic clock gene expression. These findings suggest a functional role for gut microbiota in obesity-induced disruptions to circadian metabolic function.

Irregular feeding times may also impair appetite regulatory mechanisms. Since appetite regulating hormones such as leptin, adiponectin and ghrelin exhibit rhythmic secretion patterns (Gavrila et al. 2003; Zvonic et al. 2006; Kalra et al. 2003), their levels may not be optimal when food is consumed at inappropriate times, resulting in inadequate satiety signals. Kentish and colleagues (2016) have also presented evidence for rhythmic appetite control, showing in mice that tension and mucosal receptors in gastric vagal afferents (GVAs; which signal stomach capacity to the brain) display circadian rhythmicity in their sensitivity, and this correlates with rhythmic daily fluctuations of stomach volume. This rhythmicity in GVA sensitivity and stomach volume was abolished by provision of a HF diet, which also resulted in increased incidence of irregular (light phase) feeding. Moreover, leptin potentiated GVA mucosal receptor sensitivity in control mice in a photoperiod-dependent manner, indicating that leptin may moderate rhythmic feeding patterns via circadian interactions with GVA receptors. Interestingly, leptin had no effect on GVA sensitivity in HF animals, which suggests that this regulatory capacity is disrupted by obesity (Kentish et al. 2016). In addition to its roles in regulating rhythmic feeding patterns, leptin also seems to be important for the associated weight gain noted in animals fed at irregular hours. Thus, a study by Arble et al. (2011) found that, unlike control animals, leptin deficient ob/ob mice did not experience excessive weight gain when fed at atypical hours, even when administered leptin continuously. In contrast, when leptin was administered in the typical diurnal pattern, ob/ob mice responded with increased weight gain profiles when fed at atypical hours, as was evident in wild type controls (Arble, Vitaterna & Turek 2011). This indicates that increased weight gain due to irregular temporal feeding patterns only occurs in the presence of an endogenous leptin rhythm.

### 2.4.4 Circadian dysfunction in genetic obesity models: a role for leptin?

Obesity-induced leptin disturbances likely influence the circadian regulatory system, since leptin deficient ob/ob mice display suppressed clock gene expression in peripheral tissues, including adipose tissue (Yamaoka et al. 2014; Ando et al. 2011) and liver (Ando et al. 2011). Leptin resistance also elicits circadian dysfunction, since the leptin receptor
deficient db/db mice display similar hepatic Per2 suppression, in addition to a phase advance in hepatic Bmal1 (Kudo et al. 2004), while the leptin-receptor deficient obese Zucker rat exhibits suppression of the rhythm peak in several hepatic clock genes (Motosugi et al. 2011). Interestingly, disruption to peripheral clock gene expression precedes obesity development in ob/ob mice, and leptin supplementation attenuates the clock gene irregularities in these mice, whereas a low calorie diet has no effect (Ando et al. 2011). This suggests that leptin deficiency, rather than other metabolic aberrations associated with obesity, mediates peripheral clock gene disturbances. This view is supported by evidence that hepatic and adipose tissue clock gene expression is suppressed in obese, diabetic KK-Ay mice (Ando et al. 2005; Ando et al. 2006), but is not disturbed in Goto-Kakizaki rats, which are diabetic, but not obese (Ando et al. 2009). This suggests that obesity-induced clock gene suppression in metabolic tissues is not a direct result of hyperglycaemia, but rather a consequence of other metabolic disturbances associated with obesity.

2.4.4.1 Circadian leptin function in obesity

There are variable findings as to how obesity may alter leptin rhythmicity; several studies indicate that obesity blunts leptin rhythms in humans (Saad et al. 1998; Heptulla et al. 2001; Sinha et al. 1996), while Yildiz et al. (2004) showed that leptin rhythmicity was maintained in obese men, albeit with a higher pulse height. Leptin rhythmicity was also abolished by HF feeding in rats, although absolute leptin levels were increased (Cano et al. 2009). In sheep, plasma leptin levels appeared to show an ultradian rather than circadian pattern, and obesity resulted in leptin peaks that were more frequent and higher in amplitude (Daniel et al. 2002).

2.4.5 Nuclear receptors: connecting obesity with circadian dysfunction?

As key regulators of circadian metabolic function (Section 2.3.1), nuclear receptors play direct roles in the metabolic aberrations associated with obesity. Rev-erba knockout mice exhibit increased adiposity and hyperglycaemia, and are more prone to HF diet-induced obesity (Delezie et al. 2012). Global Pparγ loss alters feeding rhythms and disturbs clock gene expression in liver and adipose tissue of mice (Yang et al. 2012). Loss of Pparδ in hepatocytes results in impaired rhythmicity in hepatic lipogenesis (Liu et al. 2013), while global loss of Pgc1α alters hepatic clock gene expression and disturbs energy metabolism (Liu et al. 2007). Conversely, diet-induced obesity alters the expression of these nuclear receptors in metabolic tissues in a circadian-dependent manner (Kohsaka et al. 2007),
which may have implications for clock gene expression or other rhythmic functions in obese individuals.

Ligands for PPARs are commonly used to treat type 2 diabetes and dyslipidaemia in humans (Staels & Fruchart 2005), but it is not known if the actions of such drugs vary in a circadian pattern. Interestingly, a recent study has demonstrated that administration of synthetic Rev-erbα ligands entrains SCN clock gene expression and behavioural rhythms in mice (Solt et al. 2012). Moreover, administration of these ligands reduced adiposity and improved lipid profiles in HF-fed mice, which implies that such molecules could be a therapeutic target for circadian disruptions such as jet lag, as well as metabolic disorders such as obesity (Solt et al. 2012).

2.5 Circadian rhythms during pregnancy

Circadian rhythms are critical for regulating numerous aspects of reproductive function, including successful pregnancy outcomes (Boden, Varcoe & Kennaway 2013). Pregnancy requires highly specialised metabolic adaptations in the mother, and recent evidence suggests that the circadian system is central in regulating these adjustments (Wharfe et al. 2016). Moreover, maternal circadian signals, such as morning cortisol elevation and nocturnal melatonin secretion, provide important temporal signals to the fetus, thereby preparing it for the external environment (Serón-Ferré et al. 2012).

The importance of a functional circadian system during pregnancy is emphasized by evidence that circadian disruption results in numerous pregnancy complications. For example, shift work (particularly overnight) has been associated with a higher risk of miscarriage, pre-term labour, and low birth weight (Zhu et al. 2004; Knutsson 2003; McDonald et al. 1988). Moreover, rodent models using altered light cycles to replicate the effects of shift work support this human evidence; pregnant rats exposed to constant light exhibit marked reductions in fetal weight, in conjunction with excessive placental oedema, leukocyte infiltration and fibrin accumulation (Gozeri et al. 2008) and disruptions to fetal adrenal development (Mendez et al. 2012). Pregnant mice exposed to a 6 h phase advance or delay every 5-6 days throughout gestation display a pronounced decline in pregnancy success rates (Summa, Vitaterna & Turek 2012), while exposure to a 12 h phase shift every 3-4 days throughout rat pregnancy leads to metabolic aberrations across maternal and fetal compartments, including disruptions to rhythmic hormone profiles and altered hepatic expression of clock and metabolic genes (Varcoe et al. 2013). Moreover, the offspring of mothers exposed to these inverted light cycles experience
programmed disruptions to glucose and insulin homeostasis in adulthood, despite being raised in normal lighting conditions themselves (Varcoe et al. 2011).

2.5.1 Maternal metabolic and circadian adaptations to pregnancy

Pregnancy is a unique physiological state that places great metabolic demand on the mother in order to sustain the rapidly growing fetus while simultaneously meeting her own energy requirements. Maternal metabolism is anabolic throughout early- to mid-gestation, whereby maternal hyperphagia and enhanced insulin sensitivity facilitate adipose tissue lipid accumulation. Later in gestation, however, metabolism converts to a catabolic state that allows for the breakdown of maternal fat stores to provide substrates for fetal growth (Lain & Catalano 2007; Herrera & Ortega-Senovilla 2010). These changes are facilitated by a late-gestational increase to maternal insulin resistance in conjunction with the action of placental lactogen, progesterone and glucocorticoids, which induce lipolytic effects in adipose tissue (Williams & Coltart 1978; Sutter-Dub et al. 1981; Xu et al. 2009). Glucose is also the major fetal substrate, and so maternal hepatic glucose production increases in late gestation (Catalano et al. 1992). Despite this increase, circulating maternal glucose decreases towards term (Catalano et al. 1991) due to rapid uptake of glucose by the fetal-placental unit (Hay 2006). Recent studies indicate that these alterations in maternal lipid and glucose metabolism are facilitated by the circadian clock, since clock gene expression in the maternal liver and adipose tissue changes substantially across mouse pregnancy. Moreover, these clock gene changes correspond to gestational adjustments to the rhythmicity of genes regulating lipogenesis and gluconeogenesis in these tissues (Wharfe 2016).

Maternal Tc rhythms are also adapted across gestation. In rodent pregnancy, maternal Tc declines over the final third of gestation, before rebounding rapidly after parturition (Fewell 1995; Gamo et al. 2013; Cairns et al. 2005). The specific mechanisms behind this adaptation are largely unknown, however it appears to be facilitated by a loss of Tc rhythmicity, whereby the normal nocturnal Tc increase is lost (Fewell 1995). Since fetal temperature is higher than maternal Tc in sheep and humans (Laburn, Mitchell & Goelst 1992; Adamsons & Towell 1965), the late gestational decline in maternal Tc may promote heat dissipation away from the fetus over the period of maximal fetal growth, and thus prevent fetal hyperthermia.
2.5.2 Circadian endocrine functions in pregnancy

Maternal endocrine function is altered substantially across gestation, although the extent to which circadian regulation of hormone synthesis is adapted across pregnancy remains poorly understood.

2.5.2.1 HPA axis

The circadian rhythmicity of the maternal HPA axis is maintained across rat and human pregnancy, but absolute glucocorticoid levels increase from mid-gestation (Atkinson & Waddell 1995; Patrick et al. 1980). This gestational increase in HPA axis function is considered important to facilitate the metabolic adaptations that promote adipose tissue lipolysis (Xu et al. 2009). Furthermore, a recent study in mice demonstrates that the rhythmic features of daily maternal corticosterone profiles are substantially altered across pregnancy, such that by late gestation absolute corticosterone levels are elevated but rhythmicity is abolished (Wharfe et al. 2016). Moreover, these changes occurred in conjunction with a gestational shift in clock gene rhythms in the maternal hypothalamus (Wharfe et al. 2016).

2.5.2.2 Progesterone

Progesterone is essential for successful pregnancy outcomes; it provides trophic support for the placenta and promotes fetal growth (Ogle, Mills & Costoff 1990; Mark, Smith & Waddell 2006), moderates maternal immunology to facilitate implantation (Joachim et al. 2003), and maintains uterine quiescence across gestation (for review, see: Brown, Leite & Strauss 2004). Progesterone levels increase steadily over human gestation due to increased placental production towards term, and there is evidence that this occurs in a circadian manner, possibly due to circadian regulation of placental steroidogenesis (Serón-Ferré, Duscay & Valenzuela 1993). In rodents, the corpus luteum is the predominant source of progesterone throughout gestation, so progesterone levels increase markedly and then decline towards term as the corpus luteum regresses (Pepe & Rothchild 1974). Progesterone secretion may be rhythmic in rodents, since clock genes are expressed in the ovary and regulate circadian aspects of fertility and luteal cell function (Boden, Varcoe & Kennaway 2013; Sellix 2015). Downregulation of Bmal1 attenuates progesterone synthesis and expression of steroidogenic genes in rat granulosa cells (Chen et al. 2013). Moreover, mice with a Bmal1 deletion specific to ovarian steroidogenic cells have markedly down-regulated plasma progesterone levels and complete pregnancy failure; this was attributed to implantation failure, since copulation and ovulation rates in...
knockout mice were comparable to those in control animals (Liu et al. 2014). Collectively, these studies indicate that circadian disruption alters ovarian progesterone synthesis, and thus fertility outcomes, but it is unknown how the circadian regulation of ovarian progesterone synthesis may be moderated across gestation, and what impact this may have on pregnancy outcomes.

2.5.2.3 Leptin

Maternal leptin levels rise across human gestation due to production from both maternal adipose tissue and the placenta (Schubring et al. 1998; Henson & Castracane 2000). Much like obesity, pregnancy is considered a state of leptin resistance, which allows for increased maternal energy intake and accumulation of maternal fat stores to provide adequate substrate for fetal growth (Grattan, Ladyman & Augustine 2007; Ladyman 2008). There is little information, however, as to how maternal leptin rhythmicity may be altered across pregnancy. One study in sheep suggests maternal leptin rhythms are maintained across gestation, while absolute levels increase as pregnancy progresses (Bertolucci et al. 2005).

2.5.3 The placenta as a rhythmic organ?

As the sole conduit between maternal and fetal systems, the placenta plays a vital role in regulating fetal exposure to maternal circadian cues. There is also evidence to suggest that the placenta exhibits functional rhythmicity, but whether this is due to an endogenous placental clock or is a response to maternal circadian signals remains unclear (Waddell et al. 2012).

Expression of clock genes has only recently been confirmed in the placenta, when Frigato et al. (2009) found that Per2 was rhythmically expressed in human trophoblast cells after serum shock in vitro. These rhythms were amplified when the cells were cultured in a chemically-induced state of hypoxia, representative of the early embryonic period, leading the authors to suggest that robust clock gene rhythms may be important for initial placentation (Frigato et al. 2009). The first evidence for placental clock gene expression in vivo was reported by Ratajczak et al. (2010), who demonstrated that clock genes were expressed in the mouse placenta and expression generally increased towards term. Moreover, placental Per1 and Cry1 exhibited some subtle time of day variation over day 17 of gestation (Ratajczak, Herzog & Muglia 2010). A shortcoming of this study was that whole placental samples were analysed, and therefore possible expression differences between the functionally distinct placental zones were not accounted for. A subsequent
study demonstrated the importance of this distinction by showing in a Per1-luciferase rat model that Per1 rhythmicity was not present in the placental labyrinth zone (Akiyama et al. 2010). More recently, all seven canonical clock genes were shown to be expressed in both the junctional and labyrinth zones of the rat placenta in a zone-specific manner (Wharfe, Mark & Waddell 2011). This study also found that while placental Bmal1, Per1 and Per2 exhibited marginal time of day variation, expression profiles did not display the typical rhythmic patterns evident in the maternal liver (e.g. Bmal1 in anti-phase to the Per genes), which suggested an absence of coordinated rhythmicity in the placenta (Wharfe, Mark & Waddell 2011).

Although clock genes appear to be largely arrhythmic in the rodent placenta, recent evidence suggests that they may be rhythmically expressed in the human placenta at term (Pérez et al. 2015). This species difference may relate to the timing of fetal circadian development (see Section 2.5.4). Furthermore, despite the lack of rhythmicity in rodent placental clock genes, other aspects of placental function do exhibit some circadian rhythmicity. For example, the rat placenta exhibits rhythmic, zone-specific expression patterns of the glucocorticoid receptor (Gr) and 11β-hsd1, pro inflammatory cytokines and nutrient transporters (Waddell et al. 2012). Given the lack of clock gene oscillations in the rodent placenta, these rhythmic placental functions are likely due to exposure to maternal circadian cues, rather than the presence of an endogenous placental clock.

### 2.5.4 Fetal circadian development: functional clock genes in fetal tissues?

The developing fetus is exposed to rhythmic aspects of maternal physiology, including core body temperature oscillations, daily hormone fluctuations, and temporal food intake cues. The nature of endogenous fetal rhythmicity, however, is not well understood; there is evidence that fetal heart rate, movement and hormone levels exhibit circadian variation that is independent of maternal rhythmicity (Serón-Ferré, Valenzuela & Torres-Farfan 2007). Despite this, it is uncertain whether the fetus or neonate exhibits a functional molecular clock comparable to that of the adult (Section 2.2.1), and at which stage of development such a system becomes fully operational.

The nature of clock gene expression within fetal tissues has been predominantly investigated using rodent models. While clock genes are expressed in whole mouse embryos from day 10 of gestation, and this expression increases from day 10 to day 19, they do not exhibit rhythmic expression patterns (Dolatshad, Cary & Davis 2010). In the rat fetus, clock genes are expressed in the SCN by gestational day 19-20, but rhythmicity
in these genes is not fully developed until postnatal day 10 (Sládek et al. 2004; Kováčiková et al. 2006). While clock genes are also expressed in the fetal liver, with the exception of Rev-erba, this expression is non-rhythmic at day 20 of rat gestation (Sládek et al. 2007). By day 21, time of day variation is evident in fetal hepatic expression of Per2, Per3 and Cry1, but expression profiles are less robust than those in the maternal liver, and do not exhibit the typical anti-phase arrangements expected of a functional circadian system (Wharfe, Mark & Waddell 2011). Rhythmicity of hepatic clock genes gradually develops in the postnatal period in rats, such that all appear fully rhythmic by postnatal day 30 (Sládek et al. 2007). This suggests that substantial development of peripheral metabolic clocks occurs during lactation. Indeed, rat offspring separated from their mothers (i.e. food restricted) during the light photoperiod in the first week after birth exhibit a complete inversion of SCN rhythms by postnatal day 6, highlighting the importance of postnatal feeding cues for central circadian development (Ohta et al. 2003). Moreover, hepatic clock gene expression in rat offspring is altered when feeding patterns shift from relatively constant daily nursing behaviour to rhythmic patterns in nocturnal food consumption following weaning (Yamazaki et al. 2009).

Interestingly, Bmal1 and Per2 expression show anti-phase rhythms in the rat fetal adrenal at day 18 of gestation, and these correspond to rhythmicity in fetal adrenal corticosterone content (Mendez et al. 2012; Torres-Farfan et al. 2011). Moreover, these fetal adrenal rhythms appear to be controlled by maternal melatonin rhythmicity, as opposed to the fetal SCN, which is still immature at this stage of gestation (Torres-Farfan et al. 2011; Mendez et al. 2012; Sládek et al. 2004). As such, it has been argued that the fetus is actually a maternal peripheral oscillator; that is, rather than developing endogenous circadian clocks entrained by the fetal SCN, circadian function in fetal organs is controlled by the maternal circadian system (Serón-Ferré, Valenzuela & Torres-Farfan 2007; Serón-Ferré et al. 2012).

Importantly, there appear to be fundamental differences in fetal circadian development between species, such that altricial species (e.g. rodents) experience more circadian development in the postnatal period. In contrast, humans and sheep have longer gestational periods and show more precocial development overall. As such, these species are likely to undergo more substantial circadian development in utero (Serón-Ferré et al. 2001b).
2.5.5 Fetal exposure to maternal circadian physiology

Maternal corticosterone rhythms are likely to provide an important temporal signal for the fetus, although this is complicated by the existence of the placental glucocorticoid barrier. As described above, the rat fetal adrenal exhibits circadian peaks in expression in Bmal1 and Per2 at day 18 of gestation, and this corresponds to rhythmicity in fetal adrenal corticosterone content (Mendez et al. 2012; Torres-Farfan et al. 2011). Rodents appear similar to humans in this regard, since a cortisol rhythm is evident in the human fetus at term (Serón-Ferré et al. 2001a). Although fetal adrenal rhythmicity is controlled by maternal melatonin, rather than an endogenous fetal clock (Torres-Farfan et al. 2011), generation of rhythmic glucocorticoid oscillations is likely valuable in preparing fetal organs for endogenous rhythmicity in postnatal life, since glucocorticoids are important signals for entraining peripheral clocks (Balsalobre et al. 2000).

Adequate fetal and neonatal leptin exposure is important for the development of a range of organs and systems, including cardiovascular and neurological development (Briffa et al. 2015). Fetal leptin levels can be maternal, placental or fetal in origin, and this varies amongst species. In humans, most fetal leptin is endogenously produced by fetal adipose tissue, with approximately 95% of placentally-derived leptin returning to maternal circulation (Lepercq et al. 2001). In rodents, the placenta produces minimal leptin (Kawai et al. 1997; Amico et al. 1998), and although fetal leptin levels rise to the equivalent of maternal levels by late gestation (Kawai et al. 1997), leptin drops substantially in offspring at birth (Smith & Waddell 2003a). This indicates that rodent fetal leptin is mostly maternal in origin, and suggests changes to fetal leptin levels are likely a result of altered transplacental passage of leptin. Indeed, the expression of the short form leptin receptor (Lepra) and the transplacental passage of leptin increases substantially over late gestation in rats (Smith & Waddell 2003b), but whether this occurs in a circadian manner is unknown.

Maternal feeding cues may also influence fetal circadian development, since restricted feeding in pregnant rats advanced Per1 expression in the fetal SCN and liver (Ohta et al. 2008). Interestingly, this phase change was not evident in the maternal SCN, which is consistent with evidence that restricted feeding entrains peripheral, but not central, clocks (Mukherji et al. 2015; Damiola et al. 2000), and also supports the notion that the fetus is essentially a maternal peripheral oscillator (Serón-Ferré, Valenzuela & Torres-Farfan 2007).
2.5.5.1 Implications for programming outcomes

Since the fetus is not exposed to the light-dark cycle in utero, it relies on maternal circadian cues to program its physiology according to the external environment. As such, normal fetal development requires exposure to rhythmic aspects of maternal physiology, and disruption to the maternal circadian system during pregnancy has programming implications for offspring. For example, as mentioned previously, exposure to a 12 h light cycle phase shift every 3-4 days throughout rat pregnancy results in programmed metabolic complications in offspring. Specifically, offspring of chronodisrupted pregnancies exhibit increased adiposity at 3 months of age, and hyperleptinaemia and altered insulin homeostasis one year postnatally (Varcoe et al. 2011). In addition, exposure of pregnant capuchin monkeys to constant light over the last trimester of pregnancy results in disrupted Tc rhythms in offspring (Serón-Ferré et al. 2013), an effect prevented when mothers are supplemented with melatonin (Serón-Ferré et al. 2013). This suggests that exposure to maternal melatonin rhythmicity is important for programming fetal physiology. Indeed, maternally-derived melatonin freely crosses the placenta, resulting in a rhythmic melatonin profiles in fetal circulation, despite no endogenous melatonin production by the fetal pineal gland in humans (Kennaway, Stamp & Goble 1992) or rats (Deguchi 1975).
Figure 2.4 Circadian rhythms during pregnancy. Schematic representation of the hierarchical arrangements between maternal central, maternal peripheral, placental and fetal clocks. The maternal SCN (1) controls rhythmic central output and entrains oscillations in maternal metabolic and endocrine organs (2). The placenta (3) then regulates fetal exposure to these maternal circadian cues, possibly by way of an endogenous placental clock. Rhythmic development occurs in fetal and neonatal tissues (4) through exposure to these maternal circadian signals in conjunction with postnatal circadian entrainment (5). The latter may be more important in altricial rodent species.
2.6 Circadian rhythmicity during obese pregnancy

Despite the clear importance of circadian rhythms in regulating pregnancy outcomes (Section 2.5) and obesity pathogenesis (Section 2.4), there is a lack of information regarding circadian function during obese pregnancy. Several recent studies in rodents demonstrate that *in utero* exposure to maternal obesity alone (Wang *et al.* 2015) or in conjunction with a postnatal obesogenic diet (Borengasser *et al.* 2014; Carter *et al.* 2014; Mouralidarane *et al.* 2015; Bruce *et al.* 2016) disrupts hepatic clock gene expression in adult offspring. Moreover, these clock gene changes often occur in conjunction with symptoms of non-alcoholic fatty liver disease (Bruce *et al.* 2016; Carter *et al.* 2016; Mouralidarane *et al.* 2015). Several of these studies attributed the programmed changes in the offspring hepatic clock to epigenetic mechanisms, noting hypermethylation of the *Bmal1* and *Per2* promotor regions (Mouralidarane *et al.* 2015) and histone modifications to *Pparα* (Borengasser *et al.* 2014) in offspring born to obese mothers. Similarly, a study in Japanese macaques reported altered histone acetylation on the *Npas2* promotor in offspring livers after maternal HF-feeding (Suter *et al.* 2011). This study also measured fetal hepatic *Npas2, Per2*, and *Rev-erba* expression in late gestation and showed that *Rev-erba* and *Npas2* expression was elevated, albeit only measured at a single time-point (Suter *et al.* 2011). There is currently no other evidence as to whether circadian disruption due to maternal obesity occurs in maternal, placental or fetal compartments.

As discussed earlier (Section 2.1), obese pregnancy is strongly associated with various maternal metabolic abnormalities, including hyperlipidaemia (Ramsay *et al.* 2002), endocrine disruptions (Jansson *et al.* 2008; Stirrat *et al.* 2016) and an altered inflammatory phenotype (Panham, Aye & Powell 2015). Whether these changes occur in a circadian manner, or are regulated by the circadian system, is largely unknown. Moreover, while maternal obesity often results in placental dysfunction (Higgins *et al.* 2011; Gaccioli *et al.* 2013), it is unclear whether rhythmic placental functions are altered during obese pregnancy and how this might influence fetal exposure to any maternal circadian disruptions.

2.6.1 PPAR function during obese pregnancy

Altered PPAR activity may also contribute to pathogenesis of obese pregnancy. Reduced expression of all three *PPAR* isoforms has been observed in the adipose tissue of obese women at term (Lappas 2014); it is unknown, however, whether maternal circadian PPAR expression profiles are altered by obesity.
Placental PPAR activity is important for adequate placental development and vascularisation (Nadra et al. 2010), and regulates transplacental fatty acid transport to the fetus (Xu et al. 2007). Maternal obesity has been shown to increase \textit{Ppar}\textsubscript{\(\gamma\)} levels in the placenta of sheep (Zhu et al. 2010) and mice (Qiao et al. 2015), and in both cases this was associated with altered placental lipid transport and fetal hyperlipidaemia. Conversely, other studies have found no change in placental \textit{Ppar} expression in obese mice (Díaz et al. 2015) and humans (Dubé et al. 2012), despite noting altered expression of placental fatty acid transporters. While these inconsistencies between studies could reflect species differences, importantly, no account was taken of circadian variation; it remains possible, therefore, that placental PPARs are altered by obesity in a circadian-dependent manner.

In addition to altered placental PPAR activity, multiple rodent studies have demonstrated that offspring born to obese mothers exhibit programmed disruptions to hepatic and adipose expression of \textit{Ppara} and \textit{Ppar}\textsubscript{\(\gamma\)}, and these changes are accompanied by other metabolic disturbances (Samuelsson et al. 2008; Yamaguchi et al. 2010; Hyatt et al. 2011; Alfaradhi et al. 2014; Borengasser et al. 2014; Desai et al. 2015). Moreover, administration of PPAR\textsubscript{\(\alpha\)} and PPAR\textsubscript{\(\gamma\)} ligands improves programmed metabolic dysfunction in offspring born to obese mice (Magliano et al. 2013; Kalanderian et al. 2013). These findings suggest that disturbances to PPAR signalling pathways are implicated in the adverse developmental programming outcomes associated with maternal obesity. The circadian regulation of these nuclear receptors has not previously been characterised in placental or fetal tissues, and so an investigation of circadian \textit{Ppar} expression during obese pregnancy is warranted.

\begin{enumerate}
\setcounter{enumi}{2}
\item \textbf{2.6.2 Leptin function during obese pregnancy}
\end{enumerate}

In accordance with their increased adiposity, obese pregnant women have elevated leptin during gestation (Farley et al. 2010; Misra & Trudeau 2011; Tessier, Ferraro & Gruslin 2013; Jansson et al. 2008). Interestingly, Misra et al. (2011) reported that although obese women were hyperleptinaemic throughout gestation, their rate of increase in plasma leptin was lower than controls, due in part to lower gestational weight gain in the obese group. This is in accordance with previous findings that metabolic and inflammatory markers converge in lean and obese subjects in late gestation (King et al. 2013; Forbes et al. 2015; Pedroni et al. 2014). While it is unknown how rhythmic leptin secretion may be altered by obesity, interestingly, a recent study reported that overweight and obese women who reported less than 5 h sleep each night had elevated leptin levels compared to those...
who reported 7-8 h sleep (Qiu et al. 2014). This relationship was not evident in normal weight controls, indicating that obesity-induced leptin changes during pregnancy may be linked to the circadian system (Qiu et al. 2014).

Maternal obesity also appears to induce placental leptin resistance, which likely modifies placental function. Thus, a study by Farley et al. (2010) reported elevated maternal leptin levels in obese women, and while there was no difference in placental leptin production or fetal leptin levels between groups, they noted decreased leptin receptor expression in the syncytiotrophoblast of obese mothers. Moreover, this apparent leptin resistance was accompanied by decreased System A sodium-dependent amino acid transporter (SNAT) expression in placental tissue of obese women (Farley et al. 2010). This is consistent with the observation that leptin treatment in cultured human placental tissue increases placental SNAT activity (Jansson et al. 2003; von Versen-Höynck et al. 2009), and suggests that placental leptin resistance during obese pregnancy may decrease fetal-placental nutrient transport. This, in turn, may contribute to the fetal growth restriction that occurs in a subset of obese pregnancies (Gardosi & Francis 2009; Radulescu et al. 2013).
Chapter 3: Experimental Objectives

The overall aim of the experiments presented in thesis was to investigate the impact of maternal obesity on rhythmic function in maternal, placental and fetal tissues during pregnancy. It is clear from the review of the literature that obese pregnancy elicits many negative and lasting effects on maternal and offspring health outcomes. It is also evident that the circadian system is vital in regulating metabolic adaptations to both obesity and pregnancy. Despite this, it is unknown whether obesity alters the molecular control of the circadian system in maternal, placental or fetal tissues during pregnancy. Moreover, there is little evidence as to whether obesity disturbs other downstream physiological processes in a circadian-dependent manner during pregnancy. As such, it was hypothesised that maternal obesity disturbs rhythmic clock gene expression in maternal, fetal and placental tissues, and alters other key downstream metabolic and physiological markers in a time-of-day dependent manner.

Study 1 (Chapter 5): Maternal obesity induced by a ‘cafeteria’ diet in the rat does not increase inflammation in maternal, placental or fetal tissues in late gestation

The objective of this study was to optimise a model for diet-induced maternal obesity in a Wistar rat cohort. This was an initial pilot study conducted at one time-point in late gestation (ZT5; day 21) to test the effectiveness of a cafeteria (CAF) feeding regime at establishing maternal obesity, compared to consumption of standard chow (CON). Previous use of a high-fat semi-pure diet in this colony of animals had been unsuccessful at inducing weight gain, due to caloric autoregulation by pregnant dams.

This study also examined the inflammatory status of maternal, placental and fetal tissues during obese pregnancy. Inflammation was chosen as an experimental endpoint because it is commonly associated with pathologies of obese pregnancy. Expression of inflammatory genes was measured in the maternal liver and adipose tissue, placental junctional and labyrinth zones, and fetal liver. Circulating inflammatory makers were also quantitated in maternal and fetal plasma to characterise the inflammatory milieu across maternal, placental and fetal compartments near term.

Study 2 (Chapter 6): Obesity disrupts the rhythmic profiles of maternal and fetal progesterone in rat pregnancy

The CAF diet successfully induced hyperphagia and subsequent weight gain (in conjunction with fetal and placental growth restriction) in the cohort of animals from
Chapter 5. Consequently, the CAF feeding manipulation was applied to a larger cohort, from which tissues were collected at six time-points (0800, 1200, 1600, 2000 and 0400 h) across days 15-16 and 21-22 of gestation (term = 23 days), thus allowing for comprehensive rhythmic analyses across these days.

Chapter 6 aimed to characterise fetal and placental growth patterns in CON and CAF animals across these two gestational days, which span the period of maximal fetal growth in rat pregnancy. Additionally, this study determined the impact of maternal obesity on the rhythmic profiles of several key plasma markers: daily profiles of steroid hormones (corticosterone, 11-DHC, progesterone and testosterone) were assessed in maternal and fetal plasma, since these steroids strongly influence fetal growth and are regulated by the circadian system. Maternal and fetal plasma lipid profiles (total cholesterol, triglyceride, HDL-C and LDL-C) were also assessed, since circulating lipids provide a key source of substrates for fetal growth. Moreover, obesity is associated with disturbances to lipid metabolism, and recent evidence implicates the circadian system in the control of lipid biology.

**Study 3 (Chapter 7): Diet-induced obesity reduces maternal core body temperature and alters the normal thermoregulatory changes of late pregnancy in the rat**

This study investigated whether CAF-induced maternal obesity alters rhythmic maternal core body temperature ($T_c$) profiles. $T_c$ exhibits distinct circadian variation which is controlled by the central circadian clock within the hypothalamus. Moreover, maternal $T_c$ is substantially adapted across rodent pregnancy, declining markedly from mid-gestation to term. This study aimed to investigate whether obesity alters the rhythmic nature of $T_c$ profiles (an indication of central circadian function), and the pregnancy-induced adaptations to maternal $T_c$. Temperature loggers were surgically implanted into the peritoneal cavity of CON and CAF non-pregnant animals and $T_c$ was recorded every 15 min for 2-3 estrous cycles and throughout gestation. Rhythmic (cosine) $T_c$ features were then assessed across the estrous cycle and pregnancy.

**Study 4 (Chapter 8): Obesity alters maternal hepatic clock gene expression and suppresses Rev-erbα in the placenta and fetal liver during rat pregnancy**

The aim of this study was to determine the impact of maternal obesity on the rhythmic expression of clock genes and associated nuclear receptors in maternal, placental and fetal tissues. Clock genes were measured in maternal liver, placental labyrinth zone and fetal liver collected from the cohort in Chapter 6. Expression of *Ppara*, *Pparδ* and *Pgc1α* was
also assessed in these tissues, since Ppars regulate the molecular clock, and are directly
involved in metabolic functions such as lipid and glucose metabolism. Maternal and fetal
insulin and glucose profiles were also measured across each day.

**Study 5 (Chapter 9): Obesity disrupts rhythmic clock gene expression in maternal
adipose tissue during rat pregnancy**

Since the adipose tissue clock is important for endocrine and metabolic adaptations to
pregnancy, the aim of the final study was to determine the impact of obesity on rhythmic
clock gene expression in maternal adipose tissue, collected from the cohort in Chapter 6.
Rhythmic adipose expression of the Ppar nuclear receptors (Ppara, Pparδ, Pparγ and
Pgc1α) was compared between CON and CAF mothers, and daily profiles of maternal
and fetal plasma leptin were also measured.
Chapter 4: Materials and Methods

4.1 Animals

4.1.1 Animals and diets

All procedures involving animals were approved by the Animal Ethics Committee of The University of Western Australia (AEC number RA/3/100/1068). Female nulliparous albino Wistar rats were obtained from the Animal Resources Centre (Murdoch, Western Australia) at three weeks of age. Females were housed three per cage in a room under controlled environmental conditions; average ambient temperature was 22°C and average humidity was 55%. The animal facility adhered to a 12:12 h light-dark cycle, with lights on at 0700 h and lights off at 1900 h. All animals had *ad libitum* access to standard rodent chow (Specialty Feeds; Glen Forrest, WA, Australia) and acidified tap water (0.008% HCL; pH 2.5-3).

Following a week of acclimatisation, animals were separated into control (CON) and cafeteria (CAF) groups. Both groups received free access to standard chow, but CAF animals were also offered four snack food items per day (e.g. cookies, cheesecake, peanut butter, hot dogs). CAF animals were given a selection of two savoury and two sweet items each day from a set list of 17 items. Food items were changed daily to maintain novelty and were provided in excess to be essentially *ad libitum*; a detailed list of CAF items and their macronutrient composition is presented in Chapter 5 (Supplementary Data). CAF items were offered 1-2 hours before lights off to minimise any atypical daytime eating patterns in CAF animals. At the time of CAF item provision, all CON cages were opened and the bedding disturbed to ensure similar environmental intervention between diet groups. In a subset of animals (*n* = 12 for each diet), food intake was measured daily by weighing the chow and each CAF item at the time of provision, and then the amount that remained in the cage 24 h later. All animals were weighed weekly throughout the pre-pregnancy period.

4.1.2 Mating and pregnancy determination

After 8 weeks of diet exposure, CON and CAF animals were monitored daily for estrous cycle stage with an estrous cycle monitor (EC40; Fine Science Tools, Vancouver, BC, Canada). Upon determination of proestrus (endometrial resistance ≥ 6,000 ohms), females were placed in a cage with a male rat overnight. Males were age-controlled (between 12 and 24 weeks old) and maintained on standard rodent chow. Mating was confirmed by
presence of spermatozoa in a vaginal smear the following morning, and this was designated day 1 of pregnancy. Pregnant animals were then caged individually and maintained on their respective diets throughout pregnancy, and maternal food intake and body weights were measured daily.

4.2 iButton temperature logger surgery

After 4 weeks of diet exposure, a subset of animals \( n = 7 \) \( \text{CON} \), \( n = 8 \) \( \text{CAF} \) underwent surgery to implant a temperature logger (DS1922L iButton Temperature Logger, Maxim Integrated, San Jose, CA, USA; dimensions: 3.3 g, 17.4 mm diameter, 5.9 mm width) into the peritoneal cavity. This allowed for the rhythmic measurement of core body temperature \( (T_c) \) across the pre-pregnancy, mating and pregnancy periods.

4.2.1 Surgical procedure

Temperature loggers were coated in three layers of inert wax (Sasol EXP986, Sasol Chemical Industries Ltd., Johannesburg, South Africa) to render them immunologically inert and impermeable to biological fluids. Loggers were then soaked overnight in a 0.5% w/v chlorhexidine gluconate in 70% w/v ethanol solution prior to implantation. All surgical equipment was sterilised by autoclave \( (121^\circ \text{C}, 20 \text{ min}, 215 \text{ kPa}; \text{Celitron Medical Technologies, Vác, Hungary}) \) prior to the procedure.

Animals were anaesthetised using 5% isoflurane \( (\text{Attane}^\text{TM}, \text{Bomac Pty Ltd, Hornsby, NSW, Australia}) \) in a nitrous oxide and oxygen mixture (0.2 and 0.8 L/min flow rate, respectively) and maintained on 2.5% isoflurane throughout the surgery. The abdomen was shaved and sterilised by swabbing with chlorhexidine surgical scrub \( (\text{Orion Laboratories, Balcatta, WA, Australia}) \) and 70% ethanol, then rinsed with a sterile saline solution. Animals were administered a subcutaneous injection of buprenorphine hydrochloride \( (0.01 \text{ mg/kg BW}; \text{Reckitt Benckister, West Ryde, NSW, Australia}) \) as a pre-emptive analgesic.

Separate midline abdominal incisions (approximately 2 cm long) were made through the skin and muscle layers, and the wax-coated temperature logger was inserted into the peritoneal cavity. The muscle layer was closed using absorbable suture and the skin layer with silk suture \( (\text{Ethicon, Somerville, NJ, USA}) \). Aseptic technique was used throughout the surgical procedure and body temperature was maintained using a warming pad. Animal recovery was monitored hourly for the first 8 h post-surgery and then twice daily for the week following.
4.2.2. Temperature data acquisition

Animals were allowed to recover for 1 week, after which temperature loggers recorded $T_c$ every 15 min for 2-3 estrous cycles and throughout pregnancy. Animals were maintained on the same diet regime as assigned for the pre-surgical period (i.e. either CON or CAF diet). Although neither dietary group gained weight in the week directly following surgery, CAF animals still maintained increased weight gain relative to CON over the study period, indicating that surgery did not prevent obesity-onset (see Chapter 7).

Loggers were recovered upon tissue collection at day 21 of gestation (see section 4.3), and calibrated against a certified mercury-in-glass thermometer (National Association of Testing Authorities, Australia) in a water bath heated from 33 - 41°C in 3°C increments. Temperature data were obtained from loggers using eTemperature software (version 8.25, OnSolution Pty Ltd, NSW, Australia).

4.3 Tissue collection

4.3.1 Timing of tissue collection

Tissue collection procedures occurred at six time-points across days 15-16 and days 21-22 of pregnancy. Gestation lasts approximately 23 days in this rat colony, and these specific gestational days were chosen to span the period of maximal fetal growth. Collection times were expressed relative to Zeitgeber time zero (ZT0), defined as when lights turned on in the animal facility at 0700 h. As such, ZT refers to the number of hours after light onset. Collection times occurred at four hourly intervals: ZT1 (0800 h), ZT5 (1200 h), ZT9 (1600 h), ZT13 (2000 h), ZT17 (2400 h) and ZT21 (0400 h).

4.3.2 Tissue collection procedure

At the appropriate time, animals ($n = 7-8$ per diet group at each ZT) were anaesthetised via inhalation of 5% isoflurane in a mixture of nitrous oxide and oxygen (0.2 and 0.8 L/min flow rate, respectively). For collections occurring in the dark hours (ZT13, ZT17 and ZT21), anaesthesia induction occurred under a 36 W, 620 nm red light lamp; this wavelength is above the spectral sensitivity of rodents ($\sim$ 360-590 nm) but remains within the visible wavelength of humans ($\sim$ 380 – 700 nm) (McCormack & Sontag 1980; McLennan & Taylor-Jeffs 2004).

The maternal abdominal cavity was opened and fetal-placental pairs were counted. Three fetal-placental pairs per uterine horn were removed via caesarean section, and fetal and
whole placental weights were recorded to the nearest mg. The placenta was then separated into the junctional (JZ) and labyrinth (LZ) zones by blunt dissection, and each zone weighed individually. Maternal liver, maternal retroperitoneal (RP) adipose tissue and fetal liver tissue samples were also collected.

Maternal blood was taken from the descending aorta and fetal blood obtained at day 21 by decapitation. Maternal and fetal blood glucose levels were measured immediately using a blood glucose monitor (Accu-Chek, Roche Diagnostics, Mannheim, Germany). Blood samples were then mixed with 10:1 (vol: vol) 0.6 M EDTA and centrifuged at 13,000 x g for 6 min to isolate plasma.

All collected tissue and plasma samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis. Following tissue collection, dams and fetuses were euthanized.

### 4.4 Body composition analysis

Maternal body composition was measured in a subset of animals (n = 23 CON, n = 22 CAF) by dual energy X-ray absorptiometry (DEXA; GE Lunar Prodigy Series, GE Lunar, Madison, WI, USA). All maternal carcasses selected for body composition analysis underwent hysterectomy following euthanasia. Care was taken to leave the mesentery and associated parametrial fat depots in place during removal of the uterus. The abdomen was then sutured closed and carcasses were frozen at -20°C for subsequent analysis. A trial performed prior to this study established that body composition measurements were comparable between fresh and frozen carcasses (data not shown).

Scans were analysed with small animal software (Encore 2004, version 8.50.093; GE Lunar) to obtain measures for total percentage body fat, bone mineral content (BMC), total lean mass (g) and total fat mass (g). In addition to whole body analysis, ‘central’ adiposity was measured by isolating a customised region of interest on the software. This ‘central’ area was defined as the region from the superior margin of the iliac crests to the inferior margin of the sternum (see Figure 4.1), and allowed for total central fat percentage, central lean mass (g) and central fat mass (g) to be calculated.
Figure 4.1 DEXA analysis of body composition. Representative soft tissue and skeletal scans of (A) CON and (B) CAF animals, with the ‘central region’ defined in boxes.
4.5 Fetal sex determination

Fetal sex at day 21-22 of pregnancy was determined by measuring anogenital distance, with a longer distance (>2mm) indicative of a male fetus (Faber & Hughes 1992). Since fetal sex is not externally distinguishable at day 15-16, sex determination in these fetuses was achieved by measuring amplification of the sex-determining region Y (Sry) gene in tail tissue via polymerase chain reaction (PCR).

Genomic DNA was extracted from approximately 15 mg of fetal tail tissue by incubation with 0.5 mg proteinase K (Promega, Sydney, NSW, Australia) and 500 µl of digestion buffer (50 mM Tris-HCL pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS) at 55°C for 3 h. Saturated NaCl (4M; 180 µl) was added and samples were centrifuged at 13,000 x g for 10 min. Genomic DNA was precipitated with 200 µl isopropanol and washed with 500 µl of 70% ethanol. Samples were air dried for 30 min, after which DNA was resuspended in 50 µl of ddH2O and assessed for concentration and purity using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Samples were analysed for the presence of genomic Sry1 using PCR on the Rotorgene Q (Qiagen, Hilden, Germany) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) at an annealing temperature of 60°C. Primers for the Sry1 gene (Forward: 5’-GAGAGAGGCAAGTTGGC-3’; Reverse: 5’-GCCTCCTGGAA AAAGGGGC-3’) were designed using Primer-BLAST (Rozen & Skaletsky 2000). Male samples amplified Sry1 product before 20 cycles (Figure 4.2).
Figure 4.2 Amplification of Sry1 from fetal DNA samples at day 15. Males consistently amplified product before 20 cycles.
4.6 Quantitative reverse transcription polymerase chain reaction

4.6.1 Background
Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a highly sensitive method for measuring mRNA concentration. RNA is extracted from a tissue sample and reverse transcribed to a stable complementary DNA (cDNA) copy. A specific gene of interest can then be quantitated in the cDNA template by amplification of the gene sequence to detectable levels. A typical PCR reaction cycle consists of denaturing, annealing and elongation phases. DNA is first denatured to become single stranded, after which primers for the gene of interest bind via complementary base pairing to the gene sequence during the annealing phase. The enzyme DNA polymerase then replicates the gene of interest, using the bound primers as an anchor; this elongation phase results in the targeted sequence becoming double stranded. This process is repeated over multiple cycles so that exponential amplification of the target gene occurs, at a level proportional to the original quantity in the sample. The concentration of the gene of interest after each amplification cycle is measured by fluorescence emitted from a fluorescent dye (SYBR green), which only activates when bound to the minor groove of double stranded DNA. The relative concentration of the gene can then be compared between samples with the aid of a standard curve.

4.6.2 RNA extraction

4.6.2.1 Liver and placental tissues
Total RNA was extracted from maternal liver, placental junctional zone, placental labyrinth zone and fetal liver samples using the QIAzol Lysis Reagent method (Cat. No 79306; QIAGEN Pty Ltd, Melbourne, VIC, Australia). Briefly, 50-100 mg of the relevant tissue was homogenized in 1 ml of QIAzol using the POLYTRON-Aggregate homogenization probe (Kinematica Inc. Bohemia, NY, USA) which had been treated with RNase-zap and washed in dimethyl dicarbonate (DMDC) water to prevent contamination by RNases. All tissues and homogenizing tubes were stored on dry ice prior to extraction to prevent RNA breakdown.

After homogenisation, 50 μl of bromoanisole was added to the homogenate mix, and the resulting solution was vortexed thoroughly and centrifuged at 4°C at 12,000 x g for 15 min to induce phase separation. The upper aqueous phase was transferred to a separate
tube and 0.5 ml of isopropanol was added to precipitate RNA. Samples were inverted
three times, left at room temperature for 10 min, and centrifuged at 4°C at 12,000 x g for
5 min. The supernatant was decanted and the remaining RNA pellet was washed twice in
75% ethanol and centrifuged at 6,000 x g for 5 min after each wash. Ethanol was removed
and the pellet was air-dried until semi-transparent, after which RNA was resuspended in
nuclease-free water.

4.6.2.2 Adipose Tissue

RNA was extracted from maternal retroperitoneal adipose tissue using an RNaseasy Lipid
Tissue Mini Kit (Cat. No 74804; QIAGEN Pty Ltd, Melbourne, VIC, Australia) as per
the manufacturer’s instructions. Adipose tissue (50-100 mg) was homogenized in 1 ml of
QIAzol Lysis Reagent (Cat. No 79306; QIAGEN Pty Ltd, Melbourne, VIC, Australia)
using the POLYTRON-Aggregate homogenization probe (Kinematica Inc. Bohemia,
NY, USA), which had been rinsed with RNase-zap and DMDC H2O to prevent RNase
contamination. The homogenate was then incubated at room temperature for 5 min after
which 200 µl of chloroform was added and the solution vortexed for 15 sec. Samples
were then centrifuged for 15 min at 12,000 x g at 4°C and the upper aqueous solution
transferred to a new tube and vortexed with 1 ml of 70% ethanol. The solution was then
filtered through an RNeasy Mini spin column by centrifugation at room temperature for
15 sec at 8,000 x g. This ensured that RNA was captured in the spin filter membrane,
which was then washed once with 700 µl of RW1 buffer and twice with 500 µl RPE
buffer to remove any impurities. Finally, 50 µl of nuclease-free water was added to the
spin filter, which was centrifuged at 8,000 x g for 1 min and the eluate collected.

All RNA samples were assessed for concentration (ng/µl) and purity (A260/A280 ≥ 1.8)
by measurement of absorbance at 260 and 280 nm using the Nanodrop ND-1000
spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Samples were stored at
-80°C until required for reverse transcription.

4.6.3 Reverse transcription

RNA (1 µg for adipose tissue and 5 µg for all other tissues) was reverse transcribed to
cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT;
Promega, Sydney, Australia). Purified RNA was combined with 0.5 µl of random
hexamer primers, 2.5 µl of Ficoll 70 (75 mg/ml), 2.5 µl of Ficoll 400 (25 mg/ml) and the
reaction made up to 14 µl with nuclease-free water (Sigma-Aldrich, St Louis, MO, USA).
Circadian adaptations to obese pregnancy

Samples were denatured at 70°C for 5 min and then placed on ice for 5 min, after which 5 μl of M-MLV 5x reaction buffer, 1.25 μl of dNTPs (10mM), 0.62 μl of RNAsin Ribonuclease Inhibitor (Promega, Sydney, Australia) and 0.75 μl M-MLV Reverse Transcriptase were added to the reaction mix. Samples were incubated in a thermal cycler (MJ Research Inc. Watertown, MA, USA) with an initial annealing reaction cycle of 25°C for 10 min, followed by reverse transcription at 42°C for 110 min. The reaction was terminated by heating to 70°C for 15 min.

The resultant cDNA was purified with an Ultra Clean PCR Cleanup Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), as per the manufacturer’s instructions. Briefly, 125 μl of Spinbind solution was added to each sample, which was then vortexed for 5 sec and centrifuged at 13,000 x g for 30 sec. The liquid flow-through was discarded and 300 μl of Spinclean solution added to the column. Samples were centrifuged at 13,000 x g for 30 sec, after which flow-through was discarded and samples were centrifuged again at 13,000 x g for 30 sec. The spin basket containing cDNA was moved to a new tube and 100 μl of elution buffer was added and incubated at room temperature for 1 min to allow for cDNA elution. Columns were then centrifuged a final time at 13,000 x g for 30 sec to collect the purified cDNA. All cDNA samples were stored at -20°C until analysis by PCR.

4.6.4 Primer design

Primer pairs were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Rozen & Skaletsky 2000) and manufactured by GeneWorks (Adelaide, SA, Australia). The exception to this was Il-1β primers, which were supplied by Qiagen (QT00181557l; Qiagen Pty Ltd, Melbourne, VIC, Australia). All primers were designed to span an intron to prevent amplification of genomic DNA. Primer specificity was confirmed by observation of a single PCR product by melt curve analysis, after which the PCR product was separated by gel electrophoresis on a 2% agarose gel for 45 min at 110 V in 1x TAE buffer. This allowed for reconfirmation of a single product (as shown by a single band) and determination of product size. The PCR product was then extracted from the gel using a QIAEX II gel extraction kit (Qiagen, Melbourne, VIC, Australia) and specificity was confirmed by sequencing using Big Dye Terminator version 3.1 (Applied Biosystems, Mulgrave, VIC, Australia). Primer sequences and product sizes are shown in Table 4.1.
Table 4.1 PCR conditions. Primer sequences, annealing temperatures, amplicon sizes and MgCl₂ concentrations for amplification reactions to measure gene expression by RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (base pairs)</th>
<th>MgCl₂ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clock genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Clock      | F5' ACAGCGCACACACAGGCCTTC 3'  
             | R5' TGGCCGGGCCTGTGATCTA 3'     | 60                         | 175         | 2          |
| Bmal1      | F5' ACACTGCACCTCGGGAGCGGA 3'  
             | R5' CGCCCCAGCTCCAGACAAAA 3'    | 60                         | 100         | 2          |
| Per1       | F5' CGCAGTGAGCTCAAACAGGCTCC ACC 3'  
             | R5' GTCCATGGCAAGGGCTCACC 3'    | 60                         | 169         | 2          |
| Per2       | F5' TGAGCTCTCTGCGTTGCCG 3'     
             | R5' ACTCAGGCCAACCTGCCACA 3'    | 60                         | 147         | 2          |
| Per3       | F5' TTTTCCCTCTCAAGACATGG 3'    
             | R5' GAAAGAGAGGGCTTGTCG 3'      | 60                         | 167         | 2          |
| Cry1       | F5' AGCTTGCCACCTGAGCTTGT 3'    
             | R5' TGCTGGCATCTTCCCCGCTT 3'    | 60                         | 158         | 2          |
| Cry2       | F5' CTGCCCAGGAGCCACCAAGC 3'    
             | R5' GCATGCACCCAGCAACGGCA 3'    | 60                         | 192         | 2          |
| Rev-erbα   | F5' ATGGCCACCGGGGCGAGAGA 3'    
             | R5' GCCAAAAGAGCCAGCGGAGGT 3'   | 60                         | 292         | 2          |
| Rorα       | F5' CCCAACCGTGCTCCATGGCGG 3'   
             | R5' CCCGTCGATCAGCTTGCGCA 3'    | 60                         | 113         | 2          |
| **Ppar and Glut2** |             |                            |                           |            |
| Pparα      | F5' AATCCACAGAAGCTACCTACCTGA 3'  
             | R5' GTCTTCTCAGCCACATGCACAA 3'  | 60                         | 132         | 2.5        |
| Pparγ      | F5' GACCCAGAGCATGGTGCCCTTG 3'   
             | R5' GCTGATTCGAGGATCTGTCAGGC 3'  | 63                         | 108         | 3          |
| Pparδ      | F5' GAGGGGTTGCAAGGGCTTCT 3'     
             | R5' CACTTTGGCGTTCTTCCTCTG 3'   | 60                         | 101         | 2.5        |
| Pgc1α      | F5' TCTGGAACCTGAGGCCTTACCTA 3'  
<pre><code>         | R5' GCAAGAGAGGCTTCCAGCTTG 3'   | 60                         | 96          | 4          |
</code></pre>
<table>
<thead>
<tr>
<th>Gene</th>
<th>F5' Primer Sequence</th>
<th>R5' Primer Sequence</th>
<th>Length (bp)</th>
<th>Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glut2</strong></td>
<td>TAGGGCGGAATGGTGCGCTCGT</td>
<td>GGGCTCCAGTCACGGAGGCT</td>
<td>61</td>
<td>102</td>
</tr>
<tr>
<td><strong>Inflammatory genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tnfα</strong></td>
<td>TACTGAACCTTGGGGTGATGGTGCGCTC</td>
<td>CAGCTTTGTGCCAGAGAACC</td>
<td>60</td>
<td>295</td>
</tr>
<tr>
<td><strong>Il-6</strong></td>
<td>TCGCAAGAGACTTGCCAGGCT</td>
<td>AGCCTCGAGATTGAAAGTGG</td>
<td>60</td>
<td>148</td>
</tr>
<tr>
<td><strong>Il-1β</strong></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><strong>Tlr2</strong></td>
<td>GCCGACATGCGGACACAGGACT</td>
<td>CTCGCTTGCAAGGCTTCCC</td>
<td>60</td>
<td>137</td>
</tr>
<tr>
<td><strong>Tlr4</strong></td>
<td>CTCACCGGCTCGGTGGTTG</td>
<td>GGGATTCATGAGGGATTTTC</td>
<td>60</td>
<td>189</td>
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<tr>
<td><strong>Cox2</strong></td>
<td>GGGGAGACCATGGTAGAAC</td>
<td>GAAGGGACACCCTTTCACAT</td>
<td>59</td>
<td>178</td>
</tr>
<tr>
<td><strong>Emr1</strong></td>
<td>CAGCTGCTCTCCCAGCTTTC</td>
<td>TAATCAGAGATTCCGGCAT</td>
<td>60</td>
<td>156</td>
</tr>
<tr>
<td><strong>Reference genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ppia</strong></td>
<td>AGCATACAGGTCCTGGACATC</td>
<td>TTCACCTTCCCAAAGACCAC</td>
<td>62</td>
<td>127</td>
</tr>
<tr>
<td><strong>Sdha</strong></td>
<td>TGGGGCCGACTGGCTTGCTTC</td>
<td>CCCCAGCTGTCAACCTAACACC</td>
<td>60</td>
<td>134</td>
</tr>
<tr>
<td><strong>Ywhaz</strong></td>
<td>GACGGAGCTGGAGGACATCTGC</td>
<td>GGCTGCAGAAGCAATGGGGATCA</td>
<td>60</td>
<td>75</td>
</tr>
</tbody>
</table>

*Qiagen QT00181657; F: Forward; R: Reverse.*
4.6.5 Quantitative polymerase chain reaction (qPCR)

4.6.5.1 Reagents

With the exception of Il-1β, all PCR reactions consisted of 1 µl of 10 x Immolase buffer, 0.2 µl of dNTPs (10 mM), 0.5 µl of a SYBR green fluorescent dye (Molecular Probes, Eugene, OR, USA; present in the reaction as a 1: 2000 dilution in DMSO), 0.05 µl of Immolase DNA polymerase (Bioline, Alexandria, NSW, Australia), and primer-specific concentrations of MgCl2, forward primer and reverse primer (detailed in Table 4.1). Reactions were made up to 9 ml with ddH2O, and 1 µl of cDNA was added to give a total reaction volume of 10 µl. PCR reactions for Il-1β were performed in a 10 µl reaction using the supplied mastermix and primers (QT00181657, Qiagen Pty Ltd, Vic, AU) and 1 µl of cDNA, according to the manufacturer’s instructions.

4.6.5.2 PCR reaction cycle

All qPCR analyses were performed on the Rotorgene Q (Qiagen, Hilden, Germany). Initial PCR runs were conducted to optimise assay conditions (i.e. MgCl2 concentrations and annealing temperatures) for each primer pair. The optimised PCR reaction cycle consisted of an initial denaturing stage at 95°C for 10 min, followed by 45 cycles comprising of 95°C for 1 sec, a primer-specific annealing temperature (detailed in Table 4.1) for 15 sec, and a 72°C extension period for 5 sec. Cycling conditions for Il-1β consisted of a two-step PCR, with incubation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 15 sec and the annealing and elongation steps combined at 60°C for 30 sec.

4.6.5.3 Melt curve analysis and quantification of transcripts

Melt peaks analyses (from 66°C to 99°C) were conducted to confirm a single PCR product; a single peak indicates that one product has been amplified (see Figure 4.3). External standards were created for each gene of interest from 10-fold serial dilutions of gel-extracted PCR product (see Section 4.6.4). These dilutions were used to generate a standard curve, which allowed relative expression of the gene of interest to be quantified using Rotorgene Q series software 1.7 (see Figure 4.3). The reference genes Ppia, Sdha and Ywhaz were used as internal controls, and expression values for each sample were standardised using the GeNorm algorithm (Vandesompele et al. 2002). No treatment or time-of-day effects were observed in reference gene expression.
Figure 4.3 RT-qPCR quantification parameters. (A) Melt curve analysis to confirm a single melt peak, (B) amplification plot, and (C) calculated standard curve for a representative gene (Bmal1 mRNA). The threshold line (B) indicates the point at which all samples fall within the log-linear range of amplification. This enables the standard curve (C) for each gene to be calculated.
4.7 Measurement of plasma steroids by LC-MS/MS

Levels of corticosterone, 11-dehydrocorticosterone (11-DHC), progesterone and testosterone were measured in maternal and day 21 fetal plasma samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The assay was optimised and conducted with the kind assistance of Asst/Prof. Michael Clarke (Centre for Metabolomics, Perth, WA, Australia).

LC-MS/MS is a highly sensitive technique which allows for the simultaneous quantification of multiple steroids within one plasma sample, while avoiding the antibody cross-reactivity that often occurs in conventional immunoassays (Stanczyk & Clarke 2010). The first phase of the LC-MS/MS technique introduces the sample to a mobile phase, which is passed through a column (stationary phase) to induce chromatographic separation of the components within the sample. This is followed by conventional mass spectrometry (MS), which identifies analytes based on their mass-to-charge ratio following ionization.

4.7.1 Steroid extraction

Steroids were extracted from plasma using a methyl tertiary butyl ether (MTBE) extraction protocol. Briefly, plasma samples (50 μl) were mixed with 50 μl internal standard mixture containing deuterium labelled steroids, namely progesterone d9 (5 ng/ml), cortisol d4 (50 ng/ml) and testosterone d5 (10 ng/ml). This mixture was incubated at room temperature for 5 min then mixed with 1 ml of MTBE. Samples were vortexed for 2 min and centrifuged for 5 min at 800 x g, after which 900 μl of supernatant was removed and dried in a centrifugal vacuum evaporator for 30 min at 40°C. Dried residue was resuspended in 70 μl of mobile phase (70% methanol, 0.1% formic acid, 29.9% H₂O) and heated for 10 min at 50°C. During method development, recovery was assessed by spiking pure standards into test serum samples and measuring the response. The recovery was between 70-90% for all analytes, however when adjusted by the labelled internal standard, the measured value was between 98-103% of the predicted value.

4.7.2 LC-MS/MS

LC-MS/MS was performed on the extracted samples (20 μl) using an Agilent 6460 Triple Quadropole MS system coupled to two 1290 UPLC Series LC pumps (Agilent Technologies). The LC system was operated in 2 dimensional (2D) mode using two columns; the first was an Agilent Poroshell 120 EC-C18 (2.1 x 50 mm x 2.7 μm; Agilent
Technologies, Santa Clara, CA, USA) and the second was a Phenomenex C18 (150 x 3.0 mm x 2.6 μm; Torrance, CA, USA). Mobile phases consisted of (A) Optima LCMS grade water (Thermo Fisher Scientific, Scoresby, VIC, Australia) and (B) LCMS grade methanol (Chem-Supply, Gillman, SA, Australia); both in solution with 0.1% formic acid. The flow rate of mobile phase was 0.2 ml/min and the column compartment temperature was 30°C. A gradient was applied to the columns as follows: t = 0, 70% B; t = 5, 80% B; t = 7, 98% B; t = 8, 98% B; t = 8.5, 60% B; t = 9, 70% B, t = 11, 70% B.

The MS system was operated in positive ion multiple reaction monitoring (MRM) mode and operating conditions were as follows: gas temperature at 275°C, gas flow at 5 L/min, nebulizer pressure at 50 psi, sheath gas temperature at 325°C, sheath gas flow at 11 L/min, capillary voltage at 4000 V and charging voltage at 500 V. The following transitions were monitored during each data acquisition: testosterone 289.2 → 109.2; testosterone d5 294.2 → 113.3; cortisol 363.2 → 121.0; cortisol d4 367.2 → 121.0; 11-DHC 345.2 → 121.0; corticosterone 347.3 → 329.1; progesterone 315.3 → 109.2; progesterone d9 324.3 → 100.2. Cortisol d4 was used as the internal standard for both 11-DHC and corticosterone. Pure metabolites were spiked in SeraCon II charcoal-stripped serum to create each standard curve. All standard curves had R² values > 0.999; a representative standard curve for progesterone is shown in Figure 4.4.

The assay precision was evaluated by including EDTA plasma samples spiked with known concentrations of corticosterone and 11-DHC. Commercial QC’s (Bio-Rad Laboratories, Hercules, CA, USA) were used for testosterone and progesterone. Inter- and intra-assay coefficients of variation (CV) were 9.7% and 1.4% respectively for corticosterone, 17.5% and 3.2% for 11-DHC, 7.7% and 0.3% for progesterone and 6.7% and 1.0% for testosterone. Mean results for the commercial QC’s were all within the quoted range expected in the QC kit inserts. Data were processed using Agilent MassHunter™ quantitative software.
Figure 4.4 Standard curve for progesterone quantitation. Values for five standards are shown, with progesterone concentration plotted against peak height ratio to internal standard.
4.8 Measurement of plasma lipids

Levels of total cholesterol (CHOL), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were measured in maternal and day 21 fetal plasma samples. These analyses were kindly performed by Dr Trevor Mori (School of Medicine and Pharmacology, The University of Western Australia). Each lipid was measured enzymatically using an analyte-specific reagent (Abbott Diagnostics, Abbott Park, IL, USA) and analysis was performed on the Architect c16000 analyser (Abbott Diagnostics, Abbott Park, IL, USA) as per the manufacturer’s instructions. The detection principles for each lipid analyte are explained briefly below and assay precision is summarised in Table 4.2.

4.8.1 Cholesterol

Cholesterol esters were hydrolysed to cholesterol and free fatty acids by cholesterol esterase, after which free cholesterol was oxidised to cholest-4-ene-3-one and hydrogen peroxide (H₂O₂). The resultant H₂O₂ then combined with hydroxybenzoic acid (HBA) and 4-aminoantipyrine to create a quinoneimine dye chromophore. This was quantitated at 500 nm to indicate cholesterol concentration within a sample.

4.8.2 Triglyceride

Triglycerides were hydrolysed to glycerol and free fatty acids by lipase. Glycerol then underwent a series of reactions to generate hydrogen peroxide (H₂O₂). Peroxidase was added to catalyse a colour reaction, whereby H₂O₂ interacted with 4-aminoantipyrine (4-AAP) and 4-chlorophenol (4-CP) to produce a red dye. The absorbance of this dye was quantitated to provide an estimate of triglyceride levels within each sample.

4.8.3 HDL Cholesterol

The Ultra HDL Reagent (Abbott Diagnostics, Abbott Park, IL, USA) was used to directly measure plasma HDL-C concentration. Non-HDL unesterified cholesterol underwent a series of reactions to generate H₂O₂ which was then consumed by peroxidase. A detergent then solubilised HDL-C, which reacted with cholesterol esterase and a chromogenic coupler to induce a colour-reaction. Colour levels produced by this reaction were used to estimate the HDL-C concentration in each sample.

4.8.4 LDL Cholesterol

The MULTIGENT Direct LDL assay (Abbott Diagnostics, Abbott Park, IL, USA) was used to measure plasma levels of LDL-C. A unique detergent was used to solubilise non-
LDL particles; cholesterol was then consumed by cholesterol esterase and cholesterol oxidase in a colourless reaction. A second detergent solubilised LDL particles, which then enzymatically reacted with a chromogenic coupler. Colour levels produced by this reaction were proportional to LDL-C concentrations within the sample.

Table 4.2 Assay precision for plasma lipid analysis.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean concentration (mmol/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>3</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>0.51</td>
</tr>
<tr>
<td>Triglyceride</td>
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<td>1.92</td>
</tr>
<tr>
<td></td>
<td>2.42</td>
<td>1.02</td>
</tr>
<tr>
<td>HDL Cholesterol</td>
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</tr>
<tr>
<td></td>
<td>2.09</td>
<td>1.94</td>
</tr>
<tr>
<td>LDL Cholesterol</td>
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<td>1.69</td>
</tr>
<tr>
<td></td>
<td>3.38</td>
<td>1.56</td>
</tr>
</tbody>
</table>

4.9 Measurement of plasma insulin and leptin

Levels of insulin and leptin were measured in maternal and day 21 fetal plasma samples using a MILLIPLEX MAP Rat Adipokine magnetic bead panel (Cat. # RADPKMAG-80K; Merck Millipore, MA, USA).

4.9.1 Background

The MILLIPLEX MAP assay utilises fluorescence-coded magnetic beads (microspheres) which are coated with a capture antibody specific to the analyte of interest (e.g. insulin). Once the analyte binds to the antibody on the bead surface, a biotinylated detection antibody is added to the reaction mix. A fluorescent reporter molecule is then introduced, which competes with the analyte for binding to the detection antibody. The microspheres are then passed through a laser which excites the fluorescent dye on the reporter molecules, the colour of which is specific to a particular analyte. The concentration of each analyte can then be quantitated by reading the fluorescence levels emitted from each bead (the degree of which indicates the amount of analyte binding). The specificity of the beads allows for the quantification of several analytes within one assay by using multiple, uniquely colour-coded microsphere groups.
4.9.2 Assay protocol

All reagents for this assay were provided with the kit and prepared according to the manufacturer’s instructions, with the exception of the Luminex Sheath Fluid, which was purchased separately (Merck Millipore, MA USA). Working standards were prepared by diluting the original reconstituted standard in assay buffer in 3-fold serial dilutions. Frozen plasma samples were thawed and centrifuged at 13,000 x g for 5 min prior to analysis.

Assay buffer (200 µl) was added to each well and the plate was sealed and mixed on a plate shaker for 10 min at room temperature. The assay buffer was then decanted and 25 µl of quality control or standard was added to the appropriate wells, while 25 µl of assay buffer was added to background and sample wells. Serum matrix solution (25 µl) was added to the background, standard and control wells and 25 µl of plasma sample was added to the appropriate sample well. The mixed beads were then vortexed and 25 µl was added to each well. The plate was sealed, wrapped in aluminium foil and incubated at 4°C on a plate shaker overnight (18 h). The following morning, fluid was decanted and the plate was washed three times with wash buffer. Detection antibodies (25 µl) were added to each well and the plate was then sealed, covered with aluminium foil and incubated for 2 h at room temperature. The fluorescent labelled receptor molecule (Streptavidin-Phycoerythrin; 50 µl) was added to each well and the plate was again sealed, covered in foil and incubated for 30 min at room temperature. Fluid was removed and the wells washed three times, after which 100 µl of sheath fluid was added to all wells. The plate was then read on a Luminex Magpix analyser (Luminex Corporation, Austin, TX, USA) and standard curves were generated for each analyte using Magpix 4.2 Software (see Figure 4.5).
Figure 4.5 Standard curves for quantitation of plasma insulin and leptin. Concentration values (pg/ml) for each standard are plotted against mean fluorescence intensity (MFI).

4.9.3 Plasma insulin ELISA
Maternal plasma insulin levels were measured using a Rat/Mouse Insulin ELISA kit (EMD Millipore Corporation, Billerica, MA, USA) for the initial cohort of animals analysed in Chapter 5. The assay was performed according to the manufacturer’s instructions, and the plate read on a BioTek Powerwave XS Spectrophotometer with KC4 software (v 3.4).

4.10 Measurement of plasma inflammatory markers
Levels of inflammatory cytokines IL-1β, IL-6, IL-10, IL-12p40, MCP1, GRO/KC, MIP-2 and TNFα were measured in a subset of maternal plasma samples using a MILLIPLEX MAP Rat Cytokine/Chemokine magnetic bead panel (Cat. # RECYTMAG-65K; EMD Millipore Corporation, Billerica, MA, USA). This assay used the same technology as the Rat Adipokine MILLIPLEX MAP assay (described in section 4.9), however the protocol details differed slightly and are described below.

4.10.1 Assay Protocol
All reagents were prepared according to the manufacturer’s instructions. Frozen plasma samples were thawed and centrifuged at 13,000 g for 5 min, after which they were diluted 1:2 in assay buffer in preparation for analysis. Working standards were created by dilution of the original reconstituted standard in assay buffer in a series of 3-fold serial dilutions.

Assay buffer (200 µl) was added to each well and the plate was sealed and incubated for 10 min at room temperature on a plate shaker. Assay buffer was then decanted and 25 µl
of standard or control was added to appropriate wells, while 25 μl of assay buffer was added to the background and sample wells. Matrix solution (25 μl) was then added to the background, standard and control wells and 25 μl of diluted plasma was added to the appropriate sample wells. The mixed beads were then vortexed and 25 μl was added to each well, after which the plate was sealed, covered in aluminium foil and incubated at room temperature on a plate shaker for 2 h. Liquid was removed from all wells and the plate was washed twice in wash buffer, after which detection antibodies (25 μl) were added to each well. The plate was again sealed and covered in foil and placed on a plate shaker for 1 h at room temperature. The reporter molecule Streptavidin-Phycoerythrin (25 μl) was then added to each well and the plate was covered and incubated for 30 min at room temperature, after which it was washed twice. Sheath fluid (125 μl) was added to each well and the plate was read on a Luminex Magpix (Luminex Corporation, Austin, TX, USA) and analysed using Magpix 4.2 Software. All quality controls fell within the expected range and the intra-assay coefficients of variation were 2.3% for IL-1β, 1.8% for IL-6, 2.2% for IL-10, 2.8% for IL-12p40, 3.0% for MCP-1, 2.4% for GRO/KC, 2.4% for MIP-2 and 2.5% for TNFα. All standard curves had R² values > 0.998; representative standard curves for IL-6 and TNFα are presented in Figure 4.6.

**Figure 4.6** Representative standard curves for quantitation of plasma inflammatory markers. Concentration values (pg/ml) for IL-6 and TNFα standards are plotted against mean fluorescence intensity (MFI).
4.11 Statistical analysis

All data are expressed as the mean ± SEM. For fetal and placental weights, each litter represents \( n=1 \). Analyses of variance (ANOVAs) were performed using Genstat version 9.0 (VSN International Ltd., Hemel Hempstead, UK). Maternal weight, food intake and \( T_c \) data were assessed by repeated measures ANOVA to test for variation due to diet and time. All measures across the six collection time points (i.e., fetal and placental weights, gene expression and plasma analytes) were assessed by three- or four-way ANOVA to account for variation by diet, gestational day, fetal sex, time of day (ZT) and/or photoperiod (light vs. dark), as appropriate. When the \( F \) test reached statistical significance \((P<0.05)\) differences were assessed by least significant difference (LSD) tests, except when significant \((P<0.05)\) interactions between factors were observed. In these cases, data were further assessed by two- or three-way ANOVAs or unpaired \( t \)-test, as appropriate.

Cosinor analysis was used to measure rhythmicity in a range of experimental endpoints measured across the day (i.e. gene expression, plasma analytes and \( T_c \)). This was achieved using non-linear regression analysis in Genstat 9.0 software (version 9.0; VSN International Ltd., Hemel Hempstead, UK), according to the following equation:

\[
y = \text{mesor} + \text{amplitude} \times \cos \left( \frac{2 \times c \pi}{24} (ZT - \text{acrophase}) \right).
\]

Cosinor analysis derived the classic rhythm features of mesor, amplitude and acrophase for each daily profile (Figure 4.7). The coefficient of determination \((r^2)\) was used as an index of cosinor rhythmicity; daily profiles were considered ‘rhythmic’ when their fit to the cosine function was significant \((P<0.05)\), as assessed by the Pearson R calculator, available at: [http://www.socscistatistics.com/pvalues/pearson-distribution.aspx](http://www.socscistatistics.com/pvalues/pearson-distribution.aspx).

The influence of diet and gestational day on these cosine curve features was assessed by two-way ANOVA (GraphPad Prism version 6.00; La Jolla, CA, USA). When the \( F \) test reached statistical significance \((P<0.05)\) differences were assessed by LSD tests, except when significant interactions occurred between diet and gestational day. In these cases differences were assessed by unpaired \( t \)-test (GraphPad Prism version 6.00; La Jolla, CA, USA).

The relationship between clock genes and downstream genes of interest (\( Ppar\delta \)) was assessed by linear regression analysis (see Chapter 8 for specific details).
Fig 4.7 Cosine curve features. Schematic representation of the typical cosine curve features: mesor (the rhythm adjusted mean), amplitude (the difference between the mesor and the rhythm peak), and acrophase (the time of the rhythm peak).
Chapter 5: Maternal obesity induced by a ‘cafeteria’ diet in the rat does not increase inflammation in maternal, placental or fetal tissues in late gestation

Preface

This chapter presents the results of what was effectively a pilot study, conducted in order to optimise the CAF feeding model prior to commencing the large-scale circadian experiment. Dietary manipulation was performed in a small group of animals, and tissues were collected at a single time-point (midday; ZT5) on day 21 of gestation. In addition to establishing the effectiveness of the CAF diet at inducing maternal obesity, this study also provided important data on the inflammatory status of maternal, fetal and placental tissues late in obese pregnancy. Inflammation was chosen as a focal point since it is commonly associated with obesity and pregnancy pathogenesis, and has ties to the circadian system.

Authorship and publication status: This chapter was co-authored by Brendan J. Waddell and Peter J. Mark, and was accepted for publication in Placenta (Vol 39, pp 33-40) in Jan 2016.

Contributions: Rachael Crew performed effectively all animal work, laboratory work and data analysis, representing almost 100% of experimental work for this chapter. She was also largely responsible for writing and submitting the manuscript, representing approximately 80% of written work for this chapter.
5.1 Abstract

Obesity during pregnancy can cause serious complications for maternal and infant health. While this has often been attributed to increased inflammation during obese pregnancy, human and animal studies exhibit variable results with respect to the inflammatory status of the mother, placenta and fetus. Cafeteria (CAF) feeding induces more inflammation than standard high-fat feeding in non-pregnant animal models. This study investigated whether maternal obesity induced by a CAF diet increases maternal, fetal or placental inflammation. Maternal obesity was established in rats by 8 weeks of pre-pregnancy CAF feeding. Maternal plasma inflammatory markers (IL-1β, IL-6, IL-10, IL-12p40, MCP1, GRO/KC, MIP-2 and TNFα) and expression of inflammatory genes (Tnfa, Il-6, Il-1β, Tlr2, Tlr4, Cox2 and Emr1) in maternal, placental and fetal tissues were measured at day 21 of gestation. Despite CAF animals having 63% more central body fat than controls at day 21 of gestation, plasma inflammatory markers were not increased; indeed, levels of IL-6, IL-12p40 and MIP2 were reduced slightly. Similarly, inflammatory gene expression remained largely unaffected by CAF feeding, except for slight reductions to Tlr4 and Emr1 expression in CAF maternal adipose tissue, and reduced Tlr4 expression in male labyrinth zone (LZ). The junctional zone (JZ) displayed increased Il-6 expression in CAF animals when fetal sexes were combined, but no inflammatory genes were affected by the CAF diet in fetal liver. Maternal obesity induced by a CAF diet before and during pregnancy does not increase the inflammatory status of the mother, placenta or fetus in late gestation.
5.2 Introduction

Obesity in pregnancy is increasingly common in Western societies and can lead to serious health complications for both the mother and fetus. Maternal obesity increases the incidence of various pregnancy complications including preeclampsia and gestational diabetes, and can result in adverse cardiometabolic programming outcomes in offspring (Sebire et al. 2001; Leddy, Power & Schulkin 2008). Whilst maternal obesity is typically linked to fetal macrosomia (Catalano et al. 2012) it is also associated with increased rates of intrauterine growth restriction (IUGR) (Gardosi & Francis 2009). Although the mechanisms underlying IUGR in obese pregnancy are not fully understood, it may reflect placental dysfunction in at least a subset of cases.

Obesity is considered a chronic inflammatory state, and increased levels of pro-inflammatory cytokines are thought to contribute to metabolic disease in obese individuals (Iyer et al. 2010). Pregnancy is also an inflammatory condition, likely due in part to placental production of pro-inflammatory cytokines in both humans (Hauguel-de Mouzon & Guerre-Millo 2006) and rats (Mark et al. 2013). Thus, the combination of obesity and pregnancy could exacerbate maternal and fetal inflammatory status and thereby exert negative effects on maternal metabolic pathways, placental function and the in utero environment (Ramsay et al. 2002; Denison et al. 2010). While some human studies have shown elevated maternal inflammatory status in obesity (Ramsay et al. 2002; Basu et al. 2011; Aye et al. 2014), others observed this effect only early in gestation (Friis et al. 2013) or not at all (Sen et al. 2013). Maternal obesity also increased pro-inflammatory cytokine expression in human term placenta (Challier et al. 2008; Roberts et al. 2011; Frias et al. 2011), but fetal plasma cytokines remained unaffected (Challier et al. 2008; Aye et al. 2014). Animal models of obese pregnancy show similarly mixed results; for example, a recent report indicates that pregnancy may actually reduce visceral adiposity and adipose tissue inflammation in mice with high fat (HF) diet-induced obesity (Pedroni et al. 2014), while others show either unchanged (Lager et al. 2014; Ingvorsen et al. 2014) or increased maternal inflammatory status (Kim et al. 2014). Even in the presence of increased maternal inflammation, two separate rodent studies show that placental expression of inflammatory cytokines can be reduced, unchanged or increased, and also vary with fetal sex and gestational age (Reynolds et al. 2015; Kim et al. 2014). Overall, these complex and often conflicting results raise significant doubt as to whether excessive inflammation is a characteristic of obese pregnancy.
Such inconsistencies in the inflammatory phenotype of obese pregnancy may reflect differential effects of obesity per se and the diet consumed during the pregnancy. Thus, while obesity in most rodent models is established by provision of a HF diet, a recent report in adult male rats suggests that obesity induced by a ‘cafeteria’ (CAF) diet (i.e. one that more closely replicates the highly palatable, energy dense foods prevalent in Western societies) generates a more robust metabolic phenotype (Sampey et al. 2011). In rat pregnancy, CAF-induced obesity has been shown to reduce placental and fetal growth, but its impact on inflammatory status is unknown (Akyol, Langley-Evans & McMullen 2009). Therefore, the present study tested the hypothesis that CAF-induced maternal obesity increases the inflammatory status of the mother, fetus and placenta. Obesity was established by provision of a CAF diet for 8 weeks prior to pregnancy, and maternal systemic inflammatory markers and inflammatory gene expression in maternal liver and retroperitoneal (RP) adipose tissue were measured at day 21 of gestation. These gene expression measures were also made in fetal liver and the junctional and labyrinth zones of the placenta. Separate analysis of placental zones was necessary because they show marked differences in inflammatory cytokine gene expression (Mark et al. 2013).

5.3 Materials & Methods

5.3.1 Animals and diets

All animal procedures were approved by the Animal Ethics Committee of The University of Western Australia. Female nulliparous albino Wistar rats, 3 weeks old, were obtained from the Animal Resources Centre (Murdoch, W.A., Australia) and housed 3 per cage under a 12:12h light-dark cycle. Following a week of acclimatisation, animals were given ad libitum access to either a control (CON; n=7) diet of standard rodent chow (total energy 14 KJ/g; 12% energy as fat, 23% as protein and 65% carbohydrate; Specialty Feeds, Glen Forrest, W.A., Australia) or a cafeteria (CAF; n=7) diet, which consisted of human snack food items (for example, cheesecake, hot dogs, cookies) in addition to standard chow. Animals were given a selection of four CAF items per day; these were provided in excess to be essentially ad libitum and changed daily to maintain novelty, although a consistent mixture of ‘sweet’ and ‘savoury’ items were offered each day. The CAF diet provided an average total energy of 15.6 KJ/g, with 41% energy as fat, 12% as protein and 47% as carbohydrate. A detailed list of items used in the CAF diet and the energy density and macronutrient distribution of each is provided in Supplementary Table 5.1. Food intake was recorded by weighing CAF item remnants daily, and animals were weighed weekly throughout the pre-pregnancy period.
After 8 weeks of diet provision, animals were mated overnight at proestrous (determined by an estrous cycle monitor, EC40; Fine Science Tools, Vancouver, British Columbia, Canada). Day 1 of pregnancy was confirmed by presence of spermatozoa in a vaginal smear the following morning, and pregnant animals were maintained on their respective diets in separate cages throughout pregnancy. Food intake and body weight were measured daily.

5.3.2 Tissue collection

Animals were anesthetised using isoflurane/nitrous oxide at 1200 h on day 21 of gestation. Fetal-placental pairs were removed via caesarean section and fetal sex determined by measurement of anogenital distance (Imperato-McGinley et al. 1986). Because fetal responses to in utero insults are often sex-dependent (Scott et al. 2009), male and female placentas and fetuses were analysed separately. Fetal and whole placental weights were recorded and placentas were then dissected into junctional (JZ) and labyrinth (LZ) zones and these were weighed separately. Maternal liver and retroperitoneal (RP) adipose tissue were also collected. Maternal blood was taken from the descending aorta and fetal trunk blood obtained by decapitation. Maternal and fetal blood glucose levels were measured immediately using an Accu-Check blood glucose monitor (Accu-Chek; Roche Diagnostics, Mannheim, Germany). Blood samples were mixed with 10:1 (vol:vol) 0.6 M EDTA and centrifuged at 13,000 x g for 6 min to isolate plasma. All collected tissue and plasma samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Dams were euthanized following tissue collection. The uterus and its contents were removed, leaving the mesentery and associated parametrial fat depot intact. Carcasses were then frozen at -20°C for subsequent measurement of total percentage body fat by dual energy X-ray absorptiometry (DEXA), using a GE Lunar Prodigy Series machine (GE Lunar, Madison, WI, USA) operated in standard mode with small animal software (Encore 2004, version 8.50.093; GE Lunar). To provide an estimate of central body fat, software was used to isolate the DEXA scan to the region demarcated by the superior iliac crests and the inferior sternum.
5.3.3 RNA and cDNA sample preparation

Total RNA was extracted from adipose tissue using the RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany). RNA was isolated from all other tissues using QIAzol (Qiagen Sciences, MD, USA), as per the manufacturer’s instructions. Total RNA was quantitated using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA (1 µg for adipose tissue and 5 µg for all other tissues) was reverse transcribed at 25°C for 10 min then 42°C for 110 min using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) RNase H Point Mutant with random hexamers (Promega, Sydney, Australia) and supplemented with 2.5 mg/ml Ficoll 400 and 7.5 mg/ml Ficoll 70 as previously described (Hewitt et al. 2006). The resultant cDNA was purified using the UltraClean PCR Cleanup Kit, according to the manufacturer’s instructions (MoBio Laboratories, Carlsbad, CA, USA).

5.3.4 RT-qPCR

The relative mRNA expression of pro-inflammatory genes Tnfα, Il-6, Il-1β, Tlr2, Tlr4, Cox2 and Emr1 and reference genes Ppia and Sdha were analysed via RT-qPCR on the Rotorgene 6000 (Corbett Industries, Sydney, Australia). With the exception of Il-1β, all primers (see Table 5.1) were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and were positioned to span an intron to prevent amplification of genomic DNA. Il-1β primers were supplied by Qiagen (Qiagen Pty Ltd, Vic, Australia).

PCR reactions consisted of 10 x Immolase buffer, 2-4 mM MgCl₂ (see Table 1), 10 mM dNTPs, SYBR green at a 1/2000 dilution in DMSO and 0.5 U Immolase DNA Polymerase (Bioline, Alexandria, NSW, Australia) and 1 µl of cDNA template per 10 µl reaction. Ficoll (2.5 mg/mL Ficoll 400 and 7.5 mg/mL Ficoll 70) was added to improve PCR efficiency for Tlr2, Tlr4 and Cox2. The PCR reaction cycle included an initial incubation at 95°C for 10 min, followed by 45 cycles each comprised of denaturation at 95°C for 1 sec, a primer-specific annealing temperature (see Table 5.1) for 15 sec and a 72°C extension period for 5 sec. Cycling conditions for Il-1β consisted of a two-step PCR with incubation at 95°C for 5 min, denaturation at 95°C for 15 sec, and the annealing and elongation steps combined at 60°C for 30 sec.

Standard curves were generated from serial, 10-fold dilutions of gel-extracted PCR product, and relative concentrations were analysed using Rotorgene 6000 series software.
All values were standardised against the reference genes *Ppia* and *Sdha* using the GeNorm algorithm (Vandesompele et al. 2002).

### 5.3.5 Inflammatory cytokine assay

Levels of inflammatory cytokines IL-1β, IL-6, IL-10, IL-12p40, MCP-1, GRO/KC, MIP-2 and TNFα were measured in 25 μl of undiluted maternal plasma using a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore Corporation, Billerica, MA, USA). The assay was performed according to the manufacturer’s instructions and the plate was read on a Luminex Magpix (Luminex Corporation, Austin, TX, USA) and analysed using Magpix 4.2 Software.

### 5.3.6 Plasma insulin assay

Insulin levels in maternal plasma were measured using a Rat/Mouse Insulin ELISA kit (EMD Millipore Corporation, Billerica, MA, USA), according to the manufacturer’s instructions. The plate was read on a BioTek Powerwave XS Spectrophotometer with KC4 software (v 3.4).

### 5.3.7 Statistical analysis

All values are expressed as the mean ± SEM with each litter representing an ‘*n*’ of one. Statistical analysis was performed using GenStat 9.0 software (Hemel Hempstead, UK). Comparisons of maternal characteristics between the two diet groups were made by Student *t*-tests. Other comparisons were made by two- or three-way ANOVA with diet, fetal sex and placental zone as factors. Where required, data were log-transformed to normalise the distribution of residuals. When the *F* test reached statistical significance (*P*<0.05), differences were assessed by LSD tests.
Table 5.1 Primer details and PCR conditions for inflammatory and reference genes measured by RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing Temperature (°C)</th>
<th>Size (bp)</th>
<th>MgCl₂ (mM)</th>
</tr>
</thead>
</table>
| Tnfα  | F 5’-TACTGAACCTCGGGGTGATTGGTCC-3’  
R 5’-CAGCCTTGTCCCTTGAAGAGAACC-3’           | 60           | 295   | 3          |
| Il-6  | F 5’-TCCGCAAGAGACTTCAGCCAGT-3’  
R 5’-AGCCTCCGATTTGTGAAGTGG-3’             | 60           | 148   | 2          |
| Il-1β | *               | *                          | *         | *          |
| Tlr2  | F 5’-GCCCAGATGGCCACAGGACT-3’  
R 5’-CCTGCGCAGAATGGCCTTCC-3’            | 60           | 137   | 2          |
| Tlr4  | F 5’-CTCACCGGCTCAGCTGTTG-3’  
R 5’-GGGATGTCATGAGGATTTGCTA-3’          | 60           | 189   | 2          |
| Cox2  | F 5’-GGGGAGACCATGTAAGA-3’  
R 5’-GAGGGAACCTTCCACAT-3’              | 59           | 178   | 4          |
| Emr1  | F 5’-CAGCTGTCTCCTCCGACTTTC-3’  
R 5’-TAAATGAGATTCCGGCCCTT-3’           | 60           | 156   | 3          |
| Ppia  | F 5’-AGCATACAGGTTCTGGCACC-3’  
R 5’-TTCACCCTCCAAAGACCAC-3’            | 62           | 127   | 3          |
| Sdha  | F 5’-TGGGGCGACTTGCAGGTCC-3’  
R 5’-CCCCGCTTGCACCTACACC-3’            | 60           | 134   | 2          |

*Qiagen QT00181657; F: Forward primer, R: Reverse primer, bp: base pairs
5.4 Results

5.4.1 Maternal characteristics

CAF animals exhibited a 48% increase in caloric intake in the first week of diet exposure, and maintained increased energy consumption throughout the pre-pregnancy feeding period (average 63% increase; \(P<0.001\); Fig 5.1A). In accordance with this increased caloric consumption, CAF animals gained 40% more total pre-gestational weight than CON animals (\(P<0.001\); \(t\)-test). The separation of weight trajectories first became evident 2 weeks after provision of the CAF diet (CAF 10% heavier; \(P=0.036\); Fig 5.1B) and after 8 weeks the CAF animals were 25% heavier (\(P<0.001\); Fig 5.1B).

The body weight differential between CON and CAF animals persisted with the onset of pregnancy; indeed, total gestational weight gain was 16% higher in CAF animals (\(P=0.035\); Fig 5.1D), consistent with their higher caloric intake across all days of pregnancy (32% higher; \(P<0.001\); Fig 5.1C). CAF animals displayed increased fat mass (\(P<0.001\); Fig 5.1E) and decreased lean mass (\(P<0.001\); Fig 5.1F) in both central and whole body regions with post-mortem DEXA analysis. This corresponded to a markedly increased fat:lean mass ratio in CAF animals (0.61 ± 0.1 CON; 1.3 ± 0.1 CAF; \(P<0.001\); \(t\)-test). CAF animals also displayed a 51% increase in total percentage adiposity (\(P<0.001\)) and a 63% increase in total percentage central adiposity (\(P<0.001\)).

Neither maternal (CON 7.5 mmol/L ± 0.2; CAF 7.9 mmol/L ± 0.2) nor fetal (CON 2.8 mmol/L ± 0.2; CAF 2.7 mmol/L ± 0.1; no significant sex differences) blood glucose levels were affected by the CAF diet. Similarly, maternal plasma insulin levels exhibited no differences between dietary groups (CON 3.1 ng/ml ± 0.7; CAF 4.6 ng/ml ± 1.1).
Figure 5.1 Energy intake, weight gain and body composition analysis in animals that consumed either control (CON; white) or cafeteria (CAF; black) diets. A) pre-gestational caloric intake, B) pre-gestational body weight, C) maternal caloric intake, D) maternal cumulative weight gain from conception, E) maternal fat mass and F) maternal lean mass. Values are the mean ± SEM (n=7 per group). † P<0.001 CON vs. CAF (repeated measures ANOVA). * P<0.05 compared to CON diet (t-test after significant Diet x Gestational Day interaction with repeated measures ANOVA); ** P<0.001 compared to CON diet (t-test).
5.4.2 Fetal and placental weights

Fertility measures, including the average time to copulation, fertility index and number of resorptions, were not different between CON and CAF animals (data not shown). The CAF diet reduced both fetal (7% males, 8% females; $P<0.05$; Fig 5.2A) and placental weights (16% males, 14% females; $P<0.05$; Fig 5.2B) at day 21 of gestation. Placental weight reductions were attributable to growth restriction in both the junctional (21% males, 17% females; $P<0.05$) and labyrinth (16% in males and females; $P<0.05$) zones. Interestingly, litter size appeared to be slightly higher in CAF (16.3 ± 0.9) versus CON (14.3 ± 0.8) mothers, and although not statistically significant ($P=0.139$) this prompted comparisons of total placental and fetal weights between the CON and CAF groups. These showed there was no diet effect on either total placental weight per litter (Fig 5.2C; $P=0.67$) or total fetal weight (Fig 5.2C; $P=0.98$), but the fetal: placental weight ratio was higher in the CAF group (7.7 ± 0.2) compared to CON (7.1 ± 0.2; $P=0.03$).
Figure 5.2 Fetal and placental weights and total litter weight at day 21 of pregnancy in mothers that consumed either control (CON; white) or cafeteria (CAF; black) diets. (A) average fetal weight (B) average placental weight and (C) total fetal and placental weight. Values are the mean ± SEM (n=7 per group). * P<0.05 compared to CON diet (t-test).
5.4.3 Maternal inflammatory status

Reduced plasma levels of IL-6 (41% lower, \(P=0.035\); Fig 5.3), IL-12p40 (42%, \(P=0.018\); Fig 5.3) and MIP2 (24%, \(P=0.045\); Fig 5.3) were observed in CAF mothers. There was also a trend for decreased plasma MCP1 (\(P=0.075\)) and GRO/KC (\(P=0.076\)) in CAF animals. The CAF diet had no effect on maternal plasma levels of IL-1β, TNFα or IL-10.

CAF mothers showed reduced RP adipose tissue expression of Tlr4 (24%, \(P=0.015\); Fig 5.4A) and the macrophage marker Emr1 (28%, \(P=0.006\); Fig 5.4A), and there was a trend for decreased Cox2 expression (\(P=0.066\); Fig 5.4A). RP adipose tissue expression of all other inflammatory genes was unaffected by the CAF diet, as was maternal liver expression of all inflammatory genes (Fig 5.4B).
Figure 5.3 Plasma levels of inflammatory markers at day 21 of gestation in mothers that consumed either control (CON; white) or cafeteria (CAF; black) diets. Values are the mean ± SEM (n=7 per group). * P<0.05 compared to CON diet (t-test).
Figure 5.4 Inflammatory gene expression in A) maternal adipose tissue and B) maternal liver at day 21 of gestation in mothers that consumed either control (CON; white) or cafeteria (CAF; black) diets. Values are the mean ± SEM (n=7 per diet group).

* P<0.05 compared to CON diet (t-test).
5.4.5 Placental and fetal inflammatory status

The effects of the CAF diet on LZ expression of inflammatory genes varied with fetal sex. Male LZ expression of Tlr4 was lower in CAF animals (51%; $P=0.015$; Fig 5.5A), and there was a trend for decreased Emr1 ($P=0.056$; Fig 5.5A) and increased Il-6 ($P=0.062$; Fig 5.5A). All other inflammatory genes (Tnfα, Il-1β, Cox2 and Tlr2) in male LZ were unaffected by diet, as were all genes measured in female LZ (results not shown).

There were no sex differences in inflammatory gene expression in junctional zone or fetal liver, so fetal sexes were combined for these tissues. The CAF diet increased expression of Il-6 in the JZ (74%; $P = 0.016$; Fig 5.5B) but no other genes were altered. Similarly, inflammatory gene expression in the fetal liver was unaffected by the diet (Fig 5.5C).
Figure 5.5 Inflammatory gene expression at day 21 of gestation in A) male labyrinth zone B) junctional zone (fetal sexes combined) and C) fetal liver (fetal sexes combined) from mothers that consumed either control (CON; white) or cafeteria (CAF; black) diets. Values are the mean ± SEM (n=7 per group). *P<0.05 compared to CON (t-test following diet x sex interaction with two-way ANOVA); †P<0.05 compared to CON diet (two-way ANOVA).
5.5 Discussion

The major finding of this study was that CAF-induced maternal obesity did not lead to increased inflammation in maternal, placental or fetal compartments near term, despite the maintenance of markedly increased central adiposity. Indeed, obese mothers showed slight reductions in some systemic inflammatory markers (i.e. circulating IL-6, IL-12p40 and MIP2) and in adipose tissue gene expression of Tlr4 and the macrophage marker Emr1. As such, our data suggest that, at least in late gestation, obesity-induced complications are not mediated by heightened inflammatory status.

The reductions in maternal plasma and adipose tissue inflammatory makers in CAF animals were somewhat unexpected, since obesity is often linked to enhanced inflammation. In this context, however, it is noteworthy that various maternal responses to obesity often seem contradictory; for example, obese women display increased risks for both pre- (McDonald et al. 2010; Shaw et al. 2014) and post-term (Arrowsmith, Wray & Quenby 2011) delivery, and for both fetal macrosomia and IUGR (Gardosi & Francis 2009). Similarly, differential inflammatory responses to obesity are apparent in studies of obese pregnancy in women and in animal models. For example, maternal obesity is reported to increase circulating maternal IL-6 in some studies (Basu et al. 2011; Challier et al. 2008; Ramsay et al. 2002; Roberts et al. 2011) but not others (Aye et al. 2014; Dao et al. 2013; Friis et al. 2013). Similarly, circulating TNFα levels were either up-regulated (Aye et al. 2014) or unchanged (Basu et al. 2011; Madan et al. 2009; Challier et al. 2008) in obese pregnancy, while one study showed reduced TNFα production by T-cells from obese pregnant women (Sen et al. 2013). Obese rodent models show similar inconsistencies, with maternal systemic inflammation either increased (Kim et al. 2014; Reynolds et al. 2015) or unchanged (Lager et al. 2014; Pedroni et al. 2014; Desai et al. 2013) by maternal obesity.

These apparent inconsistencies may reflect differences in the specific diet regimes used to induce obesity. We chose CAF feeding in our model because it appears to be more effective at inducing maternal hyperphagia (Bayol, Farrington & Stickland 2007; Akyol, Langley-Evans & McMullen 2009; Bouanane et al. 2009), possibly because of the ongoing novelty of introduced foods. In contrast, feeding of semi-pure, high-fat diets often leads to auto-regulation of caloric intake in rats (Mark et al. 2011; Howie et al. 2009; Taylor et al. 2003; Khan et al. 2005). Moreover, CAF feeding was recently shown to induce greater adiposity and adipose tissue inflammation than a HF diet in adult male
rats (Sampey et al. 2011). Our observation that CAF feeding did not increase maternal inflammation in pregnancy, despite sustained maternal hyperphagia and markedly increased central adiposity, suggests that pregnancy itself may protect against obesity-induced inflammation, at least in late gestation. Indeed, recent studies show that the inflamed phenotype observed with obesity before pregnancy in humans (Ingvorsen et al. 2014) and mice (Friis et al. 2013) is lost by late gestation. Similarly, Pedroni et al. (2014) have noted that both visceral adiposity and adipose tissue inflammation in HF-fed mice were decreased in late gestation compared to pre-pregnancy levels. Collectively, these reports and the present study suggest that maternal adaptations in late gestation may override some adverse effects of obesity, even in the presence of increased visceral adiposity. Further studies are required to determine whether early pregnancy is a more vulnerable period for obesity-induced inflammation.

Importantly, CAF-fed mothers showed normal blood glucose and plasma insulin levels at the time of tissue collection, suggesting the absence of gestational diabetes mellitus (GDM) despite the obese phenotype. While GDM has been strongly linked to increased maternal inflammation (Pantham, Aye & Powell 2015), not all obese women develop GDM. In this regard our maternal phenotype appears similar to that of King et al. (2013) and supports their contention that metabolic changes in late gestation may mask obesity-induced effects on insulin sensitivity.

We also observed minimal differences in inflammatory gene expression in either the junctional or labyrinth zones of the placenta. There was a slight decrease in Tlr4 expression in the labyrinth zone of CAF male fetuses, comparable to the CAF-induced Tlr4 reduction in the maternal adipose tissue. In contrast, Il-6 expression was increased by the CAF diet in the JZ, despite the absence of maternal inflammation, whilst there was also a non-significant trend ($P=0.062$) for increased Il-6 in male LZ. Inconsistencies in obesity-induced placental inflammation are apparent in human studies, which have documented both increased (Challier et al. 2008) and no change (Lager et al. 2014) to placental inflammation with maternal obesity. Rodent HF feeding models have also noted both heightened and unchanged placental inflammation, although interestingly HF-induced reductions to placental inflammatory gene expression have also been observed in placental tissue of females (Reynolds et al. 2015; Kim et al. 2014). This reduced placental inflammation occurred in conjunction with increased maternal systemic inflammation in both studies, while males were either unaffected (Kim et al. 2014) or displayed increased placental inflammation (Reynolds et al. 2015). Our findings showed
slight reductions in maternal systemic inflammation in conjunction with sex- and zone-dependent changes in placental inflammatory gene expression. While these discrepancies may once again be due to the nature of the CAF diet, they also emphasize the adaptable nature of placenta. This further indicates that placental inflammatory gene expression effects are dependent on fetal sex and gestational stage, and do not necessarily reflect maternal inflammatory state.

Interestingly, the JZ was the only tissue to exhibit an apparent pro-inflammatory state with obesity in our model, albeit with *Il-6* the only gene affected. The restriction of this effect to the JZ highlights the importance of analysing placental zones separately. Previous studies have also shown marked zone-specific changes in inflammatory gene expression across normal rat pregnancy (Mark et al. 2013). Interpretation of inflammatory effects are further complicated by the highly dynamic nature of placental inflammatory cytokine expression across gestation; for example, JZ expression of *Il-6* increases 3-fold between days 16 and 22 in normal rat pregnancy (Mark et al. 2013). Further studies are required to determine whether obesity disturbs this normal pattern of inflammatory cytokine expression in the placenta.

Expression of inflammatory genes in the fetal liver was also unaffected by maternal obesity. This appears to be inconsistent with previous studies that show increased inflammation in fetal tissues with maternal obesity, including the gut (Yan et al. 2011), adipose tissue (Murabayashi et al. 2013) and skeletal muscle (Yan et al. 2010). In contrast, Kandadi et al. (2013) reported obesity-induced reductions in mRNA expression of several inflammatory markers in the fetal heart, but increases in the corresponding protein levels, suggesting that post-translational modifications may have occurred with obesity. Furthermore, a recent review suggests that the fetal inflammatory status does not necessarily correlate with that of the mother (Pantham, Aye & Powell 2015), possibly due to placental regulation of fetal exposure to maternal inflammatory markers.

Although individual fetal and placental weights were slightly lower in CAF animals, the marginal (but not statistically significant) increase in litter size in the CAF group meant that total fetal and placental mass were unaffected by diet. Interestingly, fetal overgrowth (Bouanane et al. 2009; Jones et al. 2009), growth restriction (Mark et al. 2011; Howie et al. 2009; Bayol, Farrington & Stickland 2007; Taylor et al. 2003) or no change to fetal weight (Akyol, Langley-Evans & McMullen 2009; Khan et al. 2005) have all been reported in different rodent models of obese pregnancy. As discussed above, differences in specific experimental interventions may account for such inconsistencies among
models. Importantly, however, maternal obesity can lead to adverse effects both during pregnancy (Lager et al. 2014) and in respect to programming outcomes (Samuelsson et al. 2008) without necessarily affecting fetal growth.

In conclusion, this study showed that CAF-induced maternal obesity had minimal effects on the inflammatory status of the mother, placenta and fetal liver late in rat pregnancy. Given that heightened inflammation has previously been observed in obese pregnancies (in both animal models and human cohorts), further studies are needed to delineate the relative impact of other inflammatory conditions (e.g. PE, GDM), gestational age and the nature of the diet consumed during obese pregnancy.

Acknowledgements

The authors wish to acknowledge Ms Celeste Wale and Mr Greg Cozens (The School of Anatomy, Physiology and Human Biology, The University of Western Australia, Australia) for technical assistance.
## 5.6 Supplemental data

**Supplementary Table 5.8.1 Cafeteria food items and their macronutrient composition.**

<table>
<thead>
<tr>
<th>Food Item</th>
<th>Total Energy KJ/g</th>
<th>% Energy From Fat</th>
<th>% Energy From Protein</th>
<th>% Energy From Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Standard rodent chow</em></td>
<td>14.00</td>
<td>23.0</td>
<td>12.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Frankfurts</td>
<td>10.4</td>
<td>72.1</td>
<td>21.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Meat pies</td>
<td>10.7</td>
<td>52.3</td>
<td>13.1</td>
<td>34.6</td>
</tr>
<tr>
<td>Pate</td>
<td>13.9</td>
<td>87.1</td>
<td>10.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Peanut butter (smooth)</td>
<td>25.6</td>
<td>75.8</td>
<td>15.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Ritz crackers</td>
<td>20.8</td>
<td>46.6</td>
<td>4.8</td>
<td>48.6</td>
</tr>
<tr>
<td>White bread</td>
<td>9.9</td>
<td>11.1</td>
<td>13.1</td>
<td>75.8</td>
</tr>
<tr>
<td>Croissants</td>
<td>15.0</td>
<td>45.3</td>
<td>8.7</td>
<td>46.0</td>
</tr>
<tr>
<td>Lamington cake</td>
<td>12.4</td>
<td>20.2</td>
<td>4.8</td>
<td>75.0</td>
</tr>
<tr>
<td>Chocolate mud cake</td>
<td>14.9</td>
<td>40.3</td>
<td>6.7</td>
<td>53.0</td>
</tr>
<tr>
<td>Wafer biscuits</td>
<td>21.7</td>
<td>45.2</td>
<td>2.3</td>
<td>52.5</td>
</tr>
<tr>
<td>Shortbread cookies</td>
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<td>48.6</td>
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<td>47.2</td>
</tr>
<tr>
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<td>37.7</td>
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</tr>
<tr>
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<tr>
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<td>41.0</td>
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</tr>
<tr>
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<td>58.0</td>
<td>3.8</td>
<td>38.2</td>
</tr>
<tr>
<td>Chocolate cream cake log</td>
<td>14.0</td>
<td>40.0</td>
<td>5.0</td>
<td>55.0</td>
</tr>
<tr>
<td>Fruit cake</td>
<td>14.2</td>
<td>37.3</td>
<td>6.3</td>
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</tr>
</tbody>
</table>
Chapter 6: **Obesity disrupts the rhythmic profiles of maternal and fetal progesterone in rat pregnancy**

**Preface**

The primary objective of this chapter was to analyse the effect of maternal obesity on fetal and placental growth trajectories across days 15-16 and 21-22 of rat pregnancy (i.e. the period of maximal fetal growth). Because steroid hormones can exhibit rhythmic secretory patterns, diurnal profiles of steroid hormones were assessed across maternal and fetal compartments. Maternal and fetal circulating lipid biomarkers were also assessed in across the day.

**Authorship and publication status:** This chapter was co-authored by Peter J. Mark, Michael C. Clarke and Brendan J. Waddell, and was accepted for publication in *Biology of Reproduction* (Vol 95 (3), pp 55; 1-10) in May 2016.

**Contributions:** Lipid analysis for this chapter was kindly performed Dr Trevor Mori, and LC-MS/MS assay optimisation was performed by Dr Michael Clarke. Rachael Crew performed all remaining animal work, laboratory work and data analysis, representing approximately 80% of experimental work for this chapter. She was also largely responsible for writing and submitting the manuscript, representing approximately 80% of written work for this chapter.
Maternal obesity increases the risk of abnormal fetal growth, but the underlying mechanisms remain unclear. Since steroid hormones regulate fetal growth, and both pregnancy and obesity markedly alter circadian biology, we hypothesized that maternal obesity disrupts the normal rhythmic profiles of steroid hormones in rat pregnancy. Obesity was established by cafeteria (CAF) feeding for 8 weeks prior to mating and throughout pregnancy. Control (CON) animals had *ad libitum* access to chow. Daily profiles of plasma corticosterone, 11-dehydrocorticosterone, progesterone and testosterone were measured at days 15 and 21 of gestation (term = 23 days) in maternal (both days) and fetal (day 21) plasma. CAF mothers exhibited increased adiposity relative to CON and showed fetal and placental growth restriction. There was no change, however, in total fetal or placental mass due to slightly larger litter sizes in CAF. Nocturnal declines in progesterone were observed in maternal (39% lower) and fetal (45% lower) plasma in CON animals, but were absent in CAF animals. CAF mothers were hyperlipidemic at both days of gestation, but this effect was isolated to the dark period at day 21. CAF maternal testosterone was slightly lower at day 15 (8%), but increased above CON by day 21 (16%). Despite elevated maternal testosterone, male fetal testosterone was suppressed by obesity on day 21. Neither maternal nor fetal glucocorticoid profiles were affected by obesity. In conclusion, obesity disrupts rhythmic profiles of maternal and fetal progesterone, preventing the normal nocturnal decline. Obesity subtly changed testosterone profiles, but did not alter maternal and fetal glucocorticoids.
6.2 Introduction

Maternal obesity is recognized as a significant risk factor for a range pregnancy complications, including preeclampsia (Marchi et al. 2015; Sebire et al. 2001) and abnormal fetal growth (either macrosomia or growth restriction) (Gardosi & Francis 2009). In association with these altered fetal growth trajectories, offspring born to obese pregnancies carry a greater risk for cardiometabolic complications in later life (Drake & Reynolds 2010; Penfold & Ozanne 2015). Although these links between maternal obesity, fetal growth and developmental programming are well established, the underlying mechanisms remain poorly understood.

Fetal and placental growth are both strongly influenced by steroid hormones, including glucocorticoids and progesterone. Maternal glucocorticoids potently inhibit fetal and placental growth (Mark et al. 2013; Hewitt, Mark & Waddell 2006; Vaughan, Sferruzzi-Perri & Fowden 2012) and lead to adverse programming outcomes in offspring (Seckl 1997; Fowden & Forhead 2015). Similarly, elevated testosterone can reduce fetal growth, in part via suppression of placental amino acid transport (Sathishkumar et al. 2011). In contrast, progesterone supports placental growth and function (Ogle, Mills & Costoff 1990; Mark, Smith & Waddell 2006), possibly by impeding the action of glucocorticoids (Patel, Funder & Challis 2003). Glucocorticoid levels are regulated by the hypothalamic-pituitary-adrenal (HPA) axis, and recent evidence suggests that activity of this axis in pregnancy is altered by maternal obesity. For example, there are reports of both reduced (Luiza, Gallaher & Powers 2015; Stirrat et al. 2016) and increased (Aubuchon-Endsley, Bublitz & Stroud 2014) maternal HPA activity in obese women, and in each of these studies the effects were dependent on time of day. Moreover, maternal progesterone is reduced in severely obese women (Stirrat et al. 2016), whereas in rodent models both corticosterone (Bellisario et al. 2015; Desai et al. 2014) and progesterone (Muir et al. 2015; Parker et al. 2014) are elevated in obese pregnancy. While some of these inconsistencies likely reflect species differences, the timing of sample collection may also be important since circadian variation is a key feature of the HPA axis (Gamble et al. 2014) and sex steroid levels can vary with time of day (Magiakou et al. 1996). Importantly, previous studies in non-pregnant mice show that obesity disrupts various aspects of circadian biology (Hsieh et al. 2009; Kohsaka et al. 2007). Therefore, we hypothesized that maternal obesity in pregnancy disturbs the daily rhythmic profiles of
glucocorticoids, progesterone and testosterone in the mother and fetus. Maternal obesity was established in a rat model prior to pregnancy by cafeteria feeding (Chapter 5) and blood samples were obtained over days 15-16 and 21-22 of gestation (term=23 days), thus spanning the period of maximal fetal growth. The impact of obesity on fetal and placental growth trajectories was also measured across the two sampling days.

6.3 Materials & Methods

6.3.1 Animals and diets
All animal work was approved by the Animal Ethics Committee of The University of Western Australia. Nulliparous female albino Wistar rats were obtained at 3 weeks of age from the Animal Resources Centre (Murdoch, WA, Australia) and housed at the Preclinical Facility at the University of Western Australia. Animals were kept 3 per cage and maintained under a constant 12:12 h light: dark cycle at an ambient temperature of 22°C. After one week of acclimatization, animals were separated into Control (CON) and Cafeteria (CAF) groups. Both groups were allowed ad libitum access to standard rodent chow (14 KJ/g total energy; 12% energy as fat, 23% protein and 65% carbohydrate; Specialty Feeds, Glen Forrest, W.A., Australia), whilst CAF animals were supplemented with a selection of four human junk food items each day (hot dogs, cookies, etc.). Cafeteria items were offered in excess to be essentially ad libitum, and items were rotated each day to maintain novelty. The CAF diet provided an overall average total energy of 16.4 KJ/g; 47% energy as fat, 8% as protein and 44% as carbohydrate; for further details of specific CAF diet components, see Chapter 5.

Animals were maintained on these diets for 8 weeks, during which time food intake was recorded each day in a subset of animals by weighing the CAF items remaining in the cage, while body weights were measured weekly in all animals. After the 8 week feeding period, animals were mated overnight at proestrus (as determined by an estrous cycle monitor; EC40; Fine Science Tools, Vancouver, Canada). Pregnancy was confirmed by the presence of spermatozoa in a vaginal smear the following morning; this was designated as day 1 of pregnancy. Animals were housed individually and maintained on their respective diets throughout gestation, and maternal caloric intake and body weights were measured daily.
6.3.2 Tissue collection procedure

Tissues were collected at four hourly intervals across each of days 15-16 and 21-22 of pregnancy. The time of each collection was expressed relative to Zeitgeber time zero (ZT0), the time of lights-on (0700h); samples were obtained at ZT1, ZT5, ZT9, ZT13, ZT17 and ZT21.

At each collection time, animals were anesthetized (n=7-8 per group) with isoflurane/nitrous oxide. Fetal-placental pairs were removed via caesarean section and fetal and whole placental weights were recorded, after which placentas were dissected into labyrinth (LZ; the site of maternal-fetal exchange) and junctional (JZ) zones and weighed individually (Jones et al. 2013a). Since fetal sex is not externally distinguishable at day 15, fetal tail tissue was collected for later sex determination by amplification of the Sry gene via RT-qPCR, as previously described (Jones et al. 2013b). Fetal sex was determined by anogenital distance at day 21.

Maternal blood was taken from the descending aorta and fetal trunk blood was collected at day 21 via decapitation. Plasma was isolated by mixing samples with 10:1 (vol:vol) 0.6 M EDTA and centrifuging at 13,000 x g for 6 min. Plasma samples were then snap frozen in liquid nitrogen and stored at -80 °C until subsequent analysis.

6.3.3 Body composition analysis

Animals were euthanized following tissue collection, and a subset of maternal carcasses (n=23 CON, n=22 CAF) underwent body composition analysis by dual energy X-ray absorptiometry (DEXA) using a GE Lunar Prodigy Series machine (GE Lunar, Madison, WI, USA). Body composition was assessed as total percentage adiposity, total lean mass, total fat mass and bone mineral content using small animal software (Encore 2004, version 8.50.093; GE Lunar). Central adiposity and lean mass were also calculated by limiting scan analysis to the region between the superior iliac crests and the inferior sternum.
6.3.4 Plasma steroid measurement

Levels of corticosterone, 11-dehydrocorticosterone (11-DHC), progesterone and testosterone were measured in maternal and day 21 fetal plasma samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with isotope dilution. This involved the addition of labeled compounds to correct for differences in steroid recovery and to account for signal variability during LCMS data acquisition. Samples (50 µl) were mixed with 50 µl of deuterated internal standards and incubated for 5 min at room temperature. Samples were then vortexed for 2 min with 1 ml of methyl tertiary butyl ether to extract steroids. Extracts were centrifuged and the supernatant removed and dried in a centrifugal vacuum evaporator for 30 min at 40°C. Dried residue was resuspended in 70 µl of mobile phase (70% MeOH, 0.1% formic acid, 29.9% H2O), heated for 10 min at 50°C, and 20 µl injected onto an Agilent 6460 Triple Quadropole MS system coupled to two 1290 UPLC Series LC pumps (Agilent Technologies). The LC system was operated in 2 dimensional mode using two columns; an Agilent Poroshell 120 EC-C18 (2.1 x 50 mm x 2.7 µm; Agilent Technologies, Santa Clara, CA) and a Phenomenex C18 (150 x 3.0 mm x 2.6 µm; Torrance, CA). The mobile phase flow rate was 0.2 ml/min and the column compartment temperature was 30°C. The MS system was operated in positive ion multiple reaction-monitoring (MRM) mode and assay precision was evaluated by including EDTA plasma samples spiked with the relevant steroids (as quality controls). Inter- and intra-assay coefficients of variation (CV) were 9.7% and 1.4% respectively for corticosterone, 17.5% and 3.2% for 11-DHC, 7.7% and 0.3% for progesterone and 6.7% and 1.0% for testosterone.

6.3.5 Plasma lipid analysis

Total cholesterol (CHOL), triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were measured enzymatically in maternal and day 21 female fetal plasma samples, using reagents from Abbot Diagnostics (Abbott Laboratories, Abbott Park, IL USA). Assays were performed according to the manufacturer’s instructions and analysed on an Architect c16000. The intra-assay coefficients of variation were 0.9% for CHOL, 1.9% for TG, 2.0% for HDL-C and 1.7% for LDL-C.
6.3.6 Statistical analysis

All values are expressed as the mean ± SEM with each litter representing $n=1$. Statistical analysis was performed using GenStat 9.0 software (Hemel Hempstead, UK). Maternal weight and food intake changes were assessed by repeated measures ANOVA, while other comparisons among groups were made by ANOVA to account for variation due to diet, time of day, stage of pregnancy, and fetal sex. When significant interactions were observed, subsequent comparisons were performed by ANOVA or $t$-tests, as appropriate. When the $F$-test reached statistical significance ($P<0.05$) specific group comparisons were made by LSD tests.

Plasma steroid profiles were assessed for rhythmicity using non-linear (cosinor) regression analysis in Genstat 9.0 software (Hemel Hempstead, UK). This analysis generated the key rhythm characteristics (mesor, amplitude and acrophase) for each steroid profile, and the influence of diet and stage of pregnancy on these characteristics was determined by two-way ANOVA (GraphPad Prism version 6.00; La Jolla, California). Daily profiles were considered rhythmic when their fit to the cosine function was significant ($P<0.05$).
6.4 Results

6.4.1 Caloric consumption and weight gain in cafeteria-fed animals
Cafeteria-fed animals exhibited a marked increase in caloric intake ($P<0.001$) over the 8-week pre-pregnancy feeding period (Table 6.1), consistent with previously published data from a smaller cohort (Chapter 5). Accordingly, at day 1 of pregnancy maternal weight was 25% higher in CAF compared to CON mothers ($P<0.001$; Table 6.1). During pregnancy, CAF mothers continued to maintain greater caloric intake ($P<0.001$) such that maternal weight was 25% and 21% higher in CAF mothers at days 15 and 21 respectively (Table 6.1).

6.4.2 Maternal body composition analysis
CAF animals exhibited greater fat mass (Fig 6.1A) and higher total percentage body fat relative to CON at day 15 (65% higher) and day 21 (56% higher) of gestation ($P<0.001$); similar patterns were observed for central adiposity (results not shown). Total lean mass was lower in CAF animals at both days of gestation ($P<0.001$; Fig 6.1B). Consequently, the fat: lean mass ratio was markedly elevated in CAF animals on both days of gestation (day 15: 2.6-fold higher; day 21: 2.3 fold higher; $P<0.001$; Fig 6.1C). CAF animals also exhibited increased bone mineral content ($P<0.001$; Fig 6.1D) at both day 15 (23%) and day 21 (19%), and there was a marginal (6%) increase in bone mineral content from day 15 to day 21, regardless of diet ($P=0.049$).

6.4.3 Fertility measures
CAF animals did not differ from CON in average time to copulation, fertility index (i.e., the proportion of positive smears leading to a pregnancy), or number of resorptions (data not shown). Surprisingly, CAF mothers had larger litter sizes than controls (CON 13.4 ± 0.3, CAF 14.9 ± 0.4; $P<0.001$).
Table 6.1 Body weight and daily caloric intake in CON and CAF animals across the study period.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CAF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weaning (prior to diet exposure)</td>
<td>114 ± 1</td>
<td>115 ± 1</td>
</tr>
<tr>
<td>Pregnancy day 1</td>
<td>273 ± 2</td>
<td>342 ± 3*</td>
</tr>
<tr>
<td>Pregnancy day 15</td>
<td>338 ± 2</td>
<td>423 ± 4*</td>
</tr>
<tr>
<td>Pregnancy day 21</td>
<td>429 ± 5</td>
<td>518 ± 6*</td>
</tr>
<tr>
<td><strong>Average daily caloric intake (KJ)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-pregnancy</td>
<td>232 ± 2</td>
<td>401 ± 5*</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>326 ± 4</td>
<td>447 ± 30*</td>
</tr>
</tbody>
</table>

* $P<0.001$ compared to CON (t-test).
Figure 6.1 Body composition (as measured by DEXA analysis) in animals that consumed either control (CON) or cafeteria (CAF) diets. A) Total fat mass, B) total lean mass, C) fat: lean mass ratio, and D) bone mineral content in mothers at day 15 and 21 of gestation. Values are the mean ± SEM (n=8 per group at day 15, n=13-15 per group at day 21). * P<0.001 compared to corresponding CON group (two-way ANOVA and LSD test). † P<0.05 compared to day 15 (two-way ANOVA).
6.4.4 Fetal and placental weights

At both days of pregnancy, fetal weight was reduced by the CAF diet, but also varied with sex (male > female) and time of day (reflecting fetal growth across each 20-h period; Fig 6.2A & E). Whole placental and LZ weights were also reduced by the CAF diet at both gestational ages. Interestingly, while whole placental and LZ growth was evident across day 15, growth of these tissues had plateaued by day 21. Despite this cessation of placental growth, substantial fetal growth still occurred across day 21 (see Fig 6.2 for data from females and Table 6.2 for all P values; three-way ANOVA). Accordingly, the fetal: LZ weight ratio increased by around 37% during day 21 (from 11.3 ± 0.2 at ZT1 to 15.3 ± 0.4 at ZT21; P<0.001, t-test). JZ weight was reduced by the CAF diet at day 21, and there was also a significant time of day effect in JZ growth (P=0.049), although this did not reflect a progressive increase across the day (see Fig 6.2H).

Given the increased litter size in CAF animals, we also assessed whether total fetal, placental, JZ and LZ weights per litter differed between the two diet groups. This analysis showed that total fetal weight was slightly higher in CAF mothers at day 15 (9%; P=0.048 overall diet effect) but was unaffected by diet at day 21 (data not shown). Total placental, JZ and LZ weights were all unaffected by diet on either day of gestation (data not shown).
Figure 6.2 Female fetal and placental weights at days 15 and 21 of pregnancy in control (CON) or cafeteria (CAF) diet groups. (A) Fetal, (B) whole placental, (C) labyrinth zone (LZ) and (D) junctional zone (JZ) weights at day 15, and (E) fetal, (F) whole placental, (G) LZ and (H) JZ weights at day 21 of pregnancy. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). * $P<0.05$ overall diet effect; † $P<0.05$ overall time of day effect (three-way ANOVA).
Table 6.2 *P* values for diet, fetal sex and time of day variation in fetal and placental weights.

<table>
<thead>
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<th>Source of Variation</th>
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<th>Fetal Sex</th>
<th>Time of Day</th>
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<tr>
<td>Labyrinth zone</td>
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<td><strong>Day 21:</strong></td>
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<tr>
<td>Fetal</td>
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<tr>
<td>Labyrinth zone</td>
<td>0.006</td>
<td>&lt;0.001</td>
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6.4.5 Rhythmic profiles of plasma steroids

a) Corticosterone and 11-DHC: Cosinor analysis showed that corticosterone and 11-DHC were highly rhythmic in maternal plasma at each stage of gestation (see Fig 6.3A, B, D & E) and in male and female fetal plasma at day 21 (Fig 6.3G, H, J & K). In each case, however, the characteristics of these rhythms (mesor, amplitude and acrophase) were unaffected by diet (Fig 6.3). Interestingly, the fetal rhythm of corticosterone appeared to be phase-advanced compared to the maternal rhythm; accordingly, there were significant differences between maternal and fetal corticosterone acrophase (~8 h different for both sexes; \( P<0.05 \); \( t \)-tests). Consistent with these different daily profiles, fetal corticosterone levels at ZT1 were higher than corresponding maternal levels (\( P<0.001 \); \( t \)-test following Maternal/Fetal Status x Time of Day interaction in ANOVA). In contrast, the acrophase of fetal 11-DHC profiles was similar to those for both corticosterone and 11-DHC in maternal plasma (see Fig 6.3).

The ratio of corticosterone to 11-DHC in maternal plasma was also unaffected by diet, but did vary with time of day (\( P<0.001 \); see Fig 6.3C & F). The fetal profile was quite different to that in the mother (Fig 6.3F vs. Figs 6.3I & L), and exhibited a Diet x Time of Day interaction that was related to subtle differences in variation between the diet groups (see Figs 6.3I & L).
Figure 6.3 Rhythmic (cosinor) profiles of plasma corticosterone and 11-DHC, and average daily corticosterone: 11-DHC ratios in control (CON) or cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). * P<0.05, CON vs CAF (t-test following Diet x Time of Day interaction in two-way ANOVA).
b) Progesterone: Cosinor analysis revealed that progesterone profiles were rhythmic in CON animals but not in CAF mothers at day 15 or in CAF male fetuses at day 21. Therefore, progesterone data were analysed by conventional ANOVA, which showed a maternal decline from day 15 to 21 ($P<0.001$) and marked time of day variation. The latter largely reflected a decline in progesterone during the dark hours in CON mothers ($P<0.001$, Fig 6.4A & C). Intriguingly, this nocturnal decline, which was clearly evident at both gestational ages, was absent in CAF mothers. A similar nocturnal progesterone reduction was observed in both male and female fetuses, but again was not observed in the CAF group (see Fig 6.4E & G). Consequently, comparison of progesterone concentrations between the ‘light’ and ‘dark’ photoperiods (Light: ZT0-12; Dark: ZT12-24) showed that CAF animals maintained higher levels exclusively in the dark period ($P<0.01$; Fig 6.4B, D, F & H).

c) Corticosterone: Progesterone ratio: Because progesterone can potentially act as an anti-glucocorticoid (Patel, Funder & Challis 2003) the ratio of plasma corticosterone to progesterone may be important in the regulation of placental and fetal growth. Given the absence of the nocturnal decline in maternal and fetal progesterone in CAF animals, the corticosterone: progesterone ratio was compared between the light and dark periods. This showed no diet effects during either the light or dark at day 15, but by day 21 there was a marked reduction in the corticosterone: progesterone ratio in CAF animals during the dark hours in both the mother (CON 19 ± 5, CAF 6 ± 1; $P<0.01$) and in male (CON 103 ±16, CAF 55 ± 7; $P<0.01$) and female (CON 113 ±18, CAF 56 ±6; $P<0.01$) fetuses.
Figure 6.4 Daily profiles of progesterone and average progesterone values across the ‘light’ and ‘dark’ periods in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). * P<0.05 compared to CON (t-test following Diet x Time of Day interaction with two-way ANOVA). ** P<0.05 compared to CON (t-test following Diet x Photoperiod interaction in two-way ANOVA).
d) Testosterone: Although maternal plasma testosterone did not vary with diet overall, there was a significant Diet x Stage of Pregnancy interaction (\(P<0.002\)). Separate analyses at each stage of pregnancy showed lower testosterone levels (8%; \(P=0.017\); Fig 6.5) in CAF mothers at day 15, but higher levels at day 21 (16%; \(P=0.014\); Fig 6.5). While maternal plasma testosterone was not rhythmic at either day of gestation, significant cosinor rhythmicity was evident in CON male fetuses at day 21 (Fig 6.5). In contrast, testosterone rhythmicity was lost in male fetuses of CAF-fed mothers, which did not show the morning (ZT1) elevation evident in CON (\(P=0.008\)). Testosterone levels in female fetal plasma fell below the detection threshold (<0.05 ng/ml) in both dietary groups (data not shown).

Figure 6.5 Daily profiles of testosterone in maternal plasma at day 15 and 21, and male fetal plasma at day 21 in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (\(n=7-8\) per diet group at each ZT). * \(P<0.05\) overall diet effect (two-way ANOVA); ** \(P<0.05\) compared to CON (\(t\)-test following Diet x Time of Day interaction in two-way ANOVA).
6.4.6 Plasma lipid profiles

All maternal lipids varied overall with diet ($P<0.05$) and increased with advancing gestation ($P<0.001$). Lipid analytes were largely arrhythmic (by cosinor analysis) and did not exhibit overall time of day variation, but each exhibited $Diet \times Time$ of Day or $Diet \times Photoperiod$ interactions by ANOVA at one or both stages of pregnancy ($P<0.05$). This analysis revealed that the CAF diet reduced maternal CHOL levels during the light period at day 15 (13% lower; $P<0.001$; Fig 6.6A), but increased levels at ZT17 on day 21 ($P<0.001$; Fig 6.6E). The CAF diet also increased TG levels at both gestational days, but for day 21 this was evident only during the dark period ($P=0.02$; Fig 6.6B & F). In contrast, the CAF diet reduced maternal HDL-C on both days, but again this effect was restricted to the dark period for day 21 (Fig 6.6C & G). Finally, maternal LDL-C levels were increased by the CAF diet only during in the dark hours at both gestational days (each $P<0.01$; Fig 6.6D & H).

The CAF diet increased fetal levels of CHOL ($P=0.035$; Fig 6.6I), TG ($P<0.001$; Fig 6.6J) and LDL-C ($P<0.001$; Fig 6.6L) across day 21. While all lipid analytes varied with time of day in fetal plasma ($P<0.001$; Fig 6.6I-L), this variation appeared unaffected by the CAF diet.
Figure 6.6 Daily lipid profiles in maternal and fetal (day 21 females only) plasma of control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). Note the y-axis scales for J & D differ from those of corresponding analytes (cholesterol: CHOL; triglyceride: TG; high density lipoprotein cholesterol: HDL-C; low density lipoprotein cholesterol: LDL-C). * P<0.05 overall diet effect (two-way ANOVA); # P<0.001 compared to CON (t-test following Diet x Time of Day interaction in two-way ANOVA); ** P<0.001 CON vs. CAF (t-test following Diet x Photoperiod interaction in two-way ANOVA).
6.5 Discussion

This study demonstrates that obesity induced by cafeteria feeding disrupts maternal and fetal rhythmic profiles of progesterone during pregnancy, preventing the nocturnal decline in progesterone in both compartments. Obesity also altered maternal and fetal testosterone profiles but had minimal effects on the HPA axis. These obesity-induced changes in steroid hormone levels occurred in conjunction with maternal and fetal hyperlipidemia, and a reduction in individual, but not total, fetal and placental weights over the final third of gestation.

The CAF-induced increase in maternal progesterone at both days of pregnancy supports and extends other recent studies in rodents, in which elevated progesterone levels were observed in HF-fed mothers at both mid (Parker et al. 2014) and late (Muir et al. 2015) gestation. Importantly, these studies did not take into account the possible circadian influences on progesterone secretion; our results demonstrate that circulating progesterone in control mothers and fetuses exhibited pronounced rhythmic variation characterized by a marked nocturnal decline, and that this nocturnal decline was prevented by maternal obesity. To our knowledge, these data are the first to demonstrate rhythmic variation of progesterone in rat pregnancy (in either the mother or fetus), and its disruption by maternal obesity. Circadian variation of progesterone is consistent with the known expression of clock genes in the corpus luteum (Chu et al. 2011; Sellix 2015), the major source of progesterone during rat pregnancy. Disruption of the rhythmic profile of progesterone by obesity may be mediated by altered luteal expression of clock genes, since previous studies show marked changes to clock gene expression in metabolic tissues of obese rodents (Hsieh et al. 2009; Kohsaka et al. 2007) and clock genes appear to regulate ovarian progesterone synthesis (Chen et al. 2013; Boden, Varcoe & Kennaway 2013).

Interestingly, maternal LDL-C profiles appeared to broadly parallel those of progesterone. Given LDL-C is a precursor for progesterone synthesis (Niswender 2002), elevated LDL-C in CAF mothers may contribute to the loss of the nocturnal progesterone decline. There was no such similarity between fetal LDL-C and progesterone profiles, consistent with fetal progesterone being derived largely from luteal synthesis (Benbow & Waddell 1995). The maintenance of elevated nocturnal progesterone in obese mothers may also be mediated via metabolic hormone disturbances. For example, insulin
promotes steroidogenesis in granulosa and thecal cells (Poretsky et al. 1999) and HF-feeding in non-pregnant rats increases circulating progesterone in conjunction with hyperinsulinemia and altered insulin signaling in the ovary (Akamine et al. 2010). Similarly, leptin has been shown to stimulate progesterone production by luteal cells both alone (Galvão et al. 2014) and in the presence of IGF-1 (Nicklin et al. 2007), while adiponectin also regulates ovarian steroidogenesis, including luteal progesterone synthesis (Maleszka et al. 2014). Further studies are required to determine whether increased insulin, leptin or other adipokines contribute to the CAF-induced elevation in maternal and fetal progesterone during pregnancy. It will also be of interest to explore the extent to which progesterone is affected by obesity in human pregnancy; in contrast to the rodent response, recent human studies suggest maternal progesterone is reduced in obese pregnancy (Lassance et al. 2014; Stirrat et al. 2016), possibly due in part to species differences in the major source of progesterone (luteal in rodents, placental in humans).

Higher progesterone levels observed in obese pregnancy could potentially exert both positive and negative effects on pregnancy outcome. For example, elevated progesterone would be expected to support placental and fetal growth, possibly by countering the growth inhibitory effects of glucocorticoids (Patel, Funder & Challis 2003). Even though maternal corticosterone levels were not affected by obesity in the current study, the corticosterone:progesterone ratio in CON mothers was substantially higher in the dark hours at day 21. In contrast, the absence of the nocturnal progesterone decline in obese mothers could potentially disrupt the timing of parturition, which is initiated in part via reduced progesterone signaling (Merlino et al. 2007). Indeed, human data clearly show a diurnal pattern in the timing of labour (Olcese 2012; Serón-Ferré, Duscay & Valenzuela 1993), but further studies are required to determine whether this is affected by maternal obesity.

Contrary to our hypothesis, corticosterone and 11-DHC rhythms were largely unaffected by obesity in both maternal and fetal compartments. Previous rodent studies using only a single time point show either unchanged (Mark et al. 2011) or increased maternal corticosterone in obese pregnancy (Bellisario et al. 2015; Desai et al. 2014). Mixed responses to obesity have also been observed in human pregnancy, with reports of both decreased morning cortisol levels (Stirrat et al. 2016) and increased evening levels (Aubuchon-Endsley, Bublitz & Stroud 2014), suggestive of some degree of circadian
disruption. Our data also provide the first account of the full rhythmic profiles of fetal corticosterone and 11-DHC, but these were largely unaffected by maternal obesity.

Comparison of glucocorticoid rhythms in the mother and fetus showed some unexpected asynchrony, possibly related to circadian shifts in maternal and fetal corticosterone production rates. Thus, while maternal and fetal 11-DHC rhythms appeared synchronous at day 21 (with peaks at around the time of lights-off), the fetal corticosterone peak occurred around 8 h earlier than that in the mother. Indeed, fetal corticosterone levels exceeded maternal levels at ZT1, as previously reported for this stage of gestation (Wharfe, Mark & Waddell 2011). This suggests that fetal production of corticosterone at term is elevated at stages of the daily cycle when maternal levels are low. It seems likely, therefore, that the fetus contributes to maternal corticosterone in late gestation, and that this contribution varies across the day. Although the placental glucocorticoid barrier impedes the transfer of glucocorticoids between maternal and fetal compartments, this barrier is reduced to relatively low levels near term (Mark et al. 2009). Shifts in the rhythmic fetal production of corticosterone may also account for the observed maternal-fetal differences in the corticosterone: 11-DHC ratio. Confirmation of this, however, would require isotopic tracer studies conducted at different times across the day.

Previous studies show that elevated maternal testosterone in late gestation reduces birth weight (Voegtline et al. 2013; Carlsen, Jacobsen & Romundstad 2006); an effect possibly mediated by reduced nutrient transfer capacity in the placenta (Sathishkumar et al. 2011). Our data show that obesity slightly reduced maternal testosterone at day 15 but resulted in increased levels by day 21. In contrast, CAF male fetal testosterone levels were lower early on day 21, after which they were similar to controls. While several rodent models show that male offspring born to obese mothers have reduced testosterone and lower sperm counts (Reame et al. 2014; Rodriguez-Gonzalez et al. 2015), to our knowledge this is the first evidence for obesity-induced effects on testosterone in fetal life. Male fetal testosterone levels normally exhibit a surge at day 19 of pregnancy, fall briefly at day 21 then peak again shortly after birth, coincident with key testicular development in rats (Weisz & Ward 1980). Our data suggest that maternal obesity may suppress this normal pattern of testosterone in fetal life, potentially mediating adverse programming effects on male fertility.
CAF feeding also induced hyperlipidemia (i.e. elevated CHOL, TG and LDL-C) and reduced HDL-C levels in maternal and fetal plasma. To our knowledge, these data provide the first account of rhythmic circulating lipid profiles during obese pregnancy. Most notably, by day 21 CAF mothers showed only nocturnal hyperlipidemia, an effect likely associated with the nocturnal feeding patterns of laboratory rodents. Moreover, both intestinal lipid absorption (Pan & Hussain 2007) and mobilisation of lipids from adipose tissue display circadian rhythmicity (Shostak, Meyer-Kovac & Oster 2013), and so CAF mothers could potentially experience disruptions to the circadian control of digestive and/or adipose tissue function. Human studies show that while obese women are hyperlipidemic early in gestation, this effect is less pronounced later in gestation; indeed, some studies show that obese pregnant women have lower circulating lipids compared to their normal weight counterparts (Scifres, Catov & Simhan 2014; Vahratian A et al. 2010). Given our observation of nocturnal hyperlipidemia in obese dams near term, further analysis of circadian variation in the lipid status of obese pregnant women would seem warranted.

We also noted growth restriction of individual placentas and fetuses in CAF animals, consistent with several other rodent obesity models (Mark et al. 2011; Howie et al. 2009; Taylor et al. 2003; Bayol, Farrington & Stickland 2007). Obesity may influence fetal growth by altering various aspects of placental function, such as nutrient transport or vascular development (Higgins et al. 2011). While further studies are necessary to determine whether placental function is altered by the CAF diet, the increased litter size in the CAF group likely contributes to the lower fetal and placental weights reported in our model, since there was no difference in total fetal or placental weight per litter between dietary groups. Given that obesity is generally associated with reduced fertility in both humans and rodents (Sagae et al. 2012; Pasquali, Patton & Gambineri 2007), this increased litter size was unexpected. It most likely reflects a higher ovulation rate in CAF animals, since the number of resorptions did not differ between groups. Unfortunately corpora lutea were not collected in these experiments, so further studies are needed to confirm this suggested increase in ovulation rate. Importantly, compromised placental function (Lager et al. 2014) and programmed health complications in offspring (Varcoe et al. 2011; Samuelsson et al. 2008) can occur without overt changes to fetal growth. Moreover, larger litter size has been associated with adverse neurological and metabolic programming outcomes in rats, likely due to reduced nutrition access and consequent
disruptions to postnatal growth trajectories (Alexeev, Löönerdal & Griffin 2015). This suggests that offspring of CAF mothers may experience altered postnatal outcomes.

This study also provides an interesting insight into the daily patterns of fetal and placental growth at the two stages of pregnancy. Across the sampling period at day 15, rapid relative growth was evident for both the fetus (weight increase of 64% over 20 h) and the placenta (60% increase), with the latter attributable almost exclusively to LZ growth (72% increase). By day 21, fetal growth continued at a rapid absolute rate (increase of approximately 1.2 g per fetus over 20-h), despite the effective cessation of placental growth. This capacity for fetal weight to increase by over 30% in 20 h without any change in placental weight highlights the remarkable increase in placental efficiency that occurs at this late stage of pregnancy.

In conclusion, this study shows that maternal obesity disrupts the rhythmic profiles of progesterone in both the maternal and fetal compartments, and increases maternal testosterone levels while suppressing male fetal testosterone near term. These obesity-induced changes in the daily profiles of progesterone and testosterone occurred in conjunction with maternal and fetal hyperlipidemia, but without any overt effects on the maternal or fetal HPA axes.

Acknowledgements:
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Chapter 7: Diet-induced obesity reduces maternal core body temperature and alters the normal thermoregulatory changes of late pregnancy in the rat

Preface

The primary objective of this chapter was to examine the effects of obesity on the rhythmic profiles of core body temperature prior to and throughout gestation. A subset of animals from the larger cohort underwent surgery to implant intraperitoneal temperature loggers. These were then programmed to record $T_c$ every 15 min throughout the study period, which allowed for a comprehensive analysis of $T_c$ rhythms in obese animals.

Authorship and publication status: This chapter was co-authored by Brendan J. Waddell, Shane K. Maloney and Peter J. Mark, and has been submitted for publication.

Contributions: Rachael Crew performed effectively all animal work, laboratory work and data analysis, representing almost 100% of experimental work for this chapter. She was also largely responsible for writing and submitting the manuscript, representing approximately 80% of written work for this chapter.
7.1 Abstract

Obesity during pregnancy causes adverse maternal and fetal health outcomes and programs offspring for adult-onset diseases, including cardiovascular disease. Obesity also disrupts core body temperature ($T_c$) regulation in non-pregnant rodents, however it is unknown whether obesity alters normal maternal $T_c$ adaptations to pregnancy. Since $T_c$ is regulated by the circadian system, and both obesity and pregnancy alter circadian biology, it was hypothesized that obesity disrupts the normal rhythmic patterns of $T_c$ before and during gestation. Obesity was induced by cafeteria (CAF) feeding in female Wistar rats for 8 weeks prior to and during gestation, while control (CON) animals had free access to chow. Intraperitoneal temperature loggers measured daily $T_c$ profiles throughout the study, while maternal body composition and leptin levels were assessed near term. CAF animals exhibited increased fat mass (93%) and associated hyperleptinemia (3.2-fold increase) compared to CON animals. CAF consumption reduced the average $T_c$ (by up to 0.29°C) across the estrous cycle and most of pregnancy; however $T_c$ for CAF and CON animals converged towards the end of gestation. Obesity reduced the amplitude of $T_c$ rhythms at estrus and proestrus and on day 8 of pregnancy, but increased the amplitude at day 20 of pregnancy. Photoperiod analysis revealed that obesity reduced $T_c$ exclusively in the light period during pre-pregnancy but only during the dark period in late gestation. In conclusion, this study shows that obesity alters rhythmic $T_c$ profiles and reduces the magnitude of the $T_c$ decline late in rat gestation, which may have implications for maternal health and fetal development.
7.2 Introduction

In Western societies, the increasing incidence of obesity places a substantial burden on reproductive medicine. Around a third of women of reproductive age are obese (Flegal et al. 2010), and these women are more likely than their normal weight counterparts to experience pregnancy complications such as preeclampsia, gestational diabetes, and stillbirth (Marchi et al. 2015; Kristensen et al. 2005). These adverse effects of obesity also have implications for subsequent generations, since a sub-optimal fetal environment can lead to obesity and other cardiometabolic disorders in adulthood (Frias & Grove 2012; Blackmore & Ozanne 2013).

Although the link between maternal obesity and adverse pregnancy outcomes is well established, the specific mechanisms involved remain unclear. Changes to core body temperature ($T_c$) regulation are among a wide range of metabolic aberrations associated with obesity, but whether thermoregulatory changes occur in obese pregnancy and influence pregnancy outcomes is unknown. Genetic models of obesity such as the New Zealand obese mouse (Jürgens et al. 2006) and the ob/ob and db/db mouse strains (Trayhurn & James 1978; Trayhurn 1979) have lower $T_c$ than controls. Since the energy used to maintain $T_c$ accounts for approximately 60% of an organism’s resting metabolic rate, a lower $T_c$ may decrease energy expenditure and thus facilitate the development of obesity (Landsberg et al. 2009). The impact of diet-induced obesity on $T_c$ regulation, however, is less clear. While some models of high-fat feeding result in lower $T_c$ in obese animals (Tsushima et al. 2014), others show no change to $T_c$ (Roberts, Berger & Barnard 2002; Mills et al. 2007), or even higher $T_c$ with obesity (Gallardo et al. 2012; Perello et al. 2010). In humans, while most reports show no variation in $T_c$ with obesity (Heikens et al. 2011; Hoffmann et al. 2012), a recent study that controlled for ambient temperature, energy intake, and physical activity reported lower $T_c$ profiles in obese individuals relative to their lean counterparts exclusively in the daytime (Grimaldi et al. 2014). This suggests that obesity alters $T_c$ in a circadian-dependent manner, which is consistent with evidence that obesity disturbs various aspects of the circadian system (Kohsaka et al. 2007; Froy 2010; Mendoza, Pévet & Challet 2008).

During pregnancy, any impact of obesity on $T_c$ and its rhythmic variation is likely to be even more complex. In rodent pregnancy, maternal $T_c$ decreases near term (Fewell 1995; Gamo et al. 2013; Cairns et al. 2005; Eliason & Fewell 1997), possibly as an adaptive
response that allows the mother to meet fetal metabolic demand. Interestingly, one study showed the reduction in maternal $T_c$ was partly due to a suppression of the normal nocturnal rise in $T_c$, suggesting that pregnancy alters $T_c$ in a circadian-dependent manner (Fewell 1995). It is unknown if these pregnancy-induced alterations in $T_c$ are the same when the mother is obese, and if not, what impact this has on fetal development. Moreover, while obesity has been shown to disrupt estrous cyclicity in the rat (Glick et al. 1990), its impact on $T_c$ across the cycle is unknown. Consequently, the present study examined the impact of maternal obesity on rhythmic variation in $T_c$ across multiple estrous cycles and throughout gestation. A cafeteria (CAF) feeding protocol was used to establish an obese maternal phenotype and intraperitoneal temperature loggers were used to measure daily profiles of $T_c$. It was hypothesized that maternal obesity reduces absolute $T_c$ and disrupts $T_c$ rhythmicity during pregnancy.
7.3 Materials & Methods

7.3.1 Animals and diets
All procedures involving animals were approved by the Animal Ethics Committee of The University of Western Australia. Nulliparous female albino Wistar rats were obtained from the Animal Resources Centre (Murdoch, WA, Australia) at 3 weeks of age and housed 3 per cage. The animal facility was maintained at an ambient temperature of 22°C under a 12:12 h light-dark cycle, with lights on at Zeitgeber time zero (ZT0; 0700h). Following one week of acclimatization, the animals were allowed ad libitum access to either a control (CON) diet of standard rodent chow (14 KJ/g total energy; 12% energy as fat, 23% as protein and 65% as carbohydrate; Specialty Feeds, Glen Forrest, W.A, Australia) or a cafeteria (CAF) diet, which consisted of four different human snack food items per day (from a stock menu of 17 items) in addition to standard chow. CAF items were offered in excess to be essentially ad libitum and were rotated daily to maintain novelty and prevent autoregulation of caloric intake. The CAF diet provided an average energy density of 16.4 KJ/g (47% energy as fat, 8% as protein and 44% as carbohydrate); further dietary details are available in Chapter 5.

7.3.2 Measurement of core body temperature ($T_c$)
After 4 weeks of diet exposure, each animal ($n=7$ CON, $n=8$ CAF) was anesthetized using isoflurane/nitrous oxide and a temperature logger (DS1922L iButton Temperature Logger, Maxim Integrated, San Jose, USA) was surgically implanted into the peritoneal cavity. The temperature loggers were coated in inert wax prior to implantation to render them impervious to biological fluids (Sasol EXP986, Sasol Chemical Industries Ltd., Johannesburg, South Africa). Animals were allowed to recover from surgery for 1 week, after which temperature loggers recorded $T_c$ every 15 min for 2-3 estrous cycles and throughout pregnancy.

7.3.3 Establishment of pregnancy
After 8 weeks of diet exposure, animals were mated overnight once proestrus was determined using an estrous cycle monitor (EC40; Fine Science Tools, Vancouver, British Columbia, Canada). Day 1 of pregnancy was confirmed by the presence of spermatozoa in a vaginal smear the following morning. Pregnant animals were caged individually and maintained on their respective diets throughout pregnancy.
7.3.4 Recovery of temperature loggers

At day 21 of gestation, each animal was anesthetized using isoflurane/nitrous oxide and temperature loggers removed. Maternal blood was taken from the descending aorta and blood glucose was measured immediately using a blood glucose monitor (Accu-Chek; Roche Diagnostics, Mannheim, Germany). The remaining blood samples were mixed with 10:1 (vol:vol) 0.6M EDTA and centrifuged at 13,000 x g for 6 min to isolate plasma, which was frozen in liquid nitrogen and stored at -80°C until further analysis.

Temperature loggers were calibrated against a certified mercury-in-glass thermometer (National Association of Testing Authorities, Australia) in a water bath at a range of temperatures from 33- 41°C. Data from temperature loggers were downloaded using eTemperature software (version 8.25, OnSolution Pty Ltd., Baulkham Hills, Australia).

7.3.5 Body composition analysis

Following recovery of the temperature loggers, each dam was euthanized and the body composition of the carcass was assessed by dual energy X-ray absorptiometry (DEXA; GE Lunar Prodigy Series; GE Lunar, Madison, Wisconsin). Body composition data were analyzed with small animal software (Encore 2004, version 8.50.093; GE Lunar). A measure of ‘central’ body composition was obtained by isolating analysis to the region bounded by the superior iliac crests and the inferior sternum.

7.3.6 Plasma insulin and leptin measurement

Insulin and leptin were measured in maternal plasma samples using a Milliplex MAP Rat Adipokine Magnetic Bead Panel (Cat. # RCYTMAG-65K; EMD Millipore Corporation, Billerica, MA, USA). The assay was performed according to the manufacturer’s instructions and the plate was read on a Luminex Magpix (Luminex Corporation, Austin, Texas, USA). Data were analyzed with Magpix 4.2 Software.

7.3.7 Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis was performed using GenStat 9.0 software (Hemel Hempstead, UK). Maternal weight and Tc changes were assessed by repeated measures ANOVA to test for the effect of diet. Comparisons of Tc values between the light and dark photoperiod (light hours from ZT0-12, dark hours from ZT12-24) were made by two-way ANOVA, with diet and photoperiod as factors. When the F test reached statistical significance (P<0.05) differences were assessed by LSD.
tests. When significant ($P<0.05$) interactions occurred, differences were assessed by $t$-test.

Daily temperature profiles were assessed using non-linear (cosinor) regression analysis with Genstat 9.0 software (Hemel Hempstead, UK), to calculate mesor (the rhythm – adjusted mean), amplitude (difference from mesor to rhythm peak) and acrophase (the time of the rhythm peak) of daily $T_c$ rhythms. Comparisons of cosine curve features across different gestational days and between dietary groups were made by two-way ANOVA (GraphPad Prism version 6.00; La Jolla, California).
7.4 Results

7.4.1 Maternal characteristics

During the pre-pregnancy feeding period, CAF animals gained more weight than CON \(P=0.002\); Fig 7.1A). The exception to this was the week directly following surgery, when neither dietary group gained weight (Fig 7.1A). In accordance with their increased weight gain trajectory, CAF animals exhibited significantly increased body weight relative to CON, such that they were 11% heavier \(P=0.009\) after 4 weeks of diet exposure and 29% heavier \(P<0.001\) after 8 weeks.

The CAF animals remained heavier than CON throughout gestation \(P<0.001\), but there was no difference in gestational weight gain between diet groups (Fig 7.1B). Despite this, at the end of pregnancy the CAF mothers exhibited a 93% increase in total body fat mass \(P<0.001\); Fig 7.1C) and a 15% reduction in total lean mass \(P<0.001\); data not shown) compared to CON, as measured by post-mortem DEXA analysis. This resulted in a 58% higher total percentage body fat in CAF relative to CON \(P<0.001\) and a markedly higher fat: lean ratio in CAF animals (CON 0.51 ± 0.04; CAF 1.15 ± 0.11; \(P<0.001\)). Central body composition was similarly affected by consumption of the CAF diet (see Fig 7.1C for fat mass in the central region; remaining data not shown).

The plasma leptin level in CAF mothers was 3.2-fold higher than in CON \(P<0.001\); Fig 7.1D), consistent with increased adiposity. There was no difference in either blood glucose (CON 7.7 mmol/L ± 0.4; CAF 7.6 mmol/L ± 0.2) or plasma insulin (CON 2.0 ng/ml ± 0.4; CAF 2.1 ng/ml ± 0.7) between the diet groups.
Figure 7.1 Weight gain profiles, fat mass and plasma leptin levels in animals that consumed control (CON) or cafeteria (CAF) diets. A) Pre-gestational weight gain, B) gestational weight gain C) maternal fat mass and D) maternal plasma leptin at day 21 of gestation. Values are the mean ± SEM (n=7 CON, n=8 CAF). * P<0.01 overall diet effect (repeated measures ANOVA); ** P<0.001 compared to CON (t-test).
7.4.2 $T_c$ profiles across the estrous cycle

The CAF animals had a lower mean daily $T_c$ across all four stages of the estrous cycle ($P<0.001$; Fig 7.2 and 7.3). Cosinor analysis showed that the $T_c$ mesor was lower in CAF relative to CON animals at proestrus (0.29°C lower), estrus (0.19°C), diestrus-1 (0.14°C) and diestrus-2 (0.25°C) (all $P<0.001$; Fig 7.5A). Furthermore, the amplitude of the $T_c$ rhythm was lower in CAF animals at proestrus (21%; $P<0.05$) and estrus (37%; $P<0.05$), but not at diestrus-1 or -2 (Fig 7.5B). The CAF diet had no effect on the acrophase of $T_c$ rhythms across the estrous cycle (Fig 7.5C).

Since it has been reported that obesity alters the daily $T_c$ profile in a photoperiod-dependent manner (Grimaldi et al. 2014), analysis was also performed on $T_c$ means during the light (ZT0-12) and dark (ZT12-24) periods of the day. There was a significant Diet x Photoperiod interaction at proestrus, estrus and diestrus-2, whereby the CAF-induced reduction in $T_c$ was statistically significant only during the dark period ($P<0.05$; Fig 7.3).

Regardless of diet, changes to $T_c$ rhythmicity were also evident across the estrous cycle; in the CON animals, the $T_c$ mesor increased slightly from proestrus to reach a maximum at estrus, then declined to a minimum at diestrus-1 and -2 ($P<0.05$; Fig 7.5A). The amplitude of the $T_c$ rhythm was greatest at proestrus, declined by 62% to a minimum at estrus, then increased slightly at diestrus-1 and -2 ($P<0.05$; Fig 7.5B). The acrophase of the $T_c$ rhythm also varied across the cycle; the peak in the $T_c$ rhythm was similar at proestrus and estrus, but was advanced by around 2 h at diestrus-1 and -2 ($P<0.05$; Fig 7.5C). Similar patterns of change for mesor, amplitude and acrophase were observed in CAF animals across the estrous cycle (Fig 7.5A-C).
Figure 7.2 Mean daily $T_c$ in animals that consumed control (CON) or cafeteria (CAF) diets across the study period. Values are the mean ± SEM ($n=7$ CON, $n=8$ CAF) and day 0 is the day of mating. * $P<0.001$ CON vs. CAF (two-way ANOVA).
Figure 7.3 Daily $T_c$ profiles and average $T_c$ for light and dark periods in animals that consumed control (CON) or cafeteria (CAF) diets across the estrous cycle. Grey lines represent raw $T_c$ data; black lines represent cosine curves; black bars represent the dark period for each day. * $P<0.05$ compared to CON (t-test following Diet x Photoperiod interaction in two-way ANOVA); ** $P<0.001$ (two-way ANOVA).
7.4.3 Maternal $T_c$ profiles across pregnancy

The mean daily $T_c$ was consistently lower in CAF animals during the first half of pregnancy (day 1 to day 13; Fig 7.2), but when $T_c$ began to decline from day 14, the two groups converged (see Fig 7.2). To determine rhythmic $T_c$ features, cosinor analysis was performed on a subset of gestational days (days 1, 4, 8, 12, 16 and 20). While the mesor was lower in CAF animals across pregnancy, and decreased as gestation progressed in both dietary groups ($P<0.001$; Fig 7.5D), there was a Diet x Gestational Day interaction, whereby the $T_c$ mesor of CAF animals decreased at a lower rate than in the CON animals, and the two profiles ultimately converged at day 20 (Fig 7.5D). In both diet groups, the $T_c$ amplitude generally increased from day 1 of pregnancy to mid-gestation, before exhibiting a transient decline at day 16, then rising slightly at day 20 (Fig 7.5E). Interestingly, the CAF diet altered this pattern, such that $T_c$ amplitude was lower at day 8 of gestation in CAF animals, but was higher in CAF than CON at day 20 ($P<0.05$; Fig 7.5E). The acrophase of the $T_c$ rhythm did not vary with either gestational age or diet (Fig 7.5F).

Analysis of $T_c$ across the light and dark periods was performed on the same subset of gestational days, and at days 16 and 20 there was a significant Diet x Photoperiod interaction. Specifically, average $T_c$ in CAF animals was lower only during the light period on these days ($P=0.01$ day 16, $P=0.06$ trend day 20; Fig 7.4).
Figure 7.4 Daily $T_c$ profiles and average $T_c$ for light and dark periods in animals that consumed control (CON) or cafeteria (CAF) diets across selected days of pregnancy. Grey lines represent raw $T_c$ data; black lines represent cosine curves; black bars represent the dark period for each day. * $P<0.05$ compared to CON ($t$-test following Diet x Photoperiod interaction in two-way ANOVA); ** $P<0.001$ (two-way ANOVA).
Figure 7.5 Tc rhythm features in animals that consumed either control (CON) or cafeteria (CAF) diets. A) Mesor, B) amplitude and C) acrophase of Tc rhythms across the estrous cycle, and D) mesor, E) amplitude and F) acrophase of Tc rhythms across selected days of gestation. Values are the mean ± SEM (n=7 CON, n=8 CAF). * P<0.001 and # P=0.01 CON vs. CAF (t-test following Diet x Cycle Stage or Diet x Gestational Day interaction in two-way ANOVA).
7.5 Discussion

This study shows that diet-induced obesity reduced the average $T_c$ and altered the rhythmicity of temperature profiles in female rats before and throughout gestation. Obesity also disrupted the normal $T_c$ adaptations to pregnancy; average maternal $T_c$ declined from mid to late gestation in control animals, but this late-gestational decline occurred to a lesser extent in obese mothers, such that their $T_c$ converged with that of controls. These results indicate that obesity during pregnancy has ramifications for maternal thermoregulatory control, and this may have downstream effects on maternal metabolism and fetal development.

To our knowledge, this is the first study to show that obesity reduces maternal $T_c$ across the estrous cycle and during pregnancy. It is unlikely that the increased energy intake from the CAF diet was directly responsible for the lower $T_c$ in the CAF animals; since feeding elicits thermogenic effects (Rothwell & Stock 1979), increased food intake in the CAF animals would be expected to increase heat load, resulting in a higher $T_c$. Overfeeding (without altered body composition) dampens $T_c$ rhythmicity in sheep, but unlike our model, did not change mesor $T_c$ levels (Maloney et al. 2013). Interestingly, pregnancy appears to override the thermogenic effects of feeding in rodents, since hyperphagia in pregnant rats does not induce BAT activation, as occurs in the non-pregnant state (Abelenda & Puerta 1987). As such, the $T_c$ alterations in the CAF group likely occur independently of feeding cues. Similarly, increased adiposity alone is unlikely to moderate reduced $T_c$, since excessive adiposity would be expected to decrease thermal conductance, again increasing $T_c$ (Jéquier et al. 1974). This suggests that obesity likely alters the hypothalamic regulation of $T_c$.

Interestingly, the lower $T_c$ in CAF mothers is similar to that seen in non-pregnant models of genetic obesity, such as the ob/ob and db/db strains (Trayhurn 1979; Trayhurn & James 1978), suggesting that altered leptin signaling may be a mechanism behind obesity-induced $T_c$ reductions. Indeed, CAF mothers exhibited a 3-fold increase in plasma leptin at day 21 of gestation, indicating that leptin resistance may facilitate reduced $T_c$. Potentially, leptin could alter either thermal conductance (which sets the rate that body heat is lost to the surroundings) or metabolic heat production. Recent studies show that ob/ob mice have higher whole body thermal conductance across a range of ambient temperatures (Kaiyala et al. 2015), and that leptin treatment in ob/ob mice increases $T_c$.
by reducing heat loss from the tail, not by stimulating metabolic heat production in BAT (Fischer et al. 2016). This implies that leptin resistance in CAF animals may impair their ability to prevent heat loss, thus resulting in lower $T_c$.

In relation to metabolic heat production, it is important to note that this study was conducted in standard animal housing conditions of 22°C, well below the thermoneutral zone of 29.5-30.5°C for rats (Romanovsky, Ivanov & Shimansky 2002; Maloney et al. 2014). This consideration is significant since thermogenic responses to obesity may be altered by the ambient temperature; Kaiyala et al. (2015) reported that ob/ob mice had lower $T_c$ at room temperature and under cold exposure, but were no different to wild types under thermoneutral conditions. This suggests that obesity may impair the ability to maintain homeothermy under cold exposure. Indeed, obese humans exhibit lower rates of thermogenesis (Ooijen et al. 2006) and brown adipose tissue (BAT) activation (Orava et al. 2013) during cold exposure, and it has been reported that ob/ob mice had impaired cold-induced BAT activity (Hogan & Himms-Hagen 1980). Intriguingly, however, a recent comprehensive study by Fischer et al. (2016) demonstrated that ob/ob mice do not have altered BAT function, and are actually hypermetabolic at thermoneutrality. Moreover, they showed that leptin administration in ob/ob mice does not induce BAT thermogenesis, but rather raises $T_c$ by acting as a pyrexic agent that decreases heat loss via the tail (Fischer et al. 2016). This suggests that any impact of leptin in the current CAF-induced reductions in $T_c$ is likely to occur through heat conservation mechanisms, as opposed to thermogenesis-related pathways. Further studies are required to examine how thermogenic or heat dissipation processes may regulate obesity-induced changes in maternal $T_c$, and whether these changes persist under thermoneutral housing conditions.

In addition to the reduced $T_c$ mesor in obese mothers, the $T_c$ amplitude was altered in CAF animals at several stages of the estrous cycle and pregnancy. Although diet-induced obesity has been shown to dampen the $T_c$ rhythm in male rodents (Goh, Mark & Maloney 2016; Mendoza, Pévet & Challet 2008), our results are not entirely consistent with these studies. Indeed, CAF-induced reductions to $T_c$ amplitude were observed only at the proestrus and estrus stages of the estrous cycle, and at day 8 of pregnancy. In contrast, $T_c$ amplitude was increased in CAF animals at day 20 of gestation. These inconsistencies are possibly due to sex-specific factors, since the estrogen:progesterone ratio is correlated to mesor and amplitude of $T_c$ rhythms in humans (Cagnacci et al. 2002). Interestingly, however, a recent study showed that administration of pregnancy-equivalent levels of
estrogen and progesterone did not alter basal $T_c$ in non-pregnant rats (Finley, Zhang & Fewell 2015). This implies that sex steroids alone do not regulate the late-gestational decline in maternal $T_c$, but that other complex interactions may be involved. Evidence suggests that angiotensin signaling in the brain may mediate the maternal $T_c$ decline (Cairns et al. 2005), but whether this signaling pathway is altered by CAF feeding is unknown.

Interestingly, we found that the obesity-induced reductions in $T_c$ were often dependent on photoperiod, and that the patterns of this dependence changed with the onset and progression of pregnancy. During the estrous cycle, $T_c$ was reduced in CAF mothers specifically during the dark (active) phase. Grimaldi et al. (2014) recently reported that obese humans had lower daytime $T_c$ profiles, but were no different to lean controls during the dark hours. Taken with our data, these results suggest that obesity reduces $T_c$ during the active phase of the circadian cycle in non-pregnant humans and rats. Once pregnancy was established, however, CAF animals displayed lower $T_c$ than the CON animals across the entire day. The lower $T_c$ persisted until late gestation, when the $T_c$ of CAF animals converged with that of CON during the dark period, but remained lower in the light period. The mechanisms for these light cycle-specific changes are unclear, although the circadian regulatory system may be implicated in $T_c$ adaptations to both pregnancy and obesity; the suprachiasmatic nucleus (SCN) of the hypothalamus is the master regulator of circadian function, and recent evidence suggests that it coordinates with metabolic input from the arcuate nucleus and governs $T_c$ rhythmicity (Guzmán-Ruiz et al. 2015).

Since obesity is associated with disturbed circadian function in non-pregnant individuals (Froy 2010; Mendoza, Pévet & Challet 2008; Kohsaka et al. 2007), obesity-induced changes to the circadian regulatory system may facilitate the $T_c$ adaptations in CAF animals. Again, leptin may mediate the relationship between obesity and circadian dysfunction, since leptin administration in both wild type and $ob/ob$ mice moderates SCN function after chronodisruption (Grosbellet et al. 2015; Mendoza et al. 2011).

It is possible that the $T_c$ reduction in CAF mothers is facilitated by lower activity levels. Unfortunately, activity rhythms were unable to be measured in the current study, however reduced activity in conjunction with lower $T_c$ has been noted in the dark photoperiod in HF-fed mice (Mendoza, Pévet & Challet 2008) and in obese Zucker rats across both light and dark periods (Murakami, Horwitz & Fuller 1995). It would be of
interest for further studies to categorize whether maternal activity rhythms are altered during obese pregnancy.

The maternal central clock is also altered across pregnancy (Wharfe et al. 2016), and so the circadian system may be partially responsible for pregnancy-induced $T_c$ adaptations. A previous study in pregnant Sprague-Dawley rats found that the decline in maternal $T_c$ close to term was due mainly to a loss of circadian rhythmicity; specifically, maternal $T_c$ did not exhibit the typical nocturnal increase after day 15 of gestation (Fewell 1995). In contrast, while our results confirm this late-gestational decline in $T_c$, we found that there was a clear $T_c$ difference between light and dark photoperiods throughout gestation in both CAF and CON mothers. Gamo et al. (Gamo et al. 2013) also found that rhythmicity in maternal $T_c$ was maintained during the late-gestational decline in mice, and so any inconsistencies among these various models may be due to strain or species differences.

In conclusion, our data show that maternal obesity reduced average $T_c$ prior to and during gestation, and resulted in a less pronounced late-gestational decline in CAF maternal $T_c$, relative to CON mothers. These obesity-induced changes in $T_c$ were influenced by photoperiod during the estrous cycle and in the late stages of pregnancy, indicating that circadian-specific processes may regulate obesity-induced $T_c$ reductions. Further studies are required to determine the mechanisms behind these $T_c$ adaptations across obese pregnancy, including the role of leptin signaling and the influence of the circadian master clock in the control of maternal $T_c$. 
Chapter 8: **Obesity alters maternal hepatic clock gene expression and suppresses Rev-erbα in the placenta and fetal liver during rat pregnancy**

*Preface*

The primary objective of this chapter was to investigate the impact of maternal obesity on clock gene expression in maternal and fetal hepatic tissue, and the labyrinth zone of the placenta. Rhythmic profiles of genes involved in lipid and glucose metabolism (*Ppars* and *Glut2*) were also measured in these tissues, and daily profiles of plasma insulin and blood glucose were assessed.

*Authorship and publication status:* This chapter was co-authored by Brendan J. Waddell and Peter J. Mark, and has been submitted for publication.

*Contributions:* Rachael Crew performed effectively all animal work, laboratory work and data analysis, representing almost 100% of experimental work. She was also largely responsible for writing and submitting the manuscript, representing approximately 80% of written work for this chapter.
8.1 Abstract

Maternal obesity induces pregnancy complications and disturbs fetal development, but the specific mechanisms underlying this are unclear. Circadian rhythms are implicated in metabolic complications associated with obesity, and maternal metabolic adaptations to pregnancy. Accordingly, obesity-induced circadian dysfunction may drive adverse outcomes in obese pregnancy. This study investigated whether maternal obesity alters the rhythmic expression of clock genes and associated nuclear receptors across maternal, fetal and placental tissues. Wistar rats were maintained on a cafeteria (CAF) diet prior to and throughout gestation to induce maternal obesity. Maternal and fetal liver and placental labyrinth zone (LZ) were collected at four-hourly time-points across days 15-16 and 21-22 of gestation (term = 23 days). Gene expression was analysed by RT-qPCR. Expression of the accessory clock gene Rev-erbα was rhythmic in the maternal and fetal liver and LZ of chow-fed controls (CON), but in each case CAF feeding reduced peak Rev-erbα expression. Obesity resulted in a phase advance (approx. 1.5 h) in the rhythms of several clock genes and Pparδ in maternal liver. Aside from Rev-erbα, expression of clock genes was mostly arrhythmic in placental LZ and fetal liver, and unaffected by the CAF diet. In conclusion, maternal obesity suppressed Rev-erbα expression across maternal, fetal and placental compartments and phase-advanced the rhythms of maternal hepatic clock genes. Given the key role of Rev-erbα in regulating metabolic, vascular and inflammatory processes, our data suggest that disruptions to rhythmic Rev-erbα expression in utero may contribute to programmed health complications in offspring of obese pregnancies.
8.2 Introduction

Maternal obesity complicates approximately 20% of pregnancies in Western countries (Fisher et al. 2013) and leads to a range of adverse maternal and fetal health outcomes. Obese women are more likely to experience pregnancy complications such as gestational diabetes and miscarriage (Marchi et al. 2015; Martin et al. 2015), and their infants are at a heightened risk for either macrosomia or intrauterine growth restriction (Gardosi & Francis 2009; Marchi et al. 2015). This disturbed development in utero can have lifelong health consequences, since offspring born to obese pregnancies often experience programmed metabolic disease in adulthood (Thornburg 2015; Blackmore & Ozanne 2013). While it is clear that obese pregnancy has negative effects on maternal and offspring health, the underlying mechanisms remain poorly understood.

Circadian rhythms control a range of biological functions, but are particularly crucial in the regulation of metabolism (Bailey, Udoh & Young 2014; Froy 2010). At a molecular level, circadian rhythms are driven by positive and negative transcriptional feedback loops of clock genes (Clock, Bmal1, Per1-3, Cry1-2, Rev-erbα and Rorα) which then influence downstream metabolic pathways (Reppert & Weaver 2002). Since obesity is widely considered a metabolic disease, the circadian system is likely a key regulator of obesity-related pathologies. Indeed, clock gene knockout models display altered metabolic phenotypes, including obesity and disturbed glucose metabolism (Kennaway et al. 2013; Turek et al. 2005), while circadian disturbances (e.g. shift work) lead to obesity and metabolic disease (Figueiro & White 2013; Knutsson 2003). Moreover, diet-induced obesity disturbs hepatic clock gene expression, and thereby alters expression of downstream genes and associated hepatic function (Kohsaka et al. 2007; Hsieh et al. 2009; Eckel-Mahan et al. 2013). Thus, there appears to be a reciprocal relationship between obesity and circadian dysfunction.

Despite this evidence for obesity-induced changes to clock gene expression in the non-pregnant state, it is not known whether maternal obesity elicits similar effects during pregnancy. Maternal circadian rhythms change markedly across gestation (Wharfe et al. 2016), which likely facilitates the maternal metabolic adaptations required to sustain fetal growth. Moreover, these circadian adaptations appear essential for normal fetal development, since circadian disruption during pregnancy causes maternal and fetal metabolic dysfunction and programmes metabolic disturbances in offspring (Varcoe et
Given a functional circadian system appears vital for healthy pregnancy outcomes, compromised circadian clocks could be key contributors to obesity-induced pregnancy complications. This may include not only maternal tissue clocks, but also those in the placental and fetal tissues, which also express clock genes (Wharfe, Mark & Waddell 2011).

Consequently, this study investigated the circadian expression profiles of clock genes and key downstream regulators of glucose and lipid metabolism (Ppars and Glut2) in the maternal liver, placental labyrinth zone (the site of maternal-fetal exchange) and fetal liver in rat pregnancy. Maternal obesity was established by cafeteria (CAF) feeding (Chapter 5), and tissues were analysed across days 15-16 and 21-22 of gestation (term = 23 days), thus spanning the period of maximal fetal growth. It was hypothesised that maternal obesity alters rhythmicity of clock gene expression in hepatic and placental tissues, and that this has related effects on downstream metabolic genes.
8.3 Materials & Methods

8.3.1 Animals and diets
All animal procedures were approved by the Animal Ethics Committee of The University of Western Australia. Three week old albino Wistar rats were obtained from the Animal Resources Centre (Murdoch, Western Australia) and housed three to a cage. Animals were kept under a 12:12 h light-dark cycle at 22°C, with ad libitum access to water and standard rodent chow (14 KJ/g total energy; 12% energy as fat, 23% protein and 65% carbohydrate; Specialty Feeds, Glen Forrest, Western Australia). Following a week of acclimatisation, animals were separated into control (CON) or cafeteria (CAF) diet groups; CON animals were maintained on standard chow, while CAF animals were provided with four snack food items per day, in addition to chow. CAF items were given in excess to be essentially ad libitum and were changed daily (from a selection of 17 items) to maintain novelty. This resulted in an average energy density of 16.4 KJ/g (47% energy as fat, 8% as protein and 44% as carbohydrate). For a detailed list of CAF diet constituents, see Chapter 5.

After 8 weeks of diet exposure, animals were mated overnight after determination of proestrus by an estrous cycle monitor (EC40; Fine Science Tools, Vancouver, British Columbia, Canada). Day 1 of pregnancy was confirmed by presence of spermatozoa in a vaginal smear the following morning. Pregnant animals were separated to individual housing and maintained on their respective diets throughout pregnancy.

8.3.2 Tissue collection
The timing of each tissue sampling is expressed relative to Zeitgeber time zero (ZT0), i.e. when lights were turned on in the animal facility (0700h). Tissues were obtained at four-hourly intervals (ZT1; 0800h, ZT5; 1200h, ZT9; 1600h, ZT13; 2000h, ZT17; 2400h and ZT21; 0400h) across days 15-16 and 21-22 of gestation (term occurs at day 23). At the appropriate collection time, animals (n = 7-8 per ZT for each diet) were anaesthetised using isoflurane/nitrous oxide. Maternal liver tissue was collected and fetal-placental pairs were removed via caesarean section. Placental labyrinth zone (LZ) samples were isolated from whole placentas via blunt dissection, and fetal liver tissue was collected. Fetal sex was determined by amplification of the Sry gene in fetal tail tissue at days 15-16 (Jones et al. 2013a) or by anogenital distance at days 21-22 (Faber & Hughes 1992).
Although placental and fetal liver samples were collected from both sexes, only female tissues were analysed for gene expression. Notably, however, growth rates and steroid hormone responses to the CAF diet are similar in both sexes in this obesity model (see Chapter 6).

Maternal blood was taken from the descending aorta and fetal blood was obtained at day 21 via decapitation. Blood glucose was measured immediately in maternal and fetal blood samples (Accu-Chek blood glucose monitor; Roche Diagnostics, Mannheim, Germany). Blood samples were then mixed with 10: 1 (vol: vol) 0.6 M EDTA and centrifuged at 13,000 x g for 6 min to isolate plasma. All collected tissue and plasma samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis.

### 8.3.3 Insulin measurement

Insulin levels were measured in maternal and day 21 fetal plasma using a Milliplex MAP Rat Adipokine Magnetic Bead Panel (Cat. # RECYTMAG-65K; EMD Millipore Corporation, Billerica, MA, USA). The assay was performed according to the manufacturer’s instructions and the plate was read on a Luminex Magpix (Luminex Corporation, Austin, Texas, USA). Data were analysed with Magpix 4.2 Software. The intra-assay coefficient of variation was 8.4%.

### 8.3.4 RNA and cDNA sample preparation

Total RNA was extracted from 50 – 100 mg of tissue using the QIAzol method (Qiagen Sciences, Maryland, USA), according to the manufacturer’s instructions. RNA was assessed for concentration and purity with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 5 µg of RNA was reverse transcribed to cDNA by the mouse Moloney leukemia virus reverse transcriptase RNase H Point Mutant with random hexamers (Promega, Sydney, Australia) as previously reported (Hewitt et al. 2006). The resultant cDNA was purified using the UltraClean PCR Cleanup Kit (MoBio Laboratories, Carlsband, CA, USA).

### 8.3.5 Quantitative PCR

The relative mRNA expression of clock genes (*Clock, Bmal1, Per1, Per2, Per3, Cry1, Cry2, Rora* and *Rev-erba*) and genes involved in glucose and lipid metabolism (*Ppara, Pparδ, Pgc1α, Glut2*) were analysed via RT-qPCR on the Rotorgene Q (Qiagen, Hilden, Germany). All primers (see Table 8.1) were designed using Primer-BLAST (Rozen &
Skaletsky 2000) and were positioned to span an intron to prevent amplification from genomic DNA.

Each PCR reaction consisted of 10 x Immolase buffer, 0.5 U Immolase DNA Polymerase (Bioline, Alexandria, NSW, Australia), 10 mM dNTPs, SYBR green (Molecular Probes, Eugene, OR, USA) at a 1/2000 dilution in DMSO, and gene-specific concentrations (see Table 1) of MgCl$_2$ and forward and reverse primer. cDNA template (1 µl) was added to give a total reaction volume of 10 µl. The PCR reaction cycle included an initial denaturing stage at 95°C for 10 min, followed by 45 cycles each comprising of 95°C for 1 sec, a primer-specific annealing temperature (see Table 8.1) for 15 sec and a 72°C extension period for 5 sec.

Standard curves were generated from serial 10-fold dilutions of gel-extracted PCR product and were used to calculate the relative gene expression concentrations for each sample with Rotorgene Q series software. All values were standardised against the reference genes Ppia, Sdha and Ywhaz using the GeNorm algorithm (Vandesompele et al. 2002).
Table 8.1 Primer details and PCR conditions for clock genes, *Ppars*, *Glut2* and reference genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing Temp (°C)</th>
<th>Size (bp)</th>
<th>MgCl₂(mM)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock</td>
<td>F5’ ACAGCGCACACACACAGGCCCTTC 3’</td>
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<td></td>
<td>R5’ TGGGGGGCGCCCTGTGATCTA 3’</td>
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<td>Bmal1</td>
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<td>Cry2</td>
<td>F5’ CTGCCAGGAGCACAAGC 3’</td>
<td>60</td>
<td>192</td>
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</tr>
<tr>
<td></td>
<td>R5’ GCATGCACACGCAAACGGA 3’</td>
<td></td>
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<tr>
<td>Rev-erba</td>
<td>F5’ ATTGCCACGGGGCGAGAGA 3’</td>
<td>60</td>
<td>292</td>
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</tr>
<tr>
<td></td>
<td>R5’ GCCAAAGAGCGCGCAGGTTG 3’</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Rorα</td>
<td>F5’ CCAACCCTGTCCATGCGCGGA 3’</td>
<td>60</td>
<td>113</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5’ CCCGTCGATCGTTGCGCA 3’</td>
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### Ppars and Glut2

<table>
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<tr>
<th>Gene</th>
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<th>Reverse Primer</th>
<th>Tm</th>
<th>Length</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pparα</em></td>
<td>F5’ AATCCACGAAGCCTACCTGA 3’</td>
<td>R5’ GTCTTCTCAGCCATGCACAA 3’</td>
<td>60</td>
<td>132</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Pparδ</em></td>
<td>F5’ GAGGGGTGCAAGGGCTTCTT 3’</td>
<td>R5’ CACTTGTTGCGGTTCTCTTCCTG 3’</td>
<td>60</td>
<td>101</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Pgc1α</em></td>
<td>F5’ TCTGGAACTGCAGGCCTAACTC 3’</td>
<td>R5’ GCAAGAGGGGCCCTGCTTTG 3’</td>
<td>60</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td><em>Glut2</em></td>
<td>F5’ TAGGGCGAATGGTGCCCTCGT 3’</td>
<td>R5’ GGCTCCAGTCAAGGAGGCT 3’</td>
<td>61</td>
<td>102</td>
<td>2</td>
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### Reference genes

<table>
<thead>
<tr>
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<th>Tm</th>
<th>Length</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ppia</em></td>
<td>F5’ AGCATACAGGTCCTGGCATC 3’</td>
<td>R5’ TTCACCCCTCAAGACCAAC 3’</td>
<td>62</td>
<td>127</td>
<td>3</td>
</tr>
<tr>
<td><em>Sdha</em></td>
<td>F5’ TGGGGCGACTCGTGCGCTTT 3’</td>
<td>R5’ CCCCGCTGCACCTACACC 3’</td>
<td>60</td>
<td>134</td>
<td>2</td>
</tr>
<tr>
<td><em>Ywhaz</em></td>
<td>F5’ GACGGAGCTGAGGACATCTGC 3’</td>
<td>R5’ GGCTGGCAAGCATTGGGATCA 3’</td>
<td>60</td>
<td>75</td>
<td>2</td>
</tr>
</tbody>
</table>

F: Forward primer, R: Reverse primer, bp: base pairs
8.3.7 Statistical analysis

All values are expressed as mean ± SEM, with \( n = 7-8 \) per ZT group for each diet. Daily rhythms of gene expression or plasma hormone levels were assessed by non-linear (cosinor) regression in Genstat 9.0 software (Hemel Hempstead, UK). This calculated the typical rhythm features of mesor (the rhythm-adjusted mean), amplitude (the difference between the peak and the mesor) and acrophase (the time of the rhythm peak) for each daily profile. Comparisons of these cosine curve features between diet groups and gestational days were made by two-way ANOVA or \( t \)-test, as appropriate (GraphPad Prism version 6.00; La Jolla, California).

Other comparisons were made by ANOVA using GenStat 9.0 software (Hemel Hempstead, UK) to test for differences due to diet, time of day, or stage of pregnancy. When significant \( (P<0.05) \) interactions were observed between factors, differences were assessed by \( t \)-test or ANOVA, as appropriate. When the \( F \)-test reached statistical significance \( (P<0.05) \) specific group comparisons were made by LSD tests.
8.4 Results

Caloric intake, weight gain profiles and body composition data for this animal cohort has been previously reported (see Chapter 6). Briefly, CAF animals exhibited increased caloric intake and weight gain across the study period; this resulted in a 25% increase in body weight after 8 weeks of pre-pregnancy CAF feeding, and a 56% increase in total percentage adiposity in CAF mothers by day 21 of gestation. Fetal and placental weights were slightly reduced in the CAF group across both days of gestation, but total fetal and placental weights did not differ between groups due to a small increase in litter size in the CAF animals.

8.4.1 Profiles of blood glucose and plasma insulin

As expected, maternal blood glucose levels were relatively stable across each of days 15 and 21, although a significant, low-amplitude rhythm became evident at day 21. There was also a Diet x Stage of Pregnancy interaction, with elevated blood glucose evident in CAF mothers at day 15 ($P<0.001$; Fig 8.1A) but not at day 21. Fetal blood glucose levels increased 2.4-fold across day 21 ($P<0.001$), but were unaffected by diet (Fig 8.1A).

Maternal hyperinsulinemia was evident in the CAF group across all time-points at day 15 ($P<0.001$; Fig 8.1B), whereas at day 21 there was a significant Diet x Time of Day interaction, with hyperinsulinemia evident in CAF mothers only at ZT13 ($P= 0.02$; Fig 8.1B). Fetal insulin varied with time of day across day 21 ($P=0.01$) but was unaffected by diet (Fig 8.1B).
Figure 8.1 Daily profiles of blood glucose and plasma insulin in maternal and fetal plasma of control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). Note different scales for fetal blood glucose and plasma insulin. * P<0.001 CON vs. CAF (overall diet effect in two-way ANOVA), ** P<0.001 compared to CON (t-test following Diet x Time of Day interaction in two-way ANOVA).
8.4.2 Effects of the CAF diet on maternal, placental and fetal clock gene expression

(i) Maternal hepatic clock gene expression. The expression profiles of all clock genes in the maternal liver were clearly rhythmic (i.e. significant cosinor fit) at both gestational ages, the single exception being *Rora* at day 15, where rhythmicity was abolished by the CAF diet (see Table 8.2 for r² and P values for cosine fit significance). Obesity reduced the mesor of *Cry2* at both days of gestation (*P*<0.05; Fig 8.2G and Table 8.3), while there was a trend (*P*=0.08) for reduced *Per1* mesor in CAF mothers at day 15 (Fig 8.2 and Table 8.3). The CAF diet also reduced the amplitude of *Per2* at both days, and those of *Cry2* and *Rev-erbα* at day 21 (*P*<0.05; Fig 8.2 and Table 8.3). Interestingly, the CAF diet induced a phase advance of approximately 1.2 h in the maternal hepatic expression of *Bmal1*, *Per2*, *Per3* at both days of gestation, *Cry1* at day 15 and *Rev-erbα* at day 21 (all *P*<0.05; Fig 8.2 and Table 8.3).

Maternal hepatic clock gene expression also varied with gestational age; the mesor of *Bmal1*, *Per2*, *Cry1*, *Cry2* and *Rev-erbα* fell between days 15 and 21, while *Per1* expression increased over this period (*P*<0.001; Fig 8.2 and Table 8.3). The amplitude of *Bmal1*, *Cry1* and *Rev-erbα* was also reduced between days 15 and 21 (*P*<0.05; Fig 8.2 and Table 8.3). The acrophase for each maternal hepatic clock gene was largely unaffected by pregnancy stage, the only exception being that of *Rev-erbα*, which was delayed by 0.8 h at day 21 compared to day 15 (*P*<0.05; Fig 8.2I and Table 8.3). All of these gestational changes were similar in the two diet groups.
Table 8.2 Cosinor rhythmicity ($r^2$ and associated $P$ values) for maternal hepatic gene expression profiles

<table>
<thead>
<tr>
<th>Gene</th>
<th>Maternal Liver Day 15</th>
<th>Maternal Liver Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CAF</td>
</tr>
<tr>
<td>Clock</td>
<td>0.607 ($P&lt;0.001$)</td>
<td>0.419 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Bmal1</td>
<td>0.925 ($P&lt;0.001$)</td>
<td>0.895 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Per1</td>
<td>0.602 ($P&lt;0.001$)</td>
<td>0.597 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Per2</td>
<td>0.826 ($P&lt;0.001$)</td>
<td>0.710 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Per3</td>
<td>0.825 ($P&lt;0.001$)</td>
<td>0.682 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Cry1</td>
<td>0.857 ($P&lt;0.001$)</td>
<td>0.841 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Cry2</td>
<td>0.486 ($P&lt;0.001$)</td>
<td>0.278 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>0.538 ($P&lt;0.001$)</td>
<td>0.600 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Rorα</td>
<td>0.271 ($P&lt;0.001$)</td>
<td>0.036 (NS)</td>
</tr>
<tr>
<td>Pparaα</td>
<td>0.111 ($P=0.027$)</td>
<td>0.025 (NS)</td>
</tr>
<tr>
<td>Pparaδ</td>
<td>0.698 ($P&lt;0.001$)</td>
<td>0.559 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Glut2</td>
<td>0.542 ($P&lt;0.001$)</td>
<td>0.583 ($P&lt;0.001$)</td>
</tr>
</tbody>
</table>

NS: not significant ($P>0.05$).
Table 8.3 Rhythmic features (mesor, amplitude and acrophase) of maternal hepatic gene expression profiles.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CAF</td>
</tr>
<tr>
<td><strong>Clock</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 3</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>39 ± 5</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>Acrophase</td>
<td>0.2 ± 0.4</td>
<td>23.3 ± 0.7</td>
</tr>
<tr>
<td><strong>Bmal1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 2</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>Amplitude</td>
<td>98 ± 4</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>Acrophase</td>
<td>0.1 ± 0.2</td>
<td>22.8 ± 0.2*</td>
</tr>
<tr>
<td><strong>Per1</strong></td>
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<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 6</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>Amplitude</td>
<td>67 ± 8</td>
<td>59 ± 8</td>
</tr>
<tr>
<td>Acrophase</td>
<td>13.4 ± 0.4</td>
<td>13.1 ± 0.5</td>
</tr>
<tr>
<td><strong>Per2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 4</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>86 ± 6</td>
<td>54 ± 5*</td>
</tr>
<tr>
<td>Acrophase</td>
<td>16.3 ± 0.3</td>
<td>15.3 ± 0.4*</td>
</tr>
<tr>
<td><strong>Per3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 6</td>
<td>103 ± 8</td>
</tr>
<tr>
<td>Amplitude</td>
<td>119 ± 8</td>
<td>108 ± 11</td>
</tr>
<tr>
<td>Acrophase</td>
<td>12.4 ± 0.3</td>
<td>11.2 ± 0.4*</td>
</tr>
<tr>
<td><strong>Cry1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 3</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Amplitude</td>
<td>69 ± 4</td>
<td>69 ± 5</td>
</tr>
<tr>
<td>Acrophase</td>
<td>20.0 ± 0.2</td>
<td>18.9 ± 0.2*</td>
</tr>
<tr>
<td><strong>Cry2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 3</td>
<td>90 ± 4*</td>
</tr>
<tr>
<td>Amplitude</td>
<td>30 ± 5</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Acrophase</td>
<td>14.9 ± 0.6</td>
<td>14.6 ± 0.9</td>
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</table>
Circadian adaptations to obese pregnancy

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mesor</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th>Mesor</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th>Mesor</th>
<th>Amplitude</th>
<th>Acrophase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev-erbα</td>
<td>100 ± 15</td>
<td>91 ± 12</td>
<td>29.7 ± 0.9†</td>
<td>25.8 ± 0.7†</td>
<td>157 ± 22</td>
<td>132 ± 17</td>
<td>43.3 ± 1†</td>
<td>34.1 ± 1*†</td>
<td>8.9 ± 0.5</td>
</tr>
<tr>
<td>Rorα</td>
<td>100 ± 4</td>
<td>99 ± 7</td>
<td>101 ± 4</td>
<td>97 ± 5</td>
<td>27 ± 6</td>
<td>18 ± 10</td>
<td>17 ± 5</td>
<td>NS</td>
<td>20.9 ± 0.9</td>
</tr>
<tr>
<td>Ppara</td>
<td>100 ± 8</td>
<td>117 ± 9</td>
<td>190 ± 19</td>
<td>233 ± 24</td>
<td>30 ± 11</td>
<td>24 ± 14</td>
<td>NS</td>
<td>NS</td>
<td>12.1 ± 1.4</td>
</tr>
<tr>
<td>Pparδ</td>
<td>100 ± 7</td>
<td>83 ± 7</td>
<td>87 ± 5</td>
<td>84 ± 6</td>
<td>89 ± 11</td>
<td>71 ± 10</td>
<td>62 ± 7†</td>
<td>60 ± 8†</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>100 ± 5</td>
<td>87 ± 5</td>
<td>158 ± 7</td>
<td>138 ± 8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glut2</td>
<td>100 ± 3</td>
<td>112 ± 5*</td>
<td>110 ± 3</td>
<td>120 ± 6</td>
<td>35 ± 5</td>
<td>50 ± 7</td>
<td>45 ± 5</td>
<td>43 ± 9</td>
<td>16.0 ± 0.5</td>
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</tbody>
</table>

Values are the mean ± SEM and are expressed relative to CON mesor at day 15 (set to 100). Acrophase expressed in ZT. *P<0.05 compared to CON at corresponding gestational day (t-test). †P<0.05 overall effect compared to day 15, irrespective of diet (ANOVA). NS: Not significant for cosine fit.
Figure 8.2 Rhythmic expression profiles of maternal hepatic clock genes in control (CON) and cafeteria (CAF) diet groups at days 15 and 21 of gestation. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). The inset graph (different scale) is provided in panel I to highlight the shift in the Rev-erbα acrophase. Cosinor curves are shown only for those genes that had significant cosinor rhythmicity. Statistical differences for cosine curve features are summarised in Table 8.3.
(ii) Placental clock gene expression. In contrast to the maternal liver, less than half of the placental clock genes measured were significant for cosinor fit. *Per3* expression was non-rhythmic in the LZ across all groups; however the cosine fit significance of the remaining placental clock genes varied depending on diet or pregnancy stage (see supplementary Table 8.S1 for $r^2$ and associated $P$ values, and supplementary Table 8.S2 for cosine curve features).

Given that the majority of placental clock gene profiles were non-rhythmic, conventional ANOVA was used to examine the impact of CAF feeding on placental clock gene expression. LZ expression of *Clock* was reduced by the CAF diet at day 15 ($P=0.012$ overall diet effect, Fig 8.3A), while *Cry1* was increased in CAF at day 21 ($P=0.02$ overall diet effect, Fig 8.3F). LZ expression of *Rev-erba* exhibited a Diet x Time of Day interaction in LZ tissue, whereby its peak level was reduced by the CAF diet at both gestational days ($P<0.05$; Fig 8.3I). LZ expression of all clock genes was increased from day 15 to 21 ($P<0.001$; Fig 8.3), the sole exception being *Rev-erba*, overall expression of which fell between days 15 and 21 ($P<0.001$; Fig 8.3I).
Figure 8.3 Daily expression profiles of clock genes in the placental labyrinth zone at days 15 and 21 of gestation in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). * P<0.001 CON vs. CAF (overall diet effect in two-way ANOVA); ** P<0.001 compared to CON (t-test following Diet x Time of Day interaction in two-way ANOVA).
(iii) Fetal hepatic clock gene expression. As with the placenta, cosinor rhythmicity was not consistently observed for fetal hepatic clock gene profiles (10/18 profiles reached significance for cosinor fit; see Supplementary Table 8.S3 for r² and associated P values). The CAF diet abolished rhythmicity in Cry2 and Rev-erbα, but did not alter the rhythmic status of the other genes (see Supplementary Table 8.S4). Conventional ANOVA showed a significant Diet x Time of Day interaction for fetal hepatic Clock, Rev-erbα and Rorα indicating that CAF effects on these genes were time-of-day-specific (see Fig 8.4). Most notable among the diet effects was a suppression of peak fetal hepatic Rev-erbα expression, similar to the effects of the CAF diet on Rev-erbα expression in both maternal liver (Fig 8.2) and the placental LZ (Fig 8.3).

Figure 8.4 Daily expression profiles of fetal hepatic clock genes at day 21 of gestation in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). *P<0.001 CON vs. CAF (t-test following Diet x Time of Day interaction in two-way ANOVA).
8.4.3 Effects of the CAF diet on maternal, placental and fetal Glut2 and Ppars

(i) Maternal liver: Maternal hepatic expression of Pparδ and Glut2 was rhythmic (i.e. significant cosinor fit; see Table 8.2 for r² and P values) at both gestational ages in both diet groups. Pparα expression was also rhythmic in CON mothers on both days, but in each case this rhythmicity was abolished by the CAF diet (Table 8.3 and Fig 8.5A).

The CAF diet also increased the mesor (P=0.03) and amplitude (trend, P=0.08) of maternal hepatic Glut2 at day 15, but not at day 21 (Table 8.3 and Fig 8.5D). While Pgclα and Pparα levels were unaffected by the CAF diet, expression of both genes increased with gestational age (P<0.05), as did Glut2 (P<0.05). In contrast, the amplitude of Pparδ fell between gestational days 15 and 21 (P<0.05; Fig 8.5).

Interestingly, the Pparδ rhythm was phase advanced (approximately 1.7 h) by the CAF diet on both gestational days (P<0.01; Table 8.3 and Fig 8.5B), which was a comparable shift to that of several clock genes. To assess whether this Pparδ shift may be driven by clock genes, the relationships between Pparδ and key rhythm drivers, Bmal1 and Rev-erba, were assessed. This revealed a strong positive correlation between Pparδ and Bmal1, and a negative association between Pparδ and Rev-erba in each diet group across both days (see Fig 8.6 and Table 8.4).
Figure 8.5 Rhythmic expression profiles of metabolic genes in maternal liver tissue of control (CON) and cafeteria (CAF) diet groups at days 15 and 21 of gestation. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). Cosinor curves are shown only for those genes that had significant cosinor rhythmicity. Statistical differences for cosine curve features are summarised in Table 3.
Figure 8.6 Relationship between (A) \( Ppar\) and \( Rev-erb\alpha \) and (B) \( Ppar\) and \( Bmal1\).

Representative data from CAF day 21 animals.

Table 8.4 \( R \) and associated \( P \) values for correlations between \( Ppar\) and rhythmic drivers, \( Bmal1 \) and \( Rev-erb\alpha \)

<table>
<thead>
<tr>
<th></th>
<th>Day 15</th>
<th></th>
<th>Day 21</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CAF</td>
<td>CON</td>
<td>CAF</td>
</tr>
<tr>
<td>( Ppar) - ( Bmal1 )</td>
<td>0.780</td>
<td>0.789</td>
<td>0.800</td>
<td>0.850</td>
</tr>
<tr>
<td>( P )</td>
<td>( P&lt;0.001 )</td>
<td>( P&lt;0.001 )</td>
<td>( P&lt;0.001 )</td>
<td>( P&lt;0.001 )</td>
</tr>
<tr>
<td>( Ppar) - ( Rev-erb\alpha )</td>
<td>-0.380</td>
<td>-0.544</td>
<td>-0.440</td>
<td>-0.540</td>
</tr>
<tr>
<td>( P )</td>
<td>( P=0.01 )</td>
<td>( P&lt;0.001 )</td>
<td>( P=0.003 )</td>
<td>( P&lt;0.001 )</td>
</tr>
</tbody>
</table>
(ii) Placental LZ: Expression profiles for Pgc1α and Pparδ were not rhythmic at either day of gestation, while Ppara expression was rhythmic only in CON placentas at day 15 and CAF placentas at day 21 (Supplementary Tables 8.S1 and 8.S2). Conventional ANOVA showed that the CAF diet increased LZ Pgc1α expression at day 21 (P<0.001 overall diet effect; Fig 8.7C). LZ expression of Ppara and Pgc1α both increased substantially between days 15 and 21 (P<0.001; Fig 8.7A & C).

Figure 8.7 Daily expression profiles of the Ppar genes in placental labyrinth zone in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT). * P<0.001 CON vs. CAF (overall diet effect in two-way ANOVA).
(iii) Fetal liver: Rhythmic expression was observed for fetal hepatic *Ppar*<sub>α</sub> and *Ppar*<sub>δ</sub>, but not *Pgc1*<sub>α</sub> (Supplementary Tables 8.S3 and 8.S4), and none of these genes were affected by the CAF diet (Figure 8.8).

Figure 8.8 Daily expression profiles of the *Ppar* genes in the fetal liver in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n=7-8 per diet group at each ZT).
8.5 Discussion

This study demonstrates that maternal obesity in rat pregnancy suppresses the rhythmic expression of the accessory clock gene Rev-erba in maternal liver, placental LZ and fetal liver. CAF feeding also reduced hepatic expression of Cry2 and Per2 in the mother and phase-advanced the rhythms of Bmal1, Per2, Per3, Cry1 and Rev-erba. Importantly, these latter effects did not extend to either the placenta or fetal liver, both of which exhibited largely arrhythmic clock gene expression profiles. Our data also show that opposite changes occur with gestational age in maternal hepatic (decreased) and placental (increased) clock gene expression, irrespective of obesity. Overall, these data provide important insights to the impact of maternal obesity on rhythmic hepatic function in the mother. They further suggest that the molecular clock networks in fetal and placental tissues remain relatively underdeveloped late in rat pregnancy.

Rev-erba is a vital component of the molecular clock network; its transcription is stimulated by the CLOCK:BMAL1 heterodimer, and it acts in turn to repress Bmal1 and Clock transcription via RORE binding (Preitner et al. 2002; Cho et al. 2012). Rev-erba also plays a key role in the integration of the molecular clock with metabolic function (Duez & Staels 2008). Indeed, Rev-erba stimulates adipocyte differentiation in white adipose tissue (Fontaine et al. 2003) and regulates hepatic and adipose lipid metabolism (Delezie et al. 2012). Maternal hepatic Rev-erba expression was strongly rhythmic and was slightly reduced by the CAF diet (albeit significantly only for amplitude at day 21). But more significantly there was a marked decline in the mesor and amplitude of hepatic Rev-erba expression with gestational age, indicative of a ‘flattening’ of the overall rhythm. This change is consistent with recent observations in mouse pregnancy where a similar change appears linked to alterations in hepatic glucose metabolism (Wharfe 2016). Placental Rev-erba profiles were also rhythmic in CON animals, with a distinct peak in expression that was suppressed by the CAF diet. Importantly, Rev-erba also affects numerous other tissue functions not directly related to the molecular clock (Everett & Lazar 2014), including vascular (Li et al. 2014) and inflammatory status (Gibbs et al. 2012), both of which change dramatically in rat placenta over the last week of pregnancy (Hewitt, Mark & Waddell 2006; Mark et al. 2013). Therefore, it will be of interest to determine whether the CAF-induced reduction in LZ Rev-erba expression contributes to
The CAF diet also suppressed fetal hepatic Rev-erbα expression in a time-of-day-dependent manner, effectively blunting the transient peak evident in the control group. One previous study reported that maternal obesity actually increased fetal hepatic Rev-erbα in Rhesus macaques, but this was based on only a single time-point (Suter et al. 2011). Our data clearly show the importance of full circadian analyses, since CAF-induced suppression of Rev-erbα occurred only at a single time-point. While the implications of altered Rev-erbα signalling in fetal tissues are unknown, it could potentially contribute to the adverse metabolic programming outcomes observed in offspring of obese pregnancies (Blackmore & Ozanne 2013). Several recent studies have noted that this effect of obesity extends to offspring circadian biology; specifically, offspring of HF-fed mothers have disrupted hepatic clock gene expression (Wang et al. 2015; Borengasser et al. 2014), often in conjunction with symptoms of non-alcoholic fatty liver disease (Bruce et al. 2016; Mouralidarane et al. 2015; Carter et al. 2014). Taken together with these previous findings, our data suggest that altered Rev-erbα signalling in fetal life may contribute to these programmed metabolic abnormalities.

The slight CAF-induced reduction in maternal hepatic expression of several clock genes, and the approximately 1.5 h phase advance in their rhythms, are similar to previous observations in HF-fed, male mice (Kohsaka et al. 2007; Eckel-Mahan et al. 2013; Pendergast et al. 2013). Importantly, the hepatic clock is highly sensitive to food intake, with clock gene expression responding within one hour of food consumption (Oike et al. 2011). Moreover, both fat (Eckel-Mahan et al. 2013) and glucose (Iwanaga et al. 2005) consumption alters hepatic clock gene expression in rodents independently of adiposity. As such, hepatic clock gene changes in CAF mothers may reflect CAF diet consumption per se, rather than adiposity-related parameters. The phase-advances observed in the maternal hepatic clock may well reflect altered feeding times in CAF animals, since rodents with free access to a HF diet display increased daytime food intake (Kohsaka et al. 2007; Kentish et al. 2016; Pendergast et al. 2013). Although the overall timing of food intake was not measured in this study, novel food items were provided to CAF mothers one to two hours before lights off. As such, they are likely to have commenced daily eating earlier than the CON mothers, due to the novelty of the food items. Interestingly,
because meal timing has major implications for the development of obesity in humans (Garaulet & Gómez-Abellán 2014), atypical eating patterns may also contribute to the metabolic complications that occur in obese pregnant women.

Consistent with the phase advance in maternal hepatic clock gene expression, hepatic Pparδ was also advanced by around 1.7 h in CAF mothers. While the molecular links between Pparδ and the core clock machinery are not completely understood, recent evidence in zebrafish indicates that the Pparδ promoter does not contain E-box response elements (i.e., the binding sites for CLOCK:BMAL1), but does have three Rev-erbα binding sites (Kopp et al. 2016). This suggests that Rev-erbα has transcriptional control over Pparδ, consistent with our observation of a strong negative correlation between Pparδ and Rev-erbα. As expected, Pparδ also exhibited a strong positive correlation with Bmal1 expression in maternal hepatic tissue at both days of gestation, consistent with a previous report of a positive relationship between BMAL1 and PPARδ in leukocyte mRNA transcripts of pregnant women (Pappa et al. 2013). Pparδ promotes insulin resistance by stimulating hepatic glucose utilisation and impairing gluconeogenesis (Lee et al. 2006), and so the hepatic phase advance in Pparδ (likely driven by clock genes) may have downstream effects on glucose homeostasis in CAF mothers.

In contrast to maternal hepatic clock genes, placental and fetal clock genes were largely unresponsive to the maternal obesity insult. While fetal liver may be shielded from rhythmic maternal feeding cues, this is not the case for the placenta. It seems more likely that the fetal and placental clock gene systems do not respond to the CAF diet because their molecular networks are underdeveloped. Indeed, both fetal and placental clock gene expression profiles were largely arrhythmic and did not exhibit the anti-phase expression patterns typical of specific clock gene pairs (e.g. Bmal1 and Per2) in a functional molecular clock (Reppert & Weaver 2002). Interestingly, unlike the reduction in maternal hepatic clock gene expression near term, most placental clock genes increased over the same period. As discussed above in relation to Rev-erbα, it is possible that clock genes exert effects on placental function that are unrelated to the circadian clock. Importantly, there also appear to be differences between rodents and humans in terms of fetal and placental circadian development; rodents are altricial species, and evidence suggests that hepatic clock gene function does not become fully rhythmic in the rat until around postnatal day 30 (Sládek et al. 2007). In contrast, humans have a longer gestational length
and are comparatively precocial at birth, and so rhythmicity may be more established in the human fetus and possibly the placenta. Indeed, recent evidence suggests that clock gene expression is rhythmic in the term human placenta (Pérez et al. 2015), but it is unknown if this is disturbed by maternal obesity.

CAF mothers were hyperglycaemic and displayed increased hepatic Glut2 expression across day 15 of gestation, suggesting that obesity may elevate hepatic glucose flux at mid-gestation. The CAF-induced hyperglycaemia occurred in conjunction with elevated insulin, suggestive of insulin resistance. By day 21 of gestation there was no longer any dietary effect in blood glucose or hepatic Glut2 expression, and insulin was only elevated at the ZT13 time-point in CAF mothers. This time-specific insulin elevation may relate to the provision of novel food items, since ZT13 is the first time point after these were introduced. Interestingly, the lack of an overall diet effect on either glucose or insulin at day 21 is consistent with previous reports showing metabolic convergence between lean and obese individuals in late gestation (Forbes et al. 2015; King et al. 2013).

In conclusion, this study provides novel evidence that obesity alters rhythmic Rev-erbα expression in maternal and fetal hepatic tissue, and in the labyrinth zone of the rat placenta. The CAF diet also reduced maternal hepatic expression of several clock genes and advanced their peak expression, and that of Pparδ. Other than Rev-erbα, clock gene expression profiles in the placenta and fetal liver were largely arrhythmic, suggestive of functional immaturity of molecular clocks in these tissues.
### 8.6 Supplemental data

**Table 8.S1 Cosinor rhythmicity (r^2 and associated P values) for LZ gene expression profiles.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>LZ Day 15 CON</th>
<th>CAI</th>
<th>LZ Day 15 CON</th>
<th>CAI</th>
<th>LZ Day 21 CON</th>
<th>CAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clock</td>
<td>0 (NS)</td>
<td>0.055 (NS)</td>
<td>0.117 (P=0.02)</td>
<td>0.214 (P=0.003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmal1</td>
<td>0.041 (NS)</td>
<td>0 (NS)</td>
<td>0.153 (P=0.009)</td>
<td>0.200 (P=0.002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per1</td>
<td>0 (NS)</td>
<td>0.005 (NS)</td>
<td>0 (NS)</td>
<td>0.096 (P=0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per2</td>
<td>0.256 (P&lt;0.001)</td>
<td>0 (NS)</td>
<td>0.095 (P=0.04)</td>
<td>0.046 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per3</td>
<td>0.033 (NS)</td>
<td>0.028 (NS)</td>
<td>0 (NS)</td>
<td>0.061 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry1</td>
<td>0 (NS)</td>
<td>0.098 (P=0.04)</td>
<td>0.327 (P&lt;0.001)</td>
<td>0.183 (P=0.003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry2</td>
<td>0 (NS)</td>
<td>0.039 (NS)</td>
<td>0.145 (P=0.01)</td>
<td>0 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>0.133 (P=0.02)</td>
<td>0.019 (NS)</td>
<td>0.087 (P=0.04)</td>
<td>0 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rorα</td>
<td>0.016 (NS)</td>
<td>0.031 (NS)</td>
<td>0.185 (P=0.003)</td>
<td>0.206 (P=0.002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppara</td>
<td>0.146 (P=0.04)</td>
<td>0 (NS)</td>
<td>0.079 (NS)</td>
<td>0.214 (P=0.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pparδ</td>
<td>0 (NS)</td>
<td>0.023 (NS)</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pgc1α</td>
<td>0.004 (NS)</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
<td>0.037 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glut2</td>
<td>0 (NS)</td>
<td>0.031 (NS)</td>
<td>0.045 (NS)</td>
<td>0 (NS)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS: not significant (P>0.05).
Table 8.S2 Rhythmic features (mesor, amplitude and acrophase) for LZ gene expression profiles.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 15</th>
<th>Day 21</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CAF *</td>
<td>CON</td>
</tr>
<tr>
<td>Clock</td>
<td>Mesor</td>
<td>100 ± 5</td>
<td>85 ± 5</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Bmal1</td>
<td>Mesor</td>
<td>100 ± 5</td>
<td>102 ± 6</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Per1</td>
<td>Mesor</td>
<td>100 ± 3</td>
<td>104 ± 4</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Per2</td>
<td>Mesor</td>
<td>100 ± 4</td>
<td>102 ± 5</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>22 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>15.3 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>Per3</td>
<td>Mesor</td>
<td>100 ± 1</td>
<td>97 ± 10</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cry1</td>
<td>Mesor</td>
<td>100 ± 2</td>
<td>101 ± 3</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>NS</td>
<td>9 ± 3</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>NS</td>
<td>16.1 ± 1.6</td>
</tr>
<tr>
<td>Cry2</td>
<td>Mesor</td>
<td>100 ± 4</td>
<td>98 ± 4</td>
</tr>
<tr>
<td></td>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
### Circadian adaptations to obese pregnancy

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mesor (day 15 set to 100)</th>
<th>Amplitude</th>
<th>Acrophase (Zeitgeber time)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rev-erbα</em></td>
<td>100 ± 9</td>
<td>36 ± 13</td>
<td>12.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>71 ± 5</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>17 ± 2†</td>
<td>5 ± 2</td>
<td>8.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>15 ± 2†</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><em>Rora</em></td>
<td>100 ± 5</td>
<td>94 ± 6</td>
<td>16.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>94 ± 6</td>
<td>NS</td>
<td>15.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>120 ± 5†</td>
<td>26 ± 8</td>
<td>117 ± 5†</td>
</tr>
<tr>
<td></td>
<td>117 ± 5†</td>
<td>NS</td>
<td>25 ± 7</td>
</tr>
<tr>
<td><em>Ppara</em></td>
<td>100 ± 5</td>
<td>93 ± 5</td>
<td>10.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>93 ± 5</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>498 ± 29†</td>
<td>26 ± 8</td>
<td>90 ± 4</td>
</tr>
<tr>
<td></td>
<td>481 ± 20†</td>
<td>NS</td>
<td>93 ± 3</td>
</tr>
<tr>
<td><em>Pparδ</em></td>
<td>100 ± 4.6</td>
<td>96.6 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>96.6 ± 5</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>90 ± 4</td>
<td>NS</td>
<td>93 ± 3</td>
</tr>
<tr>
<td></td>
<td>90 ± 4</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><em>Pgc1α</em></td>
<td>100 ± 8</td>
<td>85 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>85 ± 9</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>576 ± 70†</td>
<td>60 ± 25</td>
<td>989 ± 79†</td>
</tr>
<tr>
<td></td>
<td>576 ± 70†</td>
<td>NS</td>
<td>65 ± 20</td>
</tr>
<tr>
<td><em>Glut2</em></td>
<td>100 ± 16</td>
<td>65 ± 18</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>65 ± 18</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60 ± 25</td>
<td>NS</td>
<td>65 ± 20</td>
</tr>
<tr>
<td></td>
<td>60 ± 25</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM and are expressed relative to CON mesor at day 15 (set to 100). Acrophase is expressed as Zeitgeber time. *P*<0.05 compared to CON at corresponding gestational day (t-test following Diet x Gestational Day interaction in ANOVA). †*P*<0.05 overall effect compared to day 15, irrespective of diet (ANOVA). NS: Not significant for cosine fit.
Table 8.S3 Cosinor rhythmicity ($r^2$ and associated $P$ values) for fetal hepatic gene expression profiles.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CAF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clock</strong></td>
<td>0.144 ($P$=0.025)</td>
<td>0.354 ($P$&lt;0.001)</td>
</tr>
<tr>
<td><strong>Bmal1</strong></td>
<td>0.565 ($P$&lt;0.001)</td>
<td>0.155 ($P$=0.008)</td>
</tr>
<tr>
<td><strong>Per1</strong></td>
<td>0.048 (NS)</td>
<td>0.017 (NS)</td>
</tr>
<tr>
<td><strong>Per2</strong></td>
<td>0.429 ($P$&lt;0.001)</td>
<td>0.298 ($P$&lt;0.001)</td>
</tr>
<tr>
<td><strong>Per3</strong></td>
<td>0.076 (NS)</td>
<td>0.077 (NS)</td>
</tr>
<tr>
<td><strong>Cry1</strong></td>
<td>0.131 ($P$=0.015)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td><strong>Cry2</strong></td>
<td>0.187 ($P$=0.003)</td>
<td>0.204 ($P$=0.002)</td>
</tr>
<tr>
<td><strong>Rev-erba</strong></td>
<td>0.254 ($P$&lt;0.001)</td>
<td>0.076 (NS)</td>
</tr>
<tr>
<td><strong>Rora</strong></td>
<td>0.037 (NS)</td>
<td>0.057 (NS)</td>
</tr>
<tr>
<td><strong>Ppara</strong></td>
<td>0.411 ($P$&lt;0.001)</td>
<td>0.118 ($P$=0.024)</td>
</tr>
<tr>
<td><strong>Pparδ</strong></td>
<td>0.373 ($P$&lt;0.001)</td>
<td>0.255 ($P$&lt;0.001)</td>
</tr>
<tr>
<td><strong>Pgc1α</strong></td>
<td>0.019 (NS)</td>
<td>0.059 (NS)</td>
</tr>
<tr>
<td><strong>Glut2</strong></td>
<td>0.118 ($P$=0.022)</td>
<td>0.113 ($P$=0.028)</td>
</tr>
</tbody>
</table>

NS: not significant ($P$>0.05).
Table 8. S4 Rhythmic features (mesor, amplitude and acrophase) for fetal hepatic gene expression profiles.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fetal Liver</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CAF</td>
<td></td>
</tr>
<tr>
<td><strong>Clock</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 9</td>
<td>105 ± 11</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>33 ± 12</td>
<td>77 ± 15*</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>6.2 ± 1.4</td>
<td>6.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Bmal1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 4</td>
<td>95 ± 6</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>36 ± 5</td>
<td>25 ± 8</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>21.5 ± 0.6</td>
<td>19.9 ± 1.2</td>
<td></td>
</tr>
<tr>
<td><strong>Per1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 5</td>
<td>103 ± 6</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>Per2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 4</td>
<td>102 ± 3</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>31 ± 5</td>
<td>22 ± 5</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>20.3 ± 0.7</td>
<td>19.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td><strong>Per3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 10</td>
<td>92 ± 7</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>Cry1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 4</td>
<td>109 ± 7</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>15 ± 5</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>21.5 ± 1.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>Cry2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 6</td>
<td>95 ± 8</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>31 ± 9</td>
<td>38 ± 11</td>
<td></td>
</tr>
<tr>
<td>Acrophase</td>
<td>6.0 ± 1.1</td>
<td>5.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Mesor 100 ± 16</td>
<td>Mesor 101 ± 13</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>98 ± 23</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25.4 ± 0.9</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Rora</td>
<td>100 ± 9</td>
<td>109 ± 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Pparα</td>
<td>100 ± 5</td>
<td>101 ± 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42 ± 7</td>
<td>43 ± 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.3 ± 0.7</td>
<td>20.2 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Pparδ</td>
<td>100 ± 4</td>
<td>96 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29 ± 6</td>
<td>24 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.5 ± 0.8</td>
<td>20.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Pgc1α</td>
<td>100 ± 5</td>
<td>105 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Glut2</td>
<td>100 ± 6</td>
<td>101 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 ± 8</td>
<td>18 ± 7</td>
<td></td>
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<tr>
<td></td>
<td>21.4 ± 1.4</td>
<td>19.4 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean ± SEM and are expressed relative to CON mesor (set to 100). Acrophase is expressed as Zeitgeber time. * P<0.05 compared to CON at corresponding gestational day (t-test). NS: not significant for cosine fit.
Chapter 9: **Obesity disrupts rhythmic clock gene expression in maternal adipose tissue during rat pregnancy**

**Preface**

The primary objective of this chapter was to characterise the impact of maternal obesity on the rhythmic expression of clock genes in maternal adipose tissue. Rhythmic expression profiles of nuclear receptors (*Ppar* s) were analysed in maternal adipose tissue and daily leptin profiles were also measured in maternal and fetal plasma.

**Authorship and publication status:** This chapter was co-authored by Peter J. Mark and Brendan J. Waddell, and has been submitted for publication.

**Contributions:** Rachael Crew performed effectively all animal work, laboratory work and data analysis for this chapter, representing almost 100% of experimental work. She was also responsible for writing and submitting the manuscript, representing approximately 80% of written work for this chapter.
9.1 Abstract

Obesity during pregnancy causes numerous maternal and fetal health complications, but the underlying mechanisms remain uncertain. Adipose tissue dysfunction is common in obese individuals, and may be regulated by the circadian system. Maternal adipose tissue also undergoes substantial adaptations across pregnancy, including modifications in the rhythmic expression of clock and related genes. Consequently, we hypothesised that obesity distorts rhythmic maternal adipose tissue function across pregnancy. Maternal obesity was established in a rat model by cafeteria (CAF) feeding, and maternal adipose tissue was collected at four-hourly intervals across days 15-16 and 21-22 of gestation (term = 23 days). Rhythmic expression of clock genes and associated nuclear receptors (Ppars and Pgc1α) was measured by RT-qPCR. Rhythmic leptin profiles were also measured in maternal and fetal plasma. CAF feeding suppressed the mesor and amplitude of adipose tissue clock genes (including Bmal1, Per2 and Rev-erba) relative to chow fed controls (CON) across both days of gestation. The CAF diet eliminated adipose Pparγ rhythmicity on both gestational days, while overall expression of Ppara, Pparδ and Pgc1α was reduced at day 15 but not day 21. CAF mothers were hyperleptinaemic at both days of gestation, and at day 21 this effect was time-of-day dependent. Fetal leptin was unaffected by diet but exhibited clear rhythmicity. In conclusion, our data show that maternal obesity disrupts rhythmic expression of clock and metabolic genes in maternal adipose tissue, and leads to maternal but not fetal hyperleptinemia.
9.2 Introduction

Obesity during pregnancy leads to immediate and long-term complications for maternal and fetal health. Obese women are more likely to develop pregnancy disorders such as pre-eclampsia and gestational diabetes (Leddy, Power & Schulkin 2008; Marchi et al. 2015), while offspring born to obese pregnancies often experience altered growth trajectories and cardiometabolic aberrations in later life (Drake & Reynolds 2010). Although these adverse outcomes of obese pregnancy are widely recognised, the mechanisms underlying them remain obscure.

Adipose tissue dysfunction and altered lipid metabolism are cornerstones of obesity-related pathologies in both non-pregnant (Hajer, van Haeften & Visseren 2008) and pregnant individuals (Lappas 2014; Scifres, Catov & Simhan 2014). As such, disturbances to adipose tissue biology could mediate certain negative outcomes of obese pregnancy, and a growing body of evidence suggests that altered circadian biology may be crucial in this regard. Indeed, because the timing of metabolic processes is essential for energy homeostasis, the metabolic system and circadian clock are intricately related (Froy 2010; Bailey, Udoh & Young 2014). Accordingly, healthy adipose tissue displays highly rhythmic functions driven by adipocyte expression of clock genes, the molecular regulators of the circadian system (Shimba et al. 2005; Zvonic et al. 2006). The clock gene network (consisting of Clock, Bmal1, Per1-3, Cry1-2, Rev-erbα and Rora), influences the expression of many downstream genes, among which the peroxisome-proliferator activated receptors (Ppara, Pparγ, Pparδ) and the related Pgc1α, are particularly important for integrating the circadian clock with metabolic function (Yang et al. 2006; Chen & Yang 2014).

The circadian regulation of adipose tissue function is also likely to be important during pregnancy. Maternal adipose tissue lipid metabolism undergoes vital adaptations across pregnancy to meet maternal energy requirements and provide adequate substrate supply to the rapidly growing fetus (Herrera & Ortega-Senovilla 2010). Importantly, recent evidence suggests that these adaptations occur in a circadian-dependent manner, since the onset and progression of pregnancy leads to major changes in maternal circadian biology (Wharfe et al. 2016). Moreover, circadian disruption during pregnancy (via exposure to altered light cycles) disturbs maternal and fetal metabolism, alters rhythmic maternal leptin secretion, and programs metabolic dysfunction in offspring (Varcoe et al. 2013;
Varcoe et al. 2011). As such, a functional circadian system is clearly necessary for successful pregnancy outcomes.

Despite evidence that obesity alters adipose tissue clock gene expression in non-pregnant humans (Vieira et al. 2014; Gomez-Abellan et al. 2007) and animals (Kohsaka et al. 2007; Hsieh et al. 2009), the impact of obesity on the maternal circadian biology of adipose tissue during pregnancy is unknown. Accordingly, this study tested the hypothesis that maternal obesity, induced by cafeteria (CAF) feeding in rats, disrupts the rhythmic expression profiles of adipose tissue clock genes, associated Ppar genes, and maternal and fetal leptin levels.

9.3 Materials and Methods

9.3.1 Animals and diets

All animal procedures were approved by the Animal Ethics Committee of The University of Western Australia. Nulliparous albino Wistar rats were obtained at weaning age from the Animal Resources Centre (Murdoch, WA, Australia) and housed at the Preclinical Facility at The University of Western Australia. Animals were maintained at an ambient temperature of 22°C under a constant 12:12 h light-dark cycle (lights on at 0700h, lights off at 1900h), with free access to water and standard rodent chow (14 KJ/g total energy; 12% energy as fat, 23% protein and 65% carbohydrate; Specialty Feeds, Glen Forrest, W.A., Australia). Following one week of acclimatisation, animals were separated into control (CON) and cafeteria (CAF) groups; both groups had ad libitum access to standard chow, but CAF animals were also offered four human snack food items each day, which were given in excess and rotated daily to maintain novelty. The overall average total energy density of the CAF diet was 16.4 KJ/g (47% energy as fat, 8% as protein and 44% as carbohydrate); further dietary details are available in Chapter 5.

Animals were maintained on their respective diets for 8 weeks, after which they were mated overnight following determination of proestrus by an estrous cycle monitor (EC40; Fine Science Tools, Vancouver, British Columbia, Canada). Day 1 of pregnancy was confirmed when spermatozoa were present in a vaginal smear the following morning. Animals were housed individually and maintained on their respective diets throughout pregnancy.
9.3.2 Tissue collection

The time of each tissue collection was expressed relative to Zeitgeber time zero (ZT0), which was defined as 0700h (when lights turned on in the animal facility). Adipose tissue and plasma samples were collected at four-hourly intervals (ZT1; 0800h, ZT5; 1200h, ZT9; 1600h, ZT13; 2000h, ZT17; 2400h and ZT21; 0400h) across each of days 15-16 and day 21-22 of gestation (term is 23 days).

At the appropriate time, animals were anesthetised using isoflurane/nitrous oxide (n = 7-8 per diet group at each time-point). The maternal abdominal cavity was surgically opened and a sample of retroperitoneal adipose tissue was taken from each mother, immediately frozen in liquid nitrogen and stored at -80°C. Maternal blood was collected from the descending aorta. For day 21 collections, fetuses were removed via caesarean section, sex was determined by anogenital distance (Faber & Hughes 1992), and fetal trunk blood was collected by decapitation. All blood samples were mixed with 10: 1 (vol: vol) 0.6 M EDTA and centrifuged at 13,000 x g for 6 min to obtain plasma, which was then frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

9.3.3 RNA and cDNA sample preparation

Total RNA was extracted from maternal adipose tissue using an RNeasy Lipid Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA was assessed for concentration and purity using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and 1 µg of total RNA was reversed transcribed to cDNA using the Moloney Murine Leukemia Virus Reverse Transcriptase RNase H Point Mutant with random hexamers (Promega, Sydney, Australia), as previously described (Hewitt et al. 2006). The resultant cDNA was purified using the UltraClean PCR Cleanup Kit, according to the manufacturer’s instructions (MoBio Laboratories, Carlsbad, CA, USA).

9.3.4 Quantitative PCR

The relative mRNA expression of clock genes (Clock, Bmal1, Per1, Per2, Per3, Cry1, Cry2, Rev-erba and Rora), and several nuclear receptors involved in lipid metabolism (Ppara, Pparγ, Pparδ and Pgc1α) was quantitated via RT-qPCR on the Rotorgene Q (Qiagen, Hilden, Germany). All primer pairs (see Table 9.1) were designed using Primer-
BLAST (Rozen & Skaletsky 2000) and were positioned to span an intron to prevent genomic DNA amplification.

Each PCR reaction consisted of 1 μl of 10 x Immolase buffer, 0.2 μl 10 mM dNTPs, 0.5 μl SYBR green dye (Molecular Probes, Eugene, OR, USA) at a 1/2000 dilution in DMSO, 0.05 μl Immolase DNA Polymerase (Bioline, Alexandria, NSW, Australia), and primer-specific concentrations (summarised in Table 9.1) of MgCl₂ and forward and reverse primer. Reactions were then made up to 9 μl with ddH₂O, after which 1 μl of cDNA was added to give a total reaction volume of 10 μl. The PCR reaction cycle consisted of an initial denaturing stage at 95°C for 10 min, followed by 45 cycles each comprising of 95°C for 1 sec, a primer-specific annealing temperature (see Table 1) for 15 sec and a 72°C extension period for 5 sec.

Standard curves were generated from 10-fold serial dilutions of gel-extracted PCR product and used to calculate relative mRNA concentrations with Rotorgene Q series software. All mRNA expression values were standardised against the reference genes Ppia, Sdha and Ywhaz using the GeNorm algorithm (Vandesompele et al. 2002).
### Table 9.1 Primer details and PCR conditions for genes measured in adipose tissue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing Temp (°C)</th>
<th>Size (bp)</th>
<th>MgCl₂ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clock genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock</td>
<td>F5' ACAGCGCACACACAGGCCTTC 3'</td>
<td>60</td>
<td>175</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' TGGCGGCGCCCTGTGTATCTA 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmal1</td>
<td>F5' AACTGCACCTCGGGAGCGA 3'</td>
<td>60</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' CGCCGAGCTCCAGAGCACAA 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per1</td>
<td>F5' CGCACTTGGGAGCTCAAACTTC 3'</td>
<td>60</td>
<td>169</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' GTCCATGGCACAGGGCTCAACC 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per2</td>
<td>F5' TGAGCTCCTTGGCGTTGCCG 3'</td>
<td>60</td>
<td>147</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' ACTCAGGCCCCACTGGCCACA 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per3</td>
<td>F5' TTTTCCCCCTTCAAGACATGG 3'</td>
<td>60</td>
<td>167</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' GAAAGAGAGGGCTTGTGTGC 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry1</td>
<td>F5' AGCTGGCCACTGAGCTGCTG 3'</td>
<td>60</td>
<td>158</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' TGCTGGCATCTCCAGGGCT 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry2</td>
<td>F5' CTGCCCAGGGAGCCACCCAAGC 3'</td>
<td>60</td>
<td>192</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' GCATGCACACGCAAACGGCA 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>F5' ATGCCCACGGGCGAGAGA 3'</td>
<td>60</td>
<td>292</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' GCCAAAGAGCGGGCGAGGTT 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rorα</td>
<td>F5' CCAACCGTGTCCATGGCGG 3'</td>
<td>60</td>
<td>113</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>R5' CCCGTGATGCGTTGCCGA 3'</td>
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<td></td>
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### Ppar genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length (bp)</th>
<th>Cq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pparα</td>
<td>F5’ AATCCACGAAGCCTACCTGA 3’&lt;br&gt;R5’ GTCTTCTCAGCCATGCACAA 3’</td>
<td>60&lt;br&gt;132&lt;br&gt;2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pparγ</td>
<td>F5’ GACCCAGAGCATGGTGCTCTCG 3’&lt;br&gt;R5’ GCTGATTCGAAAGTTGTTGGGCC 3’</td>
<td>63&lt;br&gt;108&lt;br&gt;3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pparδ</td>
<td>F5’ GAGGGGTGCAAGGGCTTCTT 3’&lt;br&gt;R5’ CACTTGTGGCCTTCTTCTTCTG 3’</td>
<td>60&lt;br&gt;101&lt;br&gt;2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pgc1α</td>
<td>F5’ TCTGGAACGCAAGGCCTAATCTC 3’&lt;br&gt;R5’ GCAAGAGGGCTTCAGCTTTG 3’</td>
<td>60&lt;br&gt;96&lt;br&gt;4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Reference genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length (bp)</th>
<th>Cq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ppia</td>
<td>F5’ AGCATACAGGTCTGGCATC 3’&lt;br&gt;R5’ TTCACCTCTCCCCAAAGACCAC 3’</td>
<td>62&lt;br&gt;127&lt;br&gt;3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sdha</td>
<td>F5’ TGGGGCGACTCGTGCTTTTC 3’&lt;br&gt;R5’ CCCCCGCTGCACCTCAACC 3’</td>
<td>60&lt;br&gt;134&lt;br&gt;2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ywhaz</td>
<td>F5’ GACGGAGCTGAGGGACATCTGC 3’&lt;br&gt;R5’ GGCTGCCAGACATGGGGATCA 3’</td>
<td>60&lt;br&gt;75&lt;br&gt;2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F: Forward primer, R: Reverse primer, bp: base pairs
9.3.6 Plasma leptin measurement

Leptin was measured in maternal and day 21 female fetal plasma using a Milliplex MAP Rat Adipokine Magnetic Bead Panel (Cat. # RECYTMAG-65K; EMD Millipore Corporation, Billerica, MA, USA), according to the manufacturer’s instructions. The plate was read on a Luminex Magpix (Luminex Corporation, Austin, Texas, USA), and data were analysed with Magpix 4.2 Software. The intra-assay coefficient of variation was 1.6%.

9.3.7 Statistical analysis

All values are expressed as the mean ± SEM. Daily gene expression and leptin profiles were assessed for rhythmicity by cosinor regression analysis in Genstat 9.0 software (Hemel Hempstead, UK). This generated the characteristic rhythm features of mesor (the rhythm adjusted mean), amplitude (the difference between the rhythm peak and the mesor) and acrophase (the time at which the rhythm peak occurs) for each daily profile. The influence of diet and stage of pregnancy on these features was assessed by two-way ANOVA and subsequent t-tests if interactions were observed (GraphPad Prism version 6.00; La Jolla, California). Profiles were considered ‘rhythmic’ when their fit to the cosine function was significant ($P<0.05$). When profiles were non-significant, data were assessed by ANOVA using GenStat 9.0 software (Hemel Hempstead, UK), with variation apportioned to diet, time of day, and stage of pregnancy. When significant ($P<0.05$) interactions were observed, subsequent comparisons were performed by two-way ANOVA or $t$-test, as appropriate. When the $F$-test reached statistical significance ($P<0.05$) specific group comparisons were made by LSD tests.
9.4 Results

Maternal caloric intake, weight gain and body composition data for this cohort of animals has been previously reported (Chapter 6). Briefly, CAF mothers consumed an average of 73% more calories and exhibited a 25% increase in body weight after 8 weeks of pre-pregnancy CAF feeding. Analysis of body composition at day 21 of gestation showed a 56% increase in total percentage adiposity in CAF mothers. Individual fetal and placental weights were reduced in the CAF group across both days, but total fetal and placental mass did not differ between diet groups due to a slight increase in CAF litter size.

9.4.1 Clock gene expression in maternal adipose tissue

The majority of adipose clock gene expression profiles showed significant cosinor rhythmicity in CON mothers (see Table 9.2), the only exception being *Clock* at day 15. In contrast, the CAF diet abolished the rhythmicity of *Cry2*, *Rora* and *Clock* at day 21, but all other genes remained rhythmic in CAF animals (Table 9.2). Maternal obesity also suppressed overall expression of adipose clock genes; specifically, the CAF diet reduced the mesor of *Per1*, *Per2*, and *Rev-erba* at day 15 and those of *Bmal1*, *Per2*, and *Cry1* at day 21 (all *P*<0.05; see Fig 9.1 and Table 9.3). The amplitudes of the *Bmal1* and *Rev-erba* rhythms were reduced in CAF animals across both days of gestation, while reduced amplitudes were also evident for *Per3* at day 15 and *Per2* at day 21 (*P*<0.05; Fig 9.1; Table 9.3). The CAF diet delayed the acrophase of the *Cry2* and *Rora* rhythms at day 15 (*P*<0.05; Fig 9.1 and Table 9.3), and of *Bmal1* at day 21 (*P*<0.05; Fig 9.1 and Table 9.3).

The rhythmic features of several clock genes also varied with gestational age; specifically, the mesor of *Per1*, *Per2*, *Per3* and *Rora* increased between days 15 and 21, whereas the *Clock* mesor was reduced (*P*<0.01; Fig 9.1 and Table 9.2). The amplitude of *Bmal1*, *Per2* and *Rev-erba* rhythms fell over this same period (*P*<0.001; Fig 9.1 and Table 9.2), while the acrophase of each clock gene rhythm remained unchanged with advancing gestation.
Table 9.2 Cosine fit ($r^2$ and associated $P$ values) for gene expression profiles in maternal adipose tissue.

<table>
<thead>
<tr>
<th></th>
<th>Day 15 CON</th>
<th>Day 15 CAF</th>
<th>Day 21 CON</th>
<th>Day 21 CAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clock</td>
<td>0 (NS)</td>
<td>0 (NS)</td>
<td>0.099 ($P=0.038$)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Bmal1</td>
<td>0.791 ($P&lt;0.001$)</td>
<td>0.796 ($P&lt;0.001$)</td>
<td>0.885 ($P&lt;0.001$)</td>
<td>0.816 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Per1</td>
<td>0.560 ($P&lt;0.001$)</td>
<td>0.400 ($P=0.004$)</td>
<td>0.372 ($P&lt;0.001$)</td>
<td>0.244 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Per2</td>
<td>0.791 ($P&lt;0.001$)</td>
<td>0.596 ($P&lt;0.001$)</td>
<td>0.545 ($P&lt;0.001$)</td>
<td>0.423 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Per3</td>
<td>0.815 ($P&lt;0.001$)</td>
<td>0.524 ($P&lt;0.001$)</td>
<td>0.597 ($P&lt;0.001$)</td>
<td>0.367 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Cry1</td>
<td>0.513 ($P&lt;0.001$)</td>
<td>0.401 ($P&lt;0.001$)</td>
<td>0.496 ($P&lt;0.001$)</td>
<td>0.322 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Cry2</td>
<td>0.227 ($P&lt;0.001$)</td>
<td>0.103 ($P=0.041$)</td>
<td>0.172 ($P=0.005$)</td>
<td>0.012 (NS)</td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>0.810 ($P&lt;0.001$)</td>
<td>0.821 ($P&lt;0.001$)</td>
<td>0.727 ($P&lt;0.001$)</td>
<td>0.516 ($P&lt;0.001$)</td>
</tr>
<tr>
<td>Rorα</td>
<td>0.246 ($P&lt;0.001$)</td>
<td>0.145 ($P=0.015$)</td>
<td>0.103 ($P=0.033$)</td>
<td>0 (NS)</td>
</tr>
<tr>
<td>Pparaα</td>
<td>0.063 (NS)</td>
<td>0.272 ($P&lt;0.001$)</td>
<td>0.186 ($P&lt;0.001$)</td>
<td>0.129 ($P=0.017$)</td>
</tr>
<tr>
<td>Pparγ</td>
<td>0.342 ($P&lt;0.001$)</td>
<td>0.277 (NS)</td>
<td>0.175 ($P=0.005$)</td>
<td>0.147 ($P=0.010$)</td>
</tr>
<tr>
<td>Pparδ</td>
<td>0.048 (NS)</td>
<td>0.206 ($P=0.002$)</td>
<td>0.279 ($P&lt;0.001$)</td>
<td>0.022 (NS)</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>0.062 (NS)</td>
<td>0.150 ($P=0.012$)</td>
<td>0.167 ($P=0.008$)</td>
<td>0.039 (NS)</td>
</tr>
</tbody>
</table>

NS: not significant ($P>0.05$).
Table 9.3 Mesor, amplitude and acrophase of rhythmic (cosinor) gene expression profiles in maternal adipose tissue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>CAF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Clock</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 3</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Acrophase</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Bmal1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 4</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>82 ± 6</td>
<td>65 ± 5*</td>
</tr>
<tr>
<td>Acrophase</td>
<td>0.3 ± 0.28</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td><strong>Per1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 4</td>
<td>88 ± 4*</td>
</tr>
<tr>
<td>Amplitude</td>
<td>43 ± 6</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>Acrophase</td>
<td>13.2 ± 0.5</td>
<td>13.9 ± 0.7</td>
</tr>
<tr>
<td><strong>Per2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 3</td>
<td>84 ± 4*</td>
</tr>
<tr>
<td>Amplitude</td>
<td>54 ±4</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>Acrophase</td>
<td>15.1 ± 0.3</td>
<td>14.6 ± 0.5</td>
</tr>
<tr>
<td><strong>Per3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 3</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>68 ± 5</td>
<td>41 ± 6*</td>
</tr>
<tr>
<td>Acrophase</td>
<td>12.6 ± 0.3</td>
<td>12.7 ± 0.6</td>
</tr>
<tr>
<td><strong>Cry1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 5</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>Amplitude</td>
<td>44 ± 6</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>Acrophase</td>
<td>19.5 ± 0.6</td>
<td>20.0 ± 0.7</td>
</tr>
<tr>
<td><strong>Cry2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 ± 5</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>Amplitude</td>
<td>25 ± 6</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>Acrophase</td>
<td>13.9 ± 1.0</td>
<td>17.5 ± 1.5*</td>
</tr>
<tr>
<td>Gene</td>
<td>Mesor</td>
<td>Amplitude</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>------------</td>
</tr>
<tr>
<td><em>Rev-erβ</em></td>
<td>100 ± 6</td>
<td>81 ± 4*</td>
</tr>
<tr>
<td></td>
<td>9.3 ± 0.3</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td><em>Rorα</em></td>
<td>100 ± 5</td>
<td>94 ± 4</td>
</tr>
<tr>
<td></td>
<td>14.1 ± 0.9</td>
<td>17.8 ± 1.3*</td>
</tr>
<tr>
<td><em>Pparα</em></td>
<td>100 ± 4</td>
<td>98 ± 5</td>
</tr>
<tr>
<td></td>
<td>13.3 ± 1.6</td>
<td>19.6 ± 0.9*</td>
</tr>
<tr>
<td><em>Pparδ</em></td>
<td>100 ± 4</td>
<td>96 ± 5</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>20.3 ± 1.1</td>
</tr>
<tr>
<td><em>Pparγ</em></td>
<td>100 ± 5</td>
<td>91 ± 5</td>
</tr>
<tr>
<td></td>
<td>13.6 ± 0.8</td>
<td>16 ± 0.9*</td>
</tr>
<tr>
<td><em>Pgc1α</em></td>
<td>100 ± 4</td>
<td>104 ± 7</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>11.9 ± 1.3</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM and are expressed relative to CON mesor at day 15 (set to 100). Acrophase is expressed as Zeitgeber time. * P<0.05 compared to CON at corresponding gestational day (t-test following Diet x Gestational Day interaction in ANOVA). † P<0.05 overall effect compared to day 15, irrespective of diet (ANOVA). NS: Not significant for cosine fit.
Figure 9.1 Daily expression profiles of clock genes in maternal adipose tissue of control (CON) and cafeteria (CAF) diet groups at days 15 and 21 of gestation. Shaded areas represent the dark period. Values are the mean ± SEM (n = 7-8 per diet group at each ZT). Cosinor curves are shown only for those genes that had significant cosinor rhythmicity. Statistical differences for cosine curve features are summarised in Table 9.3.
9.4.2 Rhythmic expression of PPARs in adipose tissue

Because the PPAR nuclear receptors (*Ppara*, *Pparγ*, *Pparδ*) and their co-regulator *Pgc1α* are linked to both the molecular clock and lipid metabolism (Chen & Yang 2014), we also investigated the impact of obesity on their expression in adipose tissue. While cosinor analysis showed that adipose expression profiles for *Ppara*, *Pparδ* and *Pgc1α* were arrhythmic in CON animals at day 15 of gestation, rhythmicity was induced by the CAF diet for each of these genes (see Table 9.2 and Fig 9.2). Conversely, *Pparγ* expression was rhythmic in CON mothers at day 15 but this was abolished by the CAF diet. By day 21 of gestation, all *Ppar* genes were rhythmic in both CON and CAF mothers, with the exception of *Pparδ*, which was rhythmic only in the CON group (Table 9.2 and Fig 9.2).

Since most *Ppar* expression profiles were arrhythmic at day 15, these data were also assessed by conventional ANOVA (with diet, time of day and gestational age as sources of variation). At day 15, there was a significant Diet x Time of Day interaction in the expression of *Ppara*, *Pparδ* and *Pgc1α*. Specifically, expression of these genes was suppressed in the CAF group at select times of the day, particularly the ZT13 time point (*P*<0.05; Fig 9.2). Interestingly, however, this effect did not persist as gestation progressed; by day 21 there were no diet-related differences in *Ppar* gene expression (Fig 9.2). The expression of *Ppara* and *Pgc1α* also increased with gestational age (*P*<0.05; Fig 9.2) irrespective of maternal diet.
Figure 9.2 Daily expression profiles of Ppar genes in maternal adipose tissue of control (CON) and cafeteria (CAF) diet groups at day 15 and 21 of gestation. Shaded areas represent the dark period. Values are the mean ± SEM (n = 7-8 per diet group at each ZT). * P<0.05 compared to CON (t-test following Diet x Time of Day interaction in two-way ANOVA). Cosinor curves are shown only for those genes that had significant cosinor rhythmicity. Statistical differences for cosine curve features are summarised in Table 9.3.
9.4.3 Maternal and fetal leptin profiles

Maternal hyperleptinemia was clearly evident in CAF mothers at both days of gestation ($P<0.001$; Fig 9.3), consistent with their increased adiposity (Chapter 6). While plasma leptin was elevated in CAF mothers at each time-point across day 15 ($P<0.001$; Fig 9.3), by day 21 there was a significant Diet x Time of Day interaction ($P<0.001$). Specifically, leptin levels in late gestation were higher ($P<0.05$, $t$-test) in CAF compared to CON mothers at ZT5, ZT13, ZT17 and ZT21, but not at ZT1 or ZT9 (Fig 9.3). Interestingly, unlike maternal leptin profiles, fetal leptin showed clear cosinor rhythmicity ($r^2 = 0.187$ ($P=0.004$) for CON; $r^2 = 0.161$ ($P=0.007$) for CAF) but the characteristics of these rhythms (mesor, amplitude and acrophase) were unaffected by diet (Fig 9.3).
Figure 9.3 Daily leptin profiles in (A) maternal plasma at day 15, (B) maternal plasma at day 21, and (C) fetal plasma day 21 in control (CON) and cafeteria (CAF) diet groups. Shaded areas represent the dark period. Values are the mean ± SEM (n = 7-8 per diet group at each ZT). *P<0.001 CON vs. CAF (overall diet effect in two-way ANOVA); ** P<0.05 compared to CON (t-test following Diet x Time of Day interaction in two-way ANOVA). Cosinor curves are shown only for those profiles that had significant cosinor rhythmicity.
9.5 Discussion

This study has established that obesity disturbs the rhythmic expression of clock genes and *Ppars* in maternal adipose tissue during pregnancy. Obesity reduced the mesor and amplitude of several clock gene rhythms, while the acrophase of *Cry2, Rora* and *Bmal1* were each delayed. Maternal obesity also reduced adipose expression of the nuclear receptors *Ppara, Pparδ* and *Pgc1α*, although this effect was dependent on gestational age. Maternal plasma leptin levels were elevated in obese mothers but remained arrhythmic regardless of diet. In contrast, while fetal plasma leptin levels were unaffected by obesity, they showed clear time-of-day variation suggestive of either diurnal changes in fetal leptin production or transplacental leptin passage.

Our results extend previous observations showing that obesity suppresses adipose tissue clock gene expression in non-pregnant rodents (Kohsaka *et al.* 2007; Prasai *et al.* 2013) and humans (Gomez-Abellan *et al.* 2007). Similarly, adipose tissue clock gene expression is suppressed in genetic obesity models (e.g. the leptin-resistant *ob/ob* mouse) (Ando *et al.* 2011; Yamaoka *et al.* 2014), and interestingly this suppressive effect in *ob/ob* mice precedes obesity development, which potentially implicates clock gene disturbances in the onset of the obese phenotype. Moreover, leptin treatment in *ob/ob* mice largely restores the clock gene profiles (Ando *et al.* 2011), suggesting that a deficiency in leptin signalling disrupts clock gene expression. Therefore, given that CAF mothers were hyperleptinemic and thus likely to have been leptin resistant (Sáinz *et al.* 2015), the observed disturbances in clock gene expression may well reflect reduced leptin action in adipose tissue.

The adipose clock genes predominantly affected by CAF diet (*Bmal1, Per2* and *Rev-erba*) each displayed reduced mesor or amplitude at both stages of gestation. Importantly, these three genes are thought to be the most influential in maintaining rhythmic oscillations of central and peripheral clocks. Specifically, *Bmal1* displays the most robust rhythmicity among the core clock genes, and in particular its oscillations are far greater than those of *Clock*, the other primary driver of overall rhythmicity (Yamamoto *et al.* 2004; Dunlap 1999). Similarly, among the downstream clock genes *Per2* appears to have the greatest feedback influence on *Bmal1* and *Clock* transcription within the transcriptional-translational loop; indeed, *Per3* has relatively little effect on rhythmicity, while *Per1* largely functions to moderate *Per2* expression through post-transcriptional means.
modifications (Zheng et al. 2001; Bae et al. 2001). The expression of Rev-erbα, which also shows robust rhythmicity, represses Bmal1 transcription and thus their expression profiles are inversely correlated (Preitner et al. 2002). In this context it is noteworthy that Rev-erbα also plays important roles in regulating lipid metabolism (Duez & Staels 2008) and adipogenesis (Kumar et al. 2010; Fontaine et al. 2003). Given the fundamental importance of Bmal1, Per2 and Rev-erbα within the adipose tissue clock, the observation that their expression was most affected by maternal obesity increases the likelihood that circadian variation in adipose function is also disrupted in CAF mothers.

Maternal obesity reduced adipose expression of Pparα, Pparδ and Pgc1α at day 15 of pregnancy, but this effect was no longer evident at day 21. Suppression of all three PPAR isoforms in adipose tissue of obese pregnant women at term has been reported (Lappas 2014), but our data are the first to show the comprehensive rhythmic expression profiles of these nuclear receptors in obese pregnancy. Previously, non-pregnant rodents fed a high-fat diet have been shown to lose diurnal rhythmicity in adipose Pparγ expression (Kohsaka et al. 2007), similar to our observations at day 15 of pregnancy. Interestingly, however, this CAF-induced loss of rhythmicity in Pparγ was no longer evident at day 21, possibly related to the major shifts in sex steroid profiles during late gestation. Indeed, previous studies suggest that normal clock gene profiles (Wharfe et al. 2016) and their response to a high-fat diet (Yanagihara et al. 2006) are influenced by sex steroids. In any event, obesity-induced changes in adipose PPARs may be central to the function of the adipose tissue clock given their dual roles in regulating lipid metabolism and clock genes (Chen & Yang 2014). Specifically, these nuclear receptors exhibit rhythmic expression profiles in metabolic tissues (Yang et al. 2006), and Pparα, Pparγ and Pgc1α all regulate Bmal1 and Rev-erbα transcription (Liu et al. 2007; Fontaine et al. 2003; Canaple et al. 2006). Interestingly, our results indicate that the obesity-induced suppression of the Ppar genes was most consistently observed at ZT13, possibly related to the proximity of this sampling time to provision of novel food items to CAF mothers. This further suggests that consumption of the CAF items could have an immediate effect on adipose tissue lipid metabolism mediated via Ppar gene expression changes. As shown in Chapter 6, hyperlipidaemia in CAF mothers that was specific to the nocturnal period in late gestation, and so the adipose clock gene disruptions presented in the current study further implicate the circadian clock in the regulation of these metabolic disturbances.
The apparent correction of obesity-induced disturbances in adipose Ppar gene profiles between days 15 and 21 is consistent with recent human (Forbes et al. 2015; Straughen, Trudeau & Misra 2013) and animal (King et al. 2013; Pedroni et al. 2014) studies showing that disturbances in the maternal metabolic phenotype are less severe late in obese pregnancy. For example, Forbes et al. (2015) demonstrated that insulin resistance and elevated triglyceride levels are evident during the first half of pregnancy in obese women, but not near term. Similarly, while the inflammatory status of obese women (Friis et al. 2013) and mice (Pedroni et al. 2014; Ingvorsen et al. 2014) is elevated early in gestation, this effect is no longer evident at term.

Maternal leptin profiles were arrhythmic in both diet groups, but absolute levels were elevated in obese mothers at each gestational age. While it is well-recognised that obese pregnant women are hyperleptinemic (Misra & Trudeau 2011; Briffa et al. 2015), the present study is the first to report diurnal profiles of maternal leptin in obese pregnancy. Leptin normally exhibits a rhythmic daily secretion pattern in the non-pregnant state, peaking nocturnally in both humans (Saad et al. 1998; Yildiz et al. 2004) and rodents (Cano et al. 2009; Ando et al. 2005; Kalsbeek et al. 2001). Previous studies on leptin rhythmicity in human obesity are inconsistent; one study reported maintenance of rhythmicity and an elevated peak in obese males (Yildiz et al. 2004), whereas another showed that obesity increased overall leptin levels but abolished rhythmicity (Saad et al. 1998). Similarly, leptin rhythmicity was lost in rats after high-fat feeding (Cano et al. 2009). Interestingly, despite the absence of a cosinor leptin rhythm in our study, maternal leptin exhibited marked time of day variation across day 21 in CAF mothers, such that they were only hyperleptinemic at select time points. In this context it is noteworthy that the central (hypothalamic) circadian clock regulates leptin rhythmicity independently of food intake, and that chronodisruption disturbs rhythmic leptin profiles (Kalsbeek et al. 2001; Varcoe et al. 2013; Kettner et al. 2015); as such, our data suggest that obesity may disturb the central circadian regulation of leptin function in pregnancy.

In contrast to the observed maternal hyperleptinemia in the CAF group, fetal leptin levels were unaffected. Fetal leptin is largely maternally derived in rodents, and both placental leptin receptor expression and transplacental leptin passage increase markedly over late gestation in rats (Smith & Waddell 2003b) in association with increased labyrinth zone expression of Lepra, the truncated form of the leptin receptor involved in leptin transport (Smith & Waddell 2002). Moreover, both placental leptin transport and expression of the
leptin receptor (Leprb) are reduced by glucocorticoid treatment (Smith & Waddell 2003b), suggesting that insults during pregnancy may alter fetal leptin levels via effects on placental transport mechanisms. Accordingly, Farley et al. (2010) reported that placental leptin receptor expression was reduced in obese human pregnancy, and maternal hyperleptinemia was not accompanied by fetal hyperleptinemia. Potentially, lower placental leptin receptor expression could compromise placental amino acid transport, since leptin is known to increase System A amino acid transporter activity in cultured human placental tissue (Jansson et al. 2003; von Versen-Höynck et al. 2009). Indeed, decreased placental amino acid transport has been observed in HF-fed mice (Lager et al. 2014), which could contribute to the fetal growth restriction that occurs in subsets of obese pregnancies (Radulescu et al. 2013; Gardosi & Francis 2009). We have previously reported CAF-induced fetal growth restriction in this model (Chapters 5 & 6), and so it would be of interest to explore whether placental nutrient transfer capacity is limited in CAF mothers, and whether this is linked to reduced leptin action in the placenta.

Unlike maternal leptin profiles, fetal leptin exhibited significant cosinor rhythmicity irrespective of diet. This rhythmic profile of fetal leptin has not previously been documented and indeed is somewhat surprising given the absence of comparable variation in the mother. Given the mostly maternal origin of fetal leptin in rodents (Smith & Waddell 2003b) the rhythmic profiles of fetal leptin may reflect diurnal variation in its transplacental passage. Moreover, despite the increased capacity for placental transport of leptin near term (described above), the presence of maternal, but not fetal, hyperleptinemia in obese pregnancy suggests that the placenta still limits leptin transfer to the fetus.

In conclusion, our data show that obesity disrupts adipose expression of clock and Ppar genes during the final third of rat pregnancy. Maternal leptin profiles were increased by obesity but remained arrhythmic, whereas fetal leptin profiles showed cosinor rhythmicity but were unaffected by obesity. We propose that disruption of the adipose tissue clock by diet-induced obesity may adversely affect the maternal metabolic adaptation to pregnancy and thereby compromise fetal growth and development.
Acknowledgements

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Chapter 10: General Discussion

The overall objective of the studies presented in this thesis was to investigate the effects of obesity on circadian function in maternal, placental and fetal tissues during pregnancy. Specific focus was placed on the core molecular components of the circadian regulatory system (i.e., clock genes), in conjunction with other metabolic and physiological processes that are implicated in obesity pathogenesis, pregnancy adaptations, and/or circadian regulation. The findings of each study have been presented and discussed in detail in the relevant chapters. This final chapter integrates the findings of all five studies, discusses their implications as a whole, reflects on the limitations of this work, and raises some interesting potential areas for further investigation.

Peripheral metabolic clock gene expression is altered during obese pregnancy

Chapters 8 and 9 of this thesis demonstrate that obesity alters rhythmic clock gene expression in the maternal liver and adipose tissue across mid- and late- gestation. Interestingly, while the predominant effect in the liver was a CAF-induced phase-advance to clock gene expression profiles, adipose tissue clock gene expression was mostly suppressed by the CAF diet (i.e. reduced mesor and amplitude). Most adipose clock genes did not exhibit phase changes, and those that did (Cry2, Rora and Bmal1) were marginally delayed by the CAF diet. These opposing phase responses to CAF feeding likely reflect the differing functions of these tissue-specific clocks; the hepatic clock is highly responsive to stimulus by food intake (Oike et al. 2011), whereas adipose tissue clocks may be more strongly adjusted by other downstream factors associated with obesity, such as altered leptin signalling. Alternatively, any specific dietary influence on adipose clocks may be less immediate than that in the liver.

Chapters 8 and 9 also reinforce the concept that the fetal and placental circadian systems are not fully functional during rat gestation. While the maternal liver and adipose tissue exhibited robust rhythms in clock gene expression, with typical feedback patterns evident (e.g. Bmal1 and Rev-erba in anti-phase), many placental and fetal liver clock genes were not significant for cosine fit. Of those that were, cosine significance seemed to reflect developmental (rather than circadian) changes to expression, since the typical phase arrangements were not evident between clock genes, possibly indicative of an immature clock in these organs. Clock genes were largely unaffected by CAF feeding in fetal and
placental tissues, which may be attributable to this functional immaturity. The exception to this was Rev-erbα, which exhibited remarkably similar rhythmic expression profiles between maternal, fetal and placental tissues in CON animals. Moreover, the normal peak in Rev-erbα expression was suppressed in these tissues by CAF feeding. This suggests that Rev-erbα has functional importance during in utero development and in adult life, and that in both cases it is readily disrupted by obesity.

The consistency of the Rev-erbα expression pattern (and its response to the CAF insult) between different maternal, placental and fetal tissue types indicates that Rev-erbα may be involved in fundamental aspects of metabolism, such as cellular energy status. There are strong links between basic cellular metabolism and the core clock. For example, cellular redox state affects CLOCK:BMAL1 binding efficiency, whereby CLOCK:BMAL1 binding is promoted by the presence of reduced nicotinamide adenine dinucleotide (NADH), but inhibited by oxidised NAD+ (Rutter et al. 2001). HF-feeding in mice also impairs NAD+ synthesis in metabolic tissues, through repression of its rate limiting enzyme, NAMPT (Yoshino et al. 2011). Similarly, insulin resistance induced by HF feeding in mice alters mitochondrial function and induces a more oxidised cellular redox state in skeletal muscle (as measured by glutathione disulfide/glutathione ratio) (Anderson et al. 2009), while obese Zucker rats exhibit similar effects in hypothalamic cellular redox status (Colombani et al. 2009). As such, altered cellular redox states in obese individuals may mediate obesity-induced disruptions to clock genes in metabolic tissues. Interestingly, Eckel-Mahan and colleagues (2013) reported that hepatic NAD+ signalling was disrupted in obese mice in a remarkably similar manner to the Rev-erbα profiles in this study; a distinct peak in NAD+ and Nampt expression occurred around the transition from light to dark in control animals, which was completely suppressed in the HF group. It would be interesting for future studies to characterise the oxidative status of maternal, fetal and placental tissues in obese pregnancy, and to determine whether oxidative changes may occur in a circadian manner, possibly through a Rev-erbα-mediated integration of cellular redox status with circadian and metabolic function.

Rev-erbα also regulates brown adipose tissue function, and is necessary for normal Τc rhythmicity and adequate thermoregulatory responses to cold challenges (Gerhart-Hines et al. 2013). Although BAT was not collected in the present study, it would be interesting to determine whether the Rev-erbα suppression that was apparent in white adipose tissue
of CAF mothers is evident in BAT, and how this might correspond the \( T_c \) reduction evident in CAF mothers.

**Central circadian functions during obese pregnancy**

This thesis was primarily focused on the rhythmic function of peripheral metabolic clocks, rather than central circadian function. Nevertheless, the examination of rhythmic maternal \( T_c \) and glucocorticoid profiles may offer some indirect insight into the functionality of the central circadian clock in this model. Maternal \( T_c \) mesor was lower in obese mothers prior to and throughout gestation. While \( T_c \) amplitude was altered by obesity over select days of the estrous cycle and pregnancy, the acrophase of temperature rhythms was remarkably similar between CON and CAF mothers (Chapter 7). Similarly, maternal corticosterone and 11-DHC rhythmicity was largely unchanged by obesity. Since both \( T_c \) (Saper, Scammell & Lu 2005) and glucocorticoid rhythms (Kalsbeek et al. 2012) are regulated by the SCN, the lack of \( T_c \) phase-changes with maternal obesity may suggest that the central rhythmic processes are relatively unaffected by maternal obesity. Notably, circadian \( T_c \) regulation involves rhythmic BAT function (Gerhart-Hines et al. 2013), and glucocorticoid rhythms are also partially regulated by peripheral clocks in the adrenal cortex (Oster et al. 2006). Therefore, further studies are required to determine the relative influence of central and peripheral oscillators on rhythmic glucocorticoid and \( T_c \) responses to obesity.

In any case, it would be interesting to determine whether obesity alters clock gene expression in the maternal SCN, or other important hypothalamic regions involved in circadian and metabolic integration, such as the arcuate or paraventricular nuclei. Previous findings by Kohsaka and colleagues (2007) indicate that hypothalamic clock gene expression is largely unchanged by HF-feeding in male mice, despite changes to adipose tissue and liver clocks which were similar to those noted in CAF mothers in the current studies (i.e. general attenuation and slight phase-advances to clock gene expression) (Kohsaka et al. 2007). This suggests that peripheral tissue clocks may be more responsive to obesity insults; however, given recent evidence that hypothalamic clock gene expression is distinctly altered by the onset and progression of pregnancy (Wharfe et al. 2016), the impact of obesity could change in the context of pregnancy. Interestingly, leptin also moderates circadian function by way of interaction between the SCN and medial hypothalamus (Grosbellet et al. 2015). Since CAF mothers were
hyperleptinaemic at both days of gestation, with time-of-day-dependent leptin increases at day 21, altered leptin signalling may lead to changes in these central circadian systems. This is also an interesting area for future research, particularly in light of the functional importance of leptin in moderating fetal development (Briffa et al. 2015).

**Inflammatory and endocrine adaptations to maternal obesity**

The first experimental chapter provided surprising evidence that CAF animals had slight reductions in circulating inflammatory cytokine levels and adipose tissue expression of inflammatory genes in late gestation. This is in contrast to previous reports that obese pregnant women exhibit a relatively inflamed phenotype (for reviews, see: Denison et al. 2010; Pantham, Aye & Powell 2015), although it is consistent with several other recent findings indicating that pregnancy may offer some protection against obesity-induced inflammation near term (Friis et al. 2013; Pedroni et al. 2014; Ingvorsen et al. 2014). Interestingly, the observation that obesity impairs the normal nocturnal progesterone decline (Chapter 6) may have implications for the maternal inflammatory phenotype. Since progesterone suppresses maternal immune function during gestation, the elevated progesterone in CAF mothers may have immune-modulating effects. Indeed, a study by Parker et al. (2014) found that plasma progesterone was increased in HF-fed mice at mid-gestation, and this was associated with decreased uterine natural killer cell function and suppression of innate and adaptive immune processes. Accordingly, increased progesterone may be partially responsible for the absence of an inflammatory phenotype CAF mothers. Maternal immune function facilitates spiral artery remodelling and adequate placental development in early gestation (Mor et al. 2011), and allows for the onset of parturition in late gestation (Bowen et al. 2002). Consequently, immunological disruptions in obese mothers could have serious implications for placental function, fetal growth, and labour induction.

Progesterone is also generally considered to be thermogenic, and so the nocturnal increase in progesterone in CAF mothers (Chapter 6) seems to be at odds with their reduced Tc (Chapter 7). On the other hand, a recent study by Finley and colleagues (2015) showed that administration of pregnancy-specific levels of progesterone and/or estrogen does not alter basal Tc in non-pregnant female rats (Finley, Zhang & Fewell 2015). This suggests that progesterone and estrogen do not directly moderate Tc, but that other pregnancy-induced adaptations facilitate the gestational Tc decline in rodents. One possibility in this
regard is the angiotensin signalling pathway, as suggested by Cairns and colleagues (2005).

**Convergence in obese individuals near term**

Interestingly, with the exception of clock gene expression in maternal tissues, there was a general pattern of convergence between CON and CAF mothers in late gestation for many of the biological endpoints measured throughout this thesis. Maternal Tc was lower in CAF animals throughout the estrous cycle and for most of pregnancy, but converged between diet groups in late gestation (Chapter 7). CAF mothers were also hyperglycaemic across day 15, but not day 21 (Chapter 8), while the elevated lipid (Chapter 6), insulin (Chapter 8) and leptin (Chapter 9) profiles observed in CAF mothers occurred largely across all of day 15, but only exhibited time of day- or photoperiod-dependent elevations by day 21. Similarly, CAF-induced changes to *Ppars*, *Pgc1a* and *Glut2* expression in the maternal liver and adipose were evident at select times across day 15, but were no longer present by day 21 (Chapters 8 & 9).

The pattern of late-gestational convergence between diet groups is consistent with other recent studies that report a convergence in metabolic phenotypes between lean and obese subjects near term (Misra & Trudeau 2011; King et al. 2013; Straughen, Trudeau & Misra 2013; Forbes et al. 2015). This suggests that late pregnancy may offer protective effects over many obesity-induced symptoms. While the findings of this thesis support this contention, they also provide further evidence (particularly in regard to the lipid, insulin and leptin profiles) that certain biomarkers may only be altered by obesity across select times of day in late pregnancy. This reinforces the need for longitudinal study of pregnancy outcomes, but also highlights that time-of-day considerations need to be accounted for through appropriate study design.

**Limitations and further considerations**

There are some inevitable limitations to the work presented in this thesis which require acknowledgement. Notably, while gene expression has been a major experimental end point throughout this dissertation, the corresponding protein levels have not been measured, meaning that any obesity-induced post-transcriptional modifications have not been accounted for. Because the control of the molecular clock is regulated by tightly-controlled transcriptional-translational feedback loops (Reppert & Weaver 2002), it
would be valuable for further studies to determine whether maternal obesity alters corresponding protein levels, or related post-transcriptional mechanisms in the core clock components. Interestingly, one study noted that while hepatic Bmal1 mRNA was attenuated and phase-advanced in HF-fed mice (a similar profile to that seen in the maternal liver of CAF animals), the associated protein levels of BMAL1 were not altered by HF feeding. This study did find, however, that CLOCK: BMAL1 chromatin recruitment was altered by HF-feeding, suggesting that post-translational modifications may be a key target for obesity-induced disruptions (Eckel-Mahan et al. 2013).

The non-rhythmic nature of clock gene expression in fetal and placental tissues also warrants further investigation. The stimulus for increased placental clock gene expression with advancing gestation is unknown, since the typical stimulus for transcription of clock genes (i.e. the rhythmic accumulation and degradation of clock proteins) does not appear to be present in the rat placenta. In-depth analysis is required to characterise the development of the fetal and placental clock gene machinery, in order to reveal which specific factors induce the expression of these non-rhythmic clock genes, and what their functional implications are during in utero development. Recent evidence suggests that Rev-erba elicits circadian and metabolic functions via different transcriptional pathways (Kopp et al. 2016), and so the Rev-erba rhythms noted in the fetal liver and placental labyrinth zone may facilitate other functions not related to the core clock. This would not be surprising given the numerous other important metabolic functions of Rev-erba (Everett & Lazar 2014), however it remains to be determined whether the core clock genes also act in a ‘non-circadian’ capacity during fetal development.

This study analysed two gestational days which span the period of maximal fetal growth in the rat, but it would be interesting to further characterise the circadian responses to obesity during other key phases of gestation. Because clock genes are rhythmically expressed in the rat oviduct (Kennaway, Varcoe & Mau 2003) and in decidualized endometrial stromal cells (Tasaki et al. 2013), the peri-implantation period may be regulated by the circadian system. Similarly, labour-induction may also be under circadian control (Olcese 2012), and so it would be fascinating to determine whether obesity may affect any circadian aspects of these pregnancy stages. Unfortunately, due to the large numbers of animals required for rhythmic analyses, this was outside the scope of the present work.
Inflammatory makers were measured at a single time-point on day 21 of pregnancy in Chapter 5. As was evident throughout this thesis, obesity-induced effects can occur at specific times of the day, with no difference between diet groups across the remaining daily period. This could also be true of inflammatory responses, since the circadian system controls immune function, and accordingly, circulating inflammatory markers exhibit circadian rhythmicity in their secretion patterns (Carter et al. 2016). Further studies could expand on the findings of Chapter 5 and investigate the rhythmic nature of inflammation in CAF mothers. It would also be interesting to characterise the CAF inflammatory phenotype prior to and early in gestation, to investigate the hypothesis that late pregnancy offers some protection against obesity-induced inflammation. Moreover, analysis across parturition may indicate whether either a lower inflammatory profile, or higher progesterone levels, in CAF mothers has the capacity to impair normal labour induction.

A logical extension to this thesis would be to assess the impact of obesity on maternal and fetal melatonin profiles. Melatonin is an important component of the central circadian clock, and may also be pertinent to the circadian disruptions associated with obesity. Indeed, melatonin is reduced by HF feeding in male rats (Cano et al. 2009), and supplementation has been shown to reduce weight gain and improve metabolic profiles in obese animals (Agil et al. 2011; Prunet-Marcassus et al. 2003), possibly via thermogenic mechanisms, given that melatonin also induces brown adipose tissue function (Tan et al. 2011; Jiménez-Aranda et al. 2013). Interestingly, however, melatonin may also reduce Tc by increasing peripheral vasodilation in humans, thereby promoting heat loss (Kräuchi et al. 2006; Tan et al. 2011), which highlights its multifaceted control over thermoregulation, and suggests that it may regulate the Tc reductions in CAF mothers (Chapter 7). Since melatonin also regulates fetal development and maternal circadian adaptations to pregnancy (Reiter et al. 2014), it may be implicated in numerous rhythmic adaptations in obese pregnancy. Interestingly, melatonin supplementation in undernourished pregnant rats rescues fetal growth restriction evident in the food restricted group (Richter et al. 2009). Although the specific mechanisms behind this growth restoration are unknown, it was speculated that melatonin may increase the antioxidant capacity of the placenta, resulting in increased placental blood flow, and subsequent improvement to fetal growth outcomes (Richter et al. 2009). Since maternal over- and under-nutrition can prompt similar developmental outcomes (Cunha et al. 2015), this
suggests that melatonin may be an interesting potential target for protection against fetal and placental growth restriction in obese pregnancy. Therefore, it will be interesting to see whether melatonin profiles are altered in CAF mothers, and if so, whether melatonin supplementation could correct various aspects of metabolic dysfunction evident in this model.

Since the average protein content of the CAF diet was 8% kcal (compared to 23% kcal in the CON group), the reduction in maternal lean body mass and fetal growth restriction evident in the CAF animals may be a result of lower protein consumption. Indeed, provision of a protein-restricted diet during pregnancy results in low birth weights and other postnatal complications in offspring, including metabolic and endocrine complications (Snoeck et al. 1990; Guzmán et al. 2006). It follows that other changes noted in the CAF animals may also be a result of protein restriction. For example, male offspring of protein restricted dams exhibit delayed pubertal onset and reduced fertility (Zambrano et al. 2005), which may indicate that the CAF-induced testosterone suppression in male fetuses (Chapter 6) is regulated by lower maternal protein consumption. Given that the CAF feeding model reflects a typical Western style diet, this problem is also likely to be evident in certain obese human populations. It is possible that a differential maternal protein status between populations or models may partially explain the contradictory responses noted between different obesity studies, such as the occurrence of fetal overgrowth in some studies but undergrowth in others. Whether protein restriction in obese individuals is accountable for various obesity-induced pregnancy complications is a relatively unexplored topic, and could be an interesting focus for further investigation.

In conclusion, the studies presented in this thesis establish that maternal obesity alters circadian rhythmicity in maternal metabolic tissue gene expression, hormone levels and lipid metabolism across the final third of gestation in the rat. Clock gene expression in the placenta and fetal liver was less susceptible to obesity-induced disruption, possibly due to the immature nature of the molecular clock in these tissues. In contrast, rhythmic Rev-erba expression was disrupted by obesity across all tissues, and the diet-induced changes in daily expression profiles of Rev-erba may have substantial downstream effects on numerous aspects of metabolic function. Overall, these studies highlight the
importance of time-of-day considerations when determining the impact of obesity on pregnancy.
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