Impact of Maternal Intermittent Fasting During Pregnancy on Fetal Growth and Cardio-Renal Function in Adult Rat Offspring

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ABSTRACT

Maternal undernutrition during pregnancy can impair placental and fetal development and lead to programming of the fetus, with long-lasting effects such as an increased risk of disease later on in life including cardio-renal dysfunction and metabolic disorders. Fasting is a compulsory act for all adult Muslims during the holy month Ramadan, and although pregnant women are exempt from this practice, many still observe this religious requirement, abstaining from food or drink from dawn to sunset. The impact of prolonged maternal intermittent fasting (IF) on fetal development and offspring health is not well defined, but a reduced placental weight and birth weight have been reported previously. The aims of this study were to investigate the effects of IF during pregnancy on fetal development and offspring health and to examine sex-specific differences. A rat model was used to investigate the effects of maternal IF, mimicking the repeated daily fasting pattern of Ramadan fasting, to determine effects on 1) maternal physiology; 2) fetal development and metabolic profile; 3) placental growth, morphology and metabolomics; 4) placental system A amino acid transporter activity and expression; 5) long-term effects on postnatal growth and blood pressure; 6) pattern of feeding behaviour; 7) salt appetite and aversion; 8) cardio-renal function; and 9) response of offspring to postnatal salt dietary challenge.

Food was withdrawn daily from pregnant Wistar rats from 5:00 pm to 9:00 am over 21 days of gestation; controls received food *ad libitum* and were used for comparison. Both groups had free access to water. IF dams consumed less food and gained significantly less weight, and at gestational day 21 (GD21) had reduced plasma glucose, glucagon and amino acid concentrations. At GD21, litter size was unaffected but IF fetuses of both sexes were significantly lighter and shorter with smaller head circumferences, but they demonstrated a higher brain:liver weight ratio. IF fetuses had normal plasma glucose and glucagon concentrations, but insulin and amino acid concentrations were decreased. Placental weight, and the junctional and labyrinth zone areas relative to total placental area were unchanged, but placental efficiency (fetal:placental weight ratio) was reduced in the IF group. The profile of placental metabolites was altered in the IF group, with sex-specific responses evident. Transplacental flux (measured as maternofetal clearance) of ^{14}C-labelled α-methylaminoisobutyric acid (^{14}C-MeAIB), a system A amino acid transporter substrate, was significantly reduced in both fetal sexes. However, measurement of system A activity as sodium-dependent ^{14}C-MeAIB uptake into isolated placental plasma membrane vesicles was unchanged for both fetal sexes. The gene expression of system A transporter subtypes *Slc38a1, Slc38a2* and *Slc38a4* (encoding SNAT1, SNAT2 and SNAT4 respectively) was upregulated in the placentas of IF males, but was unaltered in placentas of IF female fetuses. However, no changes were observed in placental SNAT1 and SNAT2 protein expression for either sex in the IF group.

At birth, IF neonatal weight was similar to control, but relative kidney weight in both IF sexes, and relative brain weight in IF females only, were reduced. Offspring of IF dams demonstrated altered postnatal growth and feeding behaviour, with IF females showing greater preference to salt intake. Blood pressure was similar between dietary groups at 5, 7 or 10 weeks of age with no changes in extracellular fluid volume. Glucose and insulin tolerance tests were unaltered by IF. *In vivo* renal ^{3}H-inulin clearance was similar between both dietary groups at 14 weeks of age. However, challenging the IF offspring from week 5 of postnatal age with a high-salt diet induced high blood pressure in both sexes, with renal dysfunction evident in males at 14 weeks of age.

In conclusion, a regimen of maternal IF, to model how nutrient intake may be perturbed during Ramadan fasting, was associated with several detrimental impacts on maternal physiology and fetal development, with changes also apparent in the profile of placental and fetal metabolites. Placental system A transporter activity measured *in vivo* was reduced and may contribute to the restriction of fetal growth seen in both sexes. Exposure to IF *in utero* had later consequences for the offspring, altering their growth trajectory and predisposing them to a higher risk of blood pressure and renal dysfunction in adulthood following a high-salt challenge, with sexual dimorphic responses clearly evident. Collectively, these observations suggest that Ramadan-type fasting during pregnancy, with repeated daily food deprivation, affects maternal and fetal health and that of the offspring in a sex-dependent manner, as well as placental function. Future refinements of the model to make it more translatable to human Ramadan fasting practice will provide further insights into the impact of Ramadan fasting during pregnancy and will empower pregnant Muslim women to make more informed decisions regarding their participation in the Ramadan fast.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or other Institute of Learning.

ABBREVIATIONS

11β-HSD-2	11β-hydroxysteroid dehydrogenase type 2
^{14}C-MeAIB	^{14}C- α- methylaminoisobutyric acid
AA	Amino acid
ACE	Angiotensin- converting enzyme
Ala	Alanine
Ang II	Angiotensin II
ANOVA	Analysis of variance
APC	Antigen presenting cells
ATR	Angiotensin II receptors
AUC	Area under the curve
BCAA	Branched chain amino acid
BM	Basal plasma membrane
BSC1	Bumetanide-sensitive Na-K-2Cl co-transporter
BT	Barrier thickness
Bwt	Bodyweight
cDNA	Complementary DNA
CRF	Corticotropin-releasing factor
CT	Cytrotrophoblast
CV	Connective tissue (stroma)
DBP	Diastolic blood pressure
DOHaD	Developmental Origins of Health and Disease
ECFV	Extracellular fluid volume
eNOS	Endothelial nitric oxide synthese
ERBF	Effective renal blood flow
ERPF	Effective renal plasma flow
ESA	Exchange surface area
ESL	Endothelial cells surface layer
FC	Fetal capillaries
FD	Facilitated diffusion
FE	Fractional excretion
FF	Filtration fraction
FGR	Fetal growth restriction
GBM	Glomerular basement membrane
GD	Gestational day
gDNA	Genomic DNA
GFR	Glomerular filtration rate
Gln	Glutamine
GLUT3	Glucose transporter isoform 3
Gly	Glycine
GTT	Glucose tolerance test

H & E	Haematoxylin and Eosin
HF	High cholesterol or high fat
HPA	Hypothalamic-pituitary-adrenal axis
HR	Heart rate
HRP	Horseradish peroxidase
HS	High-salt diet
ICV	Intracerebroventricular
IF	Maternal intermittent fasting
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IL	Interleukin
ITT	Insulin tolerance test
IVS	Intervillous space
JZ	Junctional zone
K_{mf}	Maternofetal clearance
LAT	L-type amino acid transporter
LBW	Low birth weight
LDL-C	Low-density lipoprotein cholesterol
LDL-C/HDL-C	Low-density versus high- density lipoprotein cholesterol ratio
Leu	Leucine
LP	Low-protein diet
LZ	Labyrinthine zone
MAP	Mean arterial pressure
MBS	Maternal blood space
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
MVM	Microvillous plasma membrane
ND	Standard chow diet
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
NOR	Novel object recognition test
PAH	Para-aminohippuric acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Postnatal day
PFA	Paraformaldehyde
PTM	Trophoblast plasma membrane
QC	Quality control
QPCR	Real time quantitative PCR
RAAS	Renin angiotensin-aldosterone system
RAS	Renin angiotensin system
RBF	Renal blood flow

RPF	Renal plasma flow
SBP	Systolic blood pressure
SCN	Suprachiasmatic nucleus
SEM	Standard error of mean
Ser	Serine
SGA	Small-for-gestational age
SHR	Spontaneously hypertensive rat
SIN-1	3-morpholinosydnonimine
SLC38A	Solute carrier 38 gene family member
SNAT	Sodium-dependent neutral amino acid transporter
SNGFR	Single nephron GFR
Sp	Spongiotrophoblast
SynTB	Syncytiotrophoblast
Sry	Sex determining region Y chromosome
TAE	Tris acetate-EDTA
Tau	Taurine
TB	Trophoblast
TBS	Tris-buffered saline
TGC	Trophoblast giant cells
TNF	Tumor necrosis factor
TSC	Thiazide-sensitive Na-Cl co-transporter
ACR	Albumin:creatinine concentration ratio
UPLC-MS	Ultra Performance Liquid Chromatography Mass Spectrometry Analysis
UV	Urine flow rate
VV	Villous volume

CONTRIBUTIONS FROM COLLABORATORS

All experiments reported in this thesis have been performed by Alla Alkhalefah other than:

Amnio acid analysis was conducted by Dr Franchesca Houghton at the University of Southampton. Metabolomic analysis was conducted by Dr Warwick Dunn and Dr Will Allwood at the University of Birmingham. Blood pressure was performed by myself and Dr Heather Eyre.

Alla Alkhalefah

May 2017

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DEDICATION

To my lovely family: Grandma, Mum, Dad, Auntie Laila and Uncle Yousef.

CHAPTER 1

INTRODUCTION

1.1 Overview

During pregnancy, maternal nutrition has a critical impact on feto-placental growth and development and also influences the disposition of the offspring to subsequent adult-onset diseases (Gluckman et al., 2008; Symonds et al., 2009). In agreement with this, a body of epidemiological evidence has shown that maternal malnutrition, such as global undernutrition or protein deprivation, in human pregnancies and in animal models causes fetal growth restriction (FGR) and low birth weight (Langley-Evans, 2009).

FGR is a condition where the fetus fails to achieve its genetic growth potential due to an insult *in utero* or a sub-optimal *in utero* environment. This condition occurs in up to 8% of pregnancies (Swanson and David, 2015). FGR can also lead to premature delivery (Cantwell et al., 2011) and results in an increased risk of perinatal mortality and morbidity (McCormick, 1985; Barker and Clark, 1997). Moreover, numerous epidemiological studies also have shown a strong link between low birth weight and catch-up growth in infancy to an increased risk of metabolic, cardiovascular and renal disease in the offspring's later life (Barker et al., 1989a, b; Godfery and Barker, 2001; Barker 2002; Barker et al., 2010b). Such associations are thought to be the consequence of 'programming', whereby exposure of a developing fetus *in utero* to an environmental insult or stressor, including sub-optimal nutrient availability, elicits fetal adaptive responses resulting in changes to fetal morphology, physiology and metabolism that influence propensity to disease later in life. Indeed, there is a wealth of evidence which shows that maternal dietary imbalance during pregnancy impairs fetal development and programs the offspring for disease in adulthood (Langley-Evans, 2006). For example, epidemiological studies on the effects of the Dutch Hunger Winter showed that offspring that were *in utero* during the famine had a greater risk of cardiometabolic and neurological disease in later life (Roseboom et al., 2011; see Section 1.2.1.1). In animal models of maternal protein restriction, the offspring's kidney morphology and function are affected adversely, with evidence of fewer nephrons and impaired renal filtration, which leads to the development of hypertension in adulthood (Ashton, 2000). Therefore, a sub-optimal maternal nutritional status during pregnancy can lead to an altered fetal growth trajectory and predisposition to adverse health outcomes later in life.

The placenta is the interface between the mother and the fetus. It is responsible for fetal nutrient provision, and as such it plays a substantial role in the growth of the fetus. Alterations in maternal nutrient availability lead to changes in placental growth, structure and function that contribute to FGR (Fowden et al., 2008; Sandovici et al., 2012). One of the main contributors to FGR is a change in the activity and expression of placental transporters, with amino acid transporters particularly affected (Glazier et al., 1997; Jansson and Powell, 2006; 2007; Sibley, 2009).

This project focuses on the impact of maternal intermittent fasting (IF), as a model of dietary perturbation and altered maternal nutritional intake during pregnancy, on fetal growth and development and its effects on the offspring. The rationale for the study arises from the practice of adult Muslims who fast intermittently during the month of Ramadan. Although pregnant Muslim women are exempt from this religious obligation, a majority of pregnant Muslim women around the world (estimated as 70% to 90%), participate in fasting throughout Ramadan for traditional or social reasons, abstaining from food and drink intake during daylight hours (Mirghani et al., 2003; Robinson and Raisler, 2005; van Ewijk et al., 2013). This pattern of maternal intermittent fasting has been reported to reduce birth weight and placental weight (Alwasel et al., 2010a; Almond and Mazumder, 2011) and led to an increased incidence of impaired mental development and learning disabilities in the children later in life (Almond and Mazumder, 2011). However, the broader impact of IF on fetal development remains poorly defined and the effects on placental function are unknown. This thesis therefore aims to investigate the effects of IF during pregnancy in a rat model, mimicking aspects of Ramadan fasting, on (a) maternal physiology, (b) fetal development (c) placental amino acid transport and morphological development in relation to fetal sex and (d) on the health of offspring as determined by postnatal growth trajectory, glucose and insulin tolerance, salt appetite, systolic blood pressure and renal function.

1.2 Fetal programming

The concept of fetal programming was first described by Barker and his colleagues, who authored a number of publications on the association between low birth weight and the risk of heart disease in adult life (Barker et al., 1989a, b; Barker, 1990; 1991). Since then, the term fetal programming has been modified more broadly into the "Developmental Origins of Health and Disease" (DOHaD) concept. This concept postulates that intrauterine environmental insults, such as altered maternal nutrition or a perturbed hormonal environment, at critical windows of fetal development, can lead to permanent changes in the structure and function of fetal organs (Figure 1.1). These changes, in turn, can persist into adulthood and influence the development of chronic disease (Barker et al., 1990; Gluckman and Hanson, 2004) including cardiovascular disease, type 2 diabetes, metabolic syndrome, osteoporosis and propensity to cancer (Gillman, 2005).

The concept of fetal programming can be explained by the thrifty phenotype hypothesis (Hales and Barker, 1992; 2001), which proposes that the fetus adapts to the prenatal nutrient-restricted environment by programming its metabolism to expect a matched postnatal environment. Thus, the offspring is well adapted (or 'programmed') for a continued, prospective nutritional environment. However, exposure of the offspring to a mismatch between the prenatal and postnatal nutritional environment will elicit metabolic responses that evoke a disease phenotype to occur in later life (Hales and Barker, 1992; 2001). The risk of disease is

greatest when a fetus adapted to a 'poor' intrauterine environment encounters a 'rich' postnatal environment (Li et al., 2010; 2011).
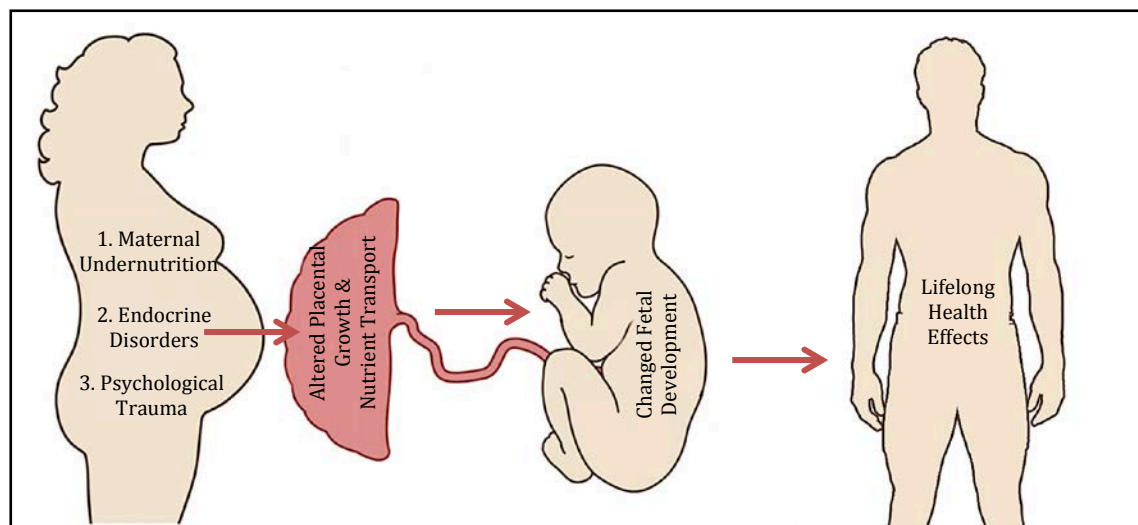
Figure 1.1 The developmental origins of adult disease hypothesis. An adverse maternal environment may permanently alter feto-placental growth and function resulting in an increased risk of disease in later life. Modified from Aye et al. (2012).

1.2.1 Maternal nutrition and fetal programming

Maternal nutritional status plays a major role in fetal programming which has been well characterised in studies on both humans and in animal models.

1.2.1.1 Human studies

Most research on maternal nutrition during pregnancy has assessed offspring cohorts that were affected *in utero* by certain dietary insults; for instance during famines (Ravelli et al., 1999).

Roseboom et al. (2001) researched the effects of the Dutch Hunger Winter on pregnancy outcomes. The Dutch Hunger Winter, a five month-long famine that occurred between 1944 and 1945 during World War II, arose from a German siege on food transport to the Netherlands. As a consequence, the mean calorific intake was reduced from 1,800 to between 400 and 800 calories per day (Roseboom et al., 2001). The studies reported that people exposed to the famine during the early period of gestation showed no difference in birth weight, but had poor lifelong health including an atherogenic lipid profile (Roseboom et al., 2000), obesity and coronary heart disease compared to non-exposed people (Roseboom et al., 2006). Additionally, those exposed to famine in early gestation had a two-fold increase in the risk of developing schizophrenia (Roseboom et al. 2006). Mid-gestation exposure had a slight effect on birth weight with an increased incidence of coronary heart disease, changes to blood

pressure (Roseboom et al., 1999) and signs of early-stage renal disease such as microalbuminuria (Painter et al., 2005). In addition, Lopuhaä et al. (2000) have found that exposure to famine during mid-gestation, a period of rapid growth for the fetal bronchial tree, was linked with an increased prevalence of obstructive pulmonary disease, potentially caused by an increase in bronchial reactivity rather than airflow blockage or atopic disease, since lung function and Immunoglobulin E serum concentration were unaffected. In contrast, exposure during late gestation resulted in a greater effect on fetal growth where babies were smaller and thinner with smaller head circumferences (Ravelli et al., 1998). This was associated with impaired glucose tolerance in offspring compared to those who were not exposed (Ravelli et al., 1998).

Therefore, the Dutch Hunger Winter provides evidence of the importance of an adequate supply of nutrients to the fetus for both the offspring's development and its risk for disease after birth. In addition, the research underlined two vital ideas. First, the influence of a dietary insult on the offspring's health in later life is dependent on the timing of the insult during the period of gestation. And second, birth weight does not always provide a picture of the conditions *in utero.* Offspring which were exposed to the Dutch Hunger Winter during the early stages of gestation did not show reduced birth weights but exhibited an increased disposition to health problems when compared with the offspring that were subjected to the famine at a later point of gestation.

Another cohort of individuals who were exposed to the Great Chinese Famine (1959 - 1961) while *in utero* showed an increased risk of hyperglycaemia compared to those who were exposed to famine in early or mid-childhood. The risk was aggravated by a rich nutritional environment in later life (Li et al., 2010). Another study also showed that exposure to famine *in utero* or during infancy was associated with an increased risk of metabolic syndrome later in life, with greater impact on overweight offspring and those who were exposed to a Western dietary pattern in adulthood (Li et al., 2011).

Contrary to these studies, the Leningrad Siege Famine study (1941 - 1944) showed no relationship between intrauterine starvation and developing risk factors for coronary heart disease in adulthood. This inconsistent result can be explained by the "thrifty phenotype" hypothesis since the postnatal environment in the Leningrad Siege cohort remained relatively poor after the famine (Stanner et al., 1997).

1.2.1.2 Animal studies

In addition to human studies, animal models have been used in an attempt to elucidate the mechanisms underlying fetal programming. In animals the environment can be strictly controlled; hence animal models overcome the major limitations of human epidemiological

studies (Bertram and Hanson, 2001). A variety of animal models have been used to test different dietary interventions, such as imposing global nutrient restriction (Ahokas et al., 1981; Belkacemi et al., 2011a, b, c), feeding a high fat diet (Holemans et al., 2004; Armitage et al., 2005; Reynolds et al., 2015) or a low protein diet (Langley and Jackson, 1994; Langley et al., 1994a; Sahajpal and Ashton, 2003; 2005; Jansson et al., 2006; Ashton et al., 2007; Alwasel and Ashton, 2009; 2012; Alwasel et al. 2010b; Fleming et al., 2015) to the pregnant animal at certain windows of gestation, to provide useful insights into the offspring's subsequent health and physiology.

Nutrient restriction models, ranging from mild (30%) (Ozaki et al., 2001), through moderate (50%) (Woodall et al., 1996) to severe (70%) (Vickers et al., 2000) restriction during pregnancy in rats and guinea pigs resulted in reduced birth weights in the offspring (Woodall et al., 1996; Ozaki et al., 2001; Kind et al., 2003). These studies showed the pathophysiological consequences of maternal nutrient restriction on the offspring, including higher blood pressure (Woodall et al., 1996; Ozaki et al., 2001) and impaired glucose tolerance (Kind et al., 2003).

Another *in utero* nutritional challenge is the protein restriction model. This approach is associated with a reduction in the offspring's birth weight in conjunction with an increased risk of metabolic disease at adulthood, including hypertension (Langley-Evans et al., 1994), higher glucose concentrations (Burdge et al., 2008) and vascular dysfunction (Torrens et al., 2009). As well as nutrient restriction, high fat diets fed to rats throughout gestation and lactation also have programming effects on the offspring. High fat diets consequently increased the offspring's birth weight and resulted in higher body fat, liver weight, serum triacylglycerol and blood glucose concentration in the offspring at weaning (Guo and Jen, 1995; Armitage et al., 2005).

Taken together, these studies provide proof of principle of the DOHaD theory and show that the *in utero* environment, in the form of sub-optimal maternal nutrition, can program adaptations in the developing fetus resulting in an altered susceptibility to chronic diseases in adult life. Such perturbations in the *in utero* environment are likely to be 'sensed' by the placenta, with altered functional responses evoked that influence fetal development (Figure 1.1). Hence, understanding how the placenta responds to changes in the maternal environment is integral to understanding the possible mechanisms by which the placenta may contribute to fetal programming (Jansson and Powel, 2007).

1.3 The structure and function of the placenta

The placenta plays a key role in the regulation of fetal development; indeed placental weight and neonatal birth weight are positively correlated (Neumann and Carroll, 1984; Hayward et al. 2016). In order to better understand this correlation between placental weight and birth weight,

placental structure and function will be outlined, with particular emphasis on the regulation of placental system A amino acid transporter, as a key transport mechanism that underscores fetal growth (Glazier et al., 1996; 1997; Cramer et al., 2002; Jansson et al., 2006). Furthermore, feto-placental adaptation to maternal malnutrition will be discussed.

1.3.1 The structure of the placenta

The original meaning of the word *placenta* in Latin is "flat cake". Indeed, this describes well the placenta of both humans and rodents which is discoid in shape. The placenta of both humans and rodents is described as haemochorial, as the trophoblast (TB) layer is in direct contact with maternal blood. In humans, the placenta is haemo-monochorial, consisting of one TB layer (syncytiotrophoblast (SynTB)), comprising two polarized plasma membranes, between the maternal and fetal circulations. The first one of these, in direct contact with maternal blood, is the microvillous plasma membrane (MVM), whereas the second one, facing the fetal endothelium, is the basal plasma membrane (BM) (Figures 1.2 and 1.3). Transporters are polarized to both MVM and BM, providing entry and exit mechanisms for solute transfer (Sibley, 2009). In contrast, the rodent placenta is haemo-trichorial, consisting of three TB layers between maternal and fetal circulations (Rossant and Cross, 2001; Figure 1.3). These layers are the main barriers for nutrient transport, which will be discussed in more detail in the section below. As development of the fetus proceeds and fetal nutrient demand increases, this barrier becomes thinner (Mayhew, 2009). At term, the human placenta weighs approximately 500 g (Raghunath et al., 2011), whereas in mice and rats it weighs around 100 mg and 500 mg respectively (Norman and Bruce, 1979; Coan et al., 2004).
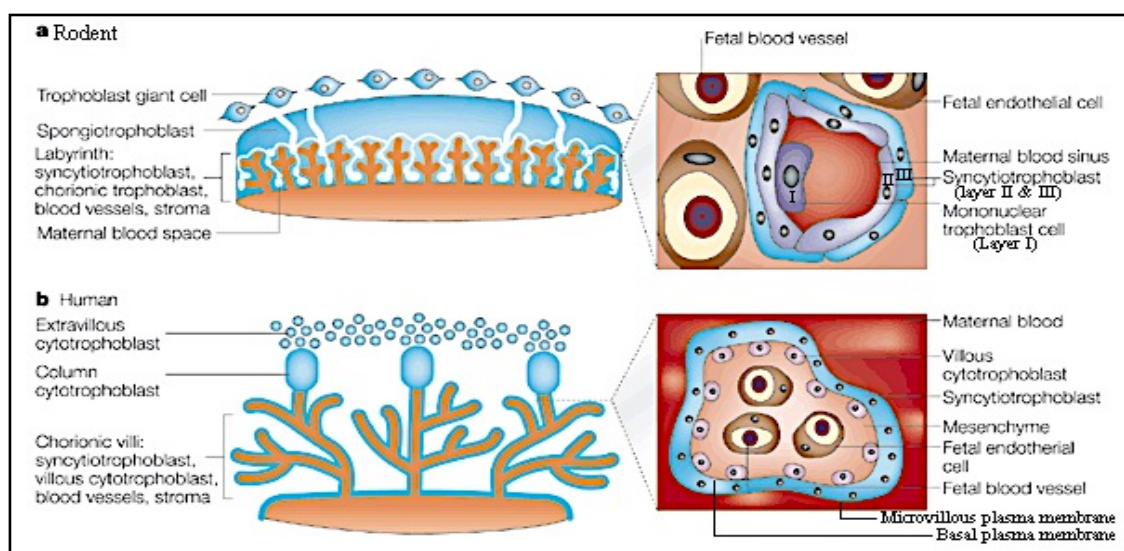
Figure 1.2 Schematic representation of the human and rodent placental structure, comparing trophoblast layers of human and rodent haemochorial placenta. Modified from Rossant and Cross (2001).
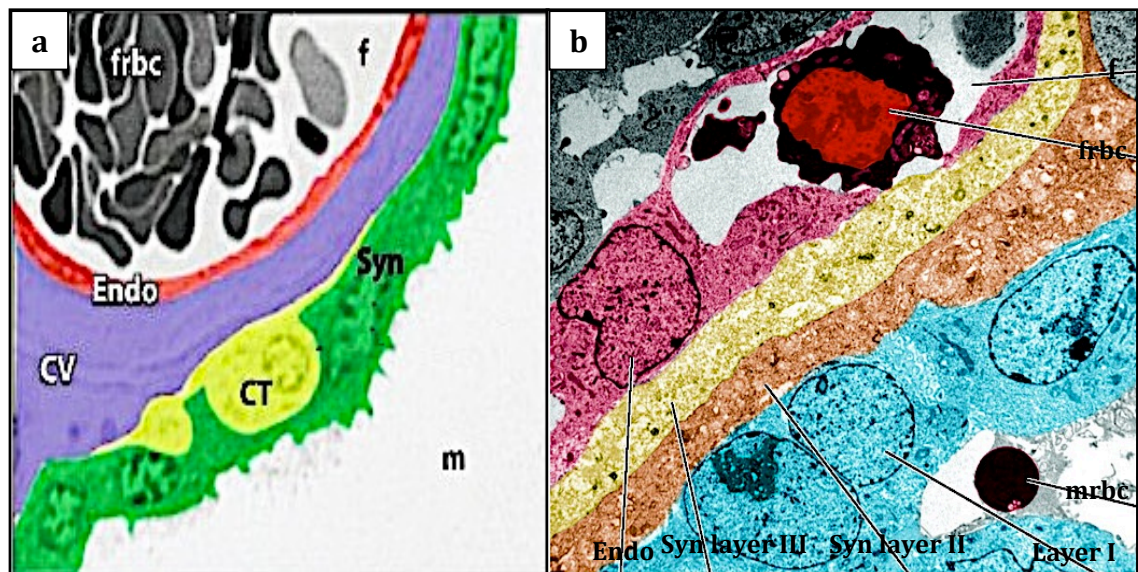
Figure 1.3 Electron micrograph of the trophoblast layers of **a.** human and **b.** rodent haemochorial placenta. CT, cytrotrophoblast; CV, connective tissue (stroma); Endo, fetal endothelial capillary; f, fetal capillary; frbc, fetal red blood cell; m, maternal blood; mrbc, maternal red blood cell; Syn, syncytiotrophoblast. Modified from Watson and Cross (2005) and Liu and Soper (2009).

1.3.1.1 The rodent placenta

The preferred animal model to study the effects of an altered *in utero* environment on placental development and function are rodents, due to the similarities between the placentas of rodents and humans (Georgiades et al., 2002; Soares et al., 2012). However, unlike humans, the mature placenta of rats and mice comprises three functionally distinct regions: the decidua, the junctional zone and the labyrinth zone (Figures 1.2 and 1.4). The decidua, which is maternally contributed, is faced by the junctional zone. The latter serves as an invasive and endocrine layer (Soares et al., 2012). The labyrinth zone is the interface across which nutrients are exchanged between the maternal and fetal circulations (Figure 1.4). Both zones are composed of TB cells (Takata et al., 1997). The junctional zone is separated from the decidua by a discontinuous layer of parietal trophoblast giant cells (Simmons et al., 2007). The junctional zone consists mainly of two types of trophoblast cells: spongiotrophoblasts and glycogen cells, with both cell types expanded in cell number as gestation advances (Coan et al., 2006). Spongiotrophoblasts and the trophoblast giant cells play a pivotal role in producing hormones that maintain pregnancy (Ain et al., 2003; Coan et al., 2006; Soares, 2004). Glycogen cells originating from the junctional zone are able to migrate into the maternal decidua (Cross, 2005; Coan et al., 2006; Simmons et al., 2007; Figure 1.4). There the cells unite to form clusters: they undergo a lytic stage, combining to form extensive lacunae which contain glycogen (Bouillot et al., 2006). These glycogen cells provide an additional energy source for the developing fetus near term or during parturition to meet the increased fetal energy demands during the later

stages of gestation (Coan et al., 2006). These cells are stimulated to break down glycogen stores into glucose by the hormone glucagon which is expressed in this cell type (Coan et al., 2006).

At the beginning of pregnancy, the labyrinth constitutes a small part of the placenta; but as development progresses, it comprises a major part of the placenta (Cross, 2005). The labyrinthine zone in rats and mice comprises trilaminar TB cells apposed between the fetal capillaries and maternal blood spaces; hence the placenta is classified as haemotrichorial (Takata et al., 1997; Figures 1.2, 1.3 and 1.4). The outer layer surrounding the maternal blood sinuses is a layer of discontinuous sinusoidal TB giant cells (Figure 1.4), denoted here as TB layer I (Watson and Cross, 2005; Simmons et al., 2007). These cells are interrupted by numerous fenestrations which make this layer highly permeable to small molecular weight solutes (Takata et al., 1997).
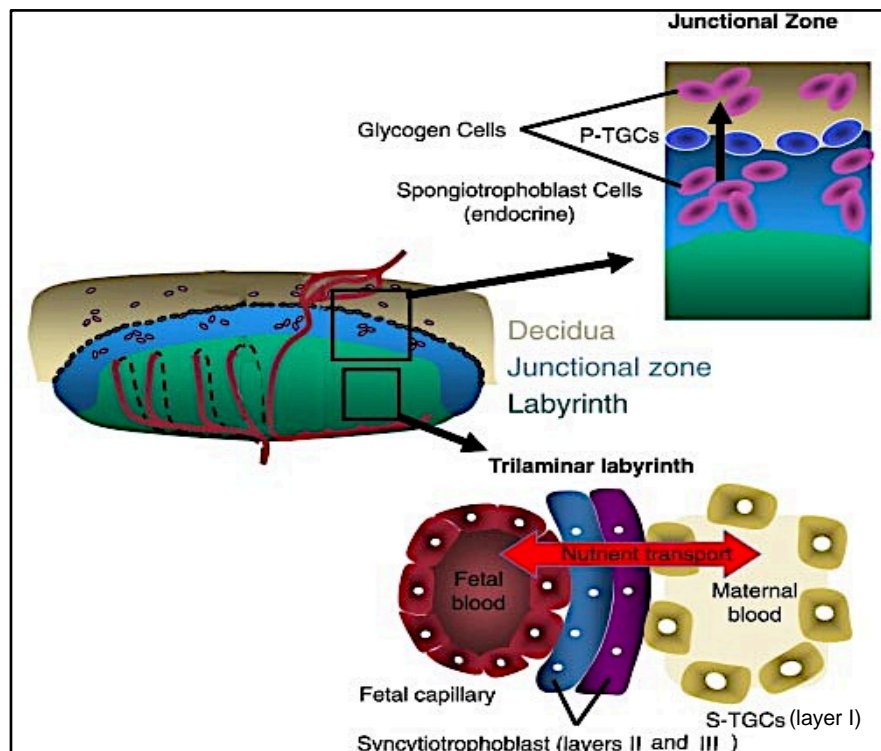
Figure 1.4 Schematic representation of labyrinth and junctional zones in rodent placenta. Within the labyrinth, the maternal and fetal blood is separated by a trilaminar layer of trophoblast cells. The trophoblast cells are the main barriers for nutrient transport. Within the junctional zone are the spongiotrophoblast cells and glycogen cells that originate in the junctional zone and can migrate into the maternal decidua. These cells engage in endocrine signalling and the glycogen cells function as an energy source to sustain pregnancy. Modified from John and Hemberger (2012).

Between TB layer I and the endothelium of the fetal capillaries are two SynTB layers. These two layers will be denoted as SynTB layer II and III for the remainder of this text (Takata et al., 1997; Hu and Cross, 2010; Figures 1.2, 1.3 and 1.4). Desmosomes, adhesive intercellular junctions, are present between the sinusoidal TB giant cells and the surface of the SynTB-II (Takata et al., 1997). The SynTB layers are true syncytium, a mass of cytoplasm with no lateral boundaries between the cells containing multinuclei, and are the main barrier for nutrient transfer between maternal and fetal circulations (Sibley, 2009; Dupressoir et al., 2009). The maternal-facing membrane of SynTB layer II is the initial plasma membrane barrier to maternofetal nutrient transport, analogous to the MVM in the human placenta (Kusinski et al., 2010; Glazier et al., 1990). Interestingly, the localization of alkaline phosphatase to the maternal-facing membrane of SynTB-II in the rodent placenta (Glazier et al., 1990; Kusinski et al., 2010; Figure 1.5) mimics its polarized distribution to the MVM of human SynTB (Jones and Fox, 1976), suggesting functional homology between the maternal-facing plasma membrane of SynTB-II of rat and mouse placenta with human placental MVM. This functional homology has been corroborated by further studies where these plasma membranes were isolated and transporter activities common to these plasma membranes were measured (Glazier et al., 1990; Jansson et al., 2006; Kusinski et al., 2010; Rosario et al., 2012). Hence, the maternal-facing plasma membrane of SynTB-II of rodent placenta, as might be expected for the first plasma membrane to confer restriction to maternofetal nutrient transfer, has an array of transporters distributed to this plasma membrane (Glazier et al., 1990; 1996; Novak et al., 1996; 2006; Jansson et al., 2006; Kusinski et al., 2010; Rosario et al., 2012).

The plasma membranes of SynTB-II and III are in close apposition, and connexin 26 gap junction proteins are localised here (Shin et al., 1996; Enders and Blankenship, 1999). These junctions allow nutrients to move between the SynTB layers. As gestation proceeds, the gap junctions increase in number (Takata et al., 1997). Thus, nutrient transport is facilitated as the fetal demand for nutrients increases. Although SynTB layers II and III have close cytoplasmic contact, they have distinct cellular compositions (Simmons and Cross, 2005; Cross et al., 2006). The basal plasma membrane of SynTB-III faces the endothelium of the fetal capillaries and is the final plasma membrane barrier for nutrient efflux (Dilworth and Sibley, 2013; Figures 1.2, 1.3 and 1.4). This corresponds to the BM in the human placenta (Enders and Blankenship, 1999; Dilworth and Sibley, 2013).
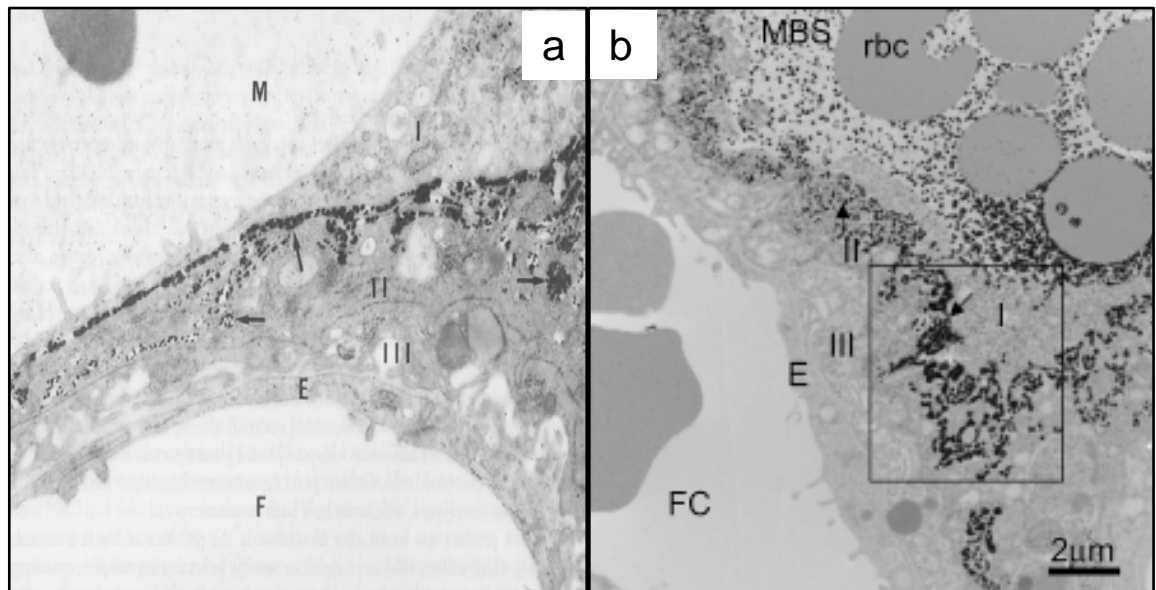
Figure 1.5 Alkaline phosphatase distribution in **a.** rat placenta and **b.** mouse placenta. Alkaline phosphatase is localized between trophoblast layer I and II as indicated by arrows and is associated predominantly with the maternal-facing plasma membrane of SynTB-II. I, II, III indicate the three trophoblast layers; F and FC, fetal capillary; E, fetal capillary endothelium; M and MBS, maternal blood space. Taken from Glazier et al. (1990); Kusinski et al. (2010).

1.3.2 The function of the placenta

The placenta performs many functions for the maintenance of fetal growth and viability including protection, metabolism, transport and endocrine functions (Donnelly and Campling, 2011). It protects the fetus against the transfer of infections and maternal diseases and provides the fetus with circulating maternal immunoglobulins. Furthermore, the placenta metabolizes various key metabolites with the capability of delivering these into the circulatory systems of the mother and/or fetus. Additionally, the placenta mediates materno-fetal nutrient transfer and removes fetal waste products. It also acts as an endocrine organ producing hormones to maintain pregnancy and fetal growth and facilitate parturition (Cross, 2006; Donnelly and Campling, 2011).

1.3.2.1 The transfer of nutrients

The transport of nutrients and metabolites across the placenta occurs mainly through the transcellular pathway, although some hydrophilic molecules, such as inulin, mannitol and Cr-EDTA, are transported by the paracellular route (Sibley et al., 2004). Transplacental transport can be limited by blood flow, the rate of transfer across the plasma membranes, thickness of the exchange barrier, or the concentration of solutes (Glazier et al., 1999). There are three modes of transplacental transport:

a) Simple diffusion: molecules like O_2, CO_2 and urea diffuse easily through plasma membranes and are therefore limited by placental blood flow.

b) Facilitated diffusion: transport of solutes down their concentration gradient mediated by a carrier with no energy requirement, such as glucose, lactate and some amino acids (Cleal et al., 2011). Hence, the solute transport will depend upon the number, affinity and activity of carriers.

c) Active transport: transfer of molecules against a concentration gradient, mediated by a carrier with energy requirement. This is divided into primary and secondary active transport. Primary active transport utilizes energy directly from the hydrolysis of ATP such as Na^+/K^+-ATPase and Ca^{+2}-ATPase. Transport of amino acids is considered to be secondary active transport, and depends on the transport of a second solute which constitutes the driving force of the transporter. The transporter can either be classified as a symporter or co-transporter, where two solutes are transferred in the same direction, or an antiporter or counter-transporter, where the transfer occurs in the opposite direction.

The following section will discuss amino acid transport across the placenta with particular emphasis on a specific transporter, system A, which is linked to fetal growth (Glazier et al., 1997; Jansson et al., 2006).

1.4 Amino acid transport

As a result of several studies conducted by Christensen (1966; 1979) and Van Winkle (1988), 20 distinct amino acid transporters have been characterised. Several classes of amino acid transporters have been identified in the placenta based on two criteria: Na^+ dependence and substrate specificity (Moe, 1995; Jansson, 2001; Cleal and Lewis, 2008). Amino acids are important for a number of reasons; for fetal growth, development and protein accretion, as precursors for synthesis of nitrogen-containing modulators, such as nitric oxide (NO), and as substrates for hormones, nucleotides and protein synthesis (Grillo et al., 2008). Furthermore, they act as insulin secretagogues, the main growth-stimulating hormone during fetal life (Aldoretta and Hay, 1995). Amino acids are categorized into essential amino acids, which are provided from nutrients and cannot be synthesized by the fetal body, and non-essential amino acids, which can be regarded as conditionally essential in the fetus due to the high demand for protein synthesis (Cleal and Lewis, 2008). Regardless of the fact that the main source of fetal energy is glucose, amino acids derived from the maternal circulation supply the fetus with 20% - 40% of the total energy requirement (Beur et al., 1998; Cramer et al., 2002).

The concentration of amino acids in the fetal plasma is higher than in the mother's plasma, whereas the concentration of amino acids in the placenta is higher than in both the mother's and the fetal plasma (Philipps et al., 1978). This provides evidence for the existence of active

transport mechanisms operating to mediate transplacental transfer of amino acids (Gude et al., 2004). In FGR, fetal plasma concentrations of certain amino acids and umbilical concentrations of total α-amino nitrogen are decreased in the second and third trimesters (Cetin et al., 1992; 1993; 1996). Thus, FGR may be a consequence of placental failure to supply the fetus with sufficient amino acids required to support normal growth (Cetin et al., 1992; Cetin, 2003).

The main barrier restricting amino acid transfer is the SynTB epithelium of the placenta (Figure 1.2). Amino acids cross the human MVM and rodent's SynTB-II apical membrane in an active manner through several transporters. Figure 1.6 shows a scheme for the transplacental transfer of neutral amino acids across human and rat placentas, emphasizing that there are some common features between these species. In rodents, amino acids taken up into SynTB-II cytoplasm then move through the gap junctions between SynTB layers II and III, thereafter effluxing across the fetal-facing plasma membrane of SynTB III, down the concentration gradient by facilitated diffusion or through exchanger efflux mechanisms. Finally, amino acids reach the fetal circulation by diffusing through fenestrated fetal endothelial capillaries (Takata et al., 1997; Cleal and Lewis, 2008).
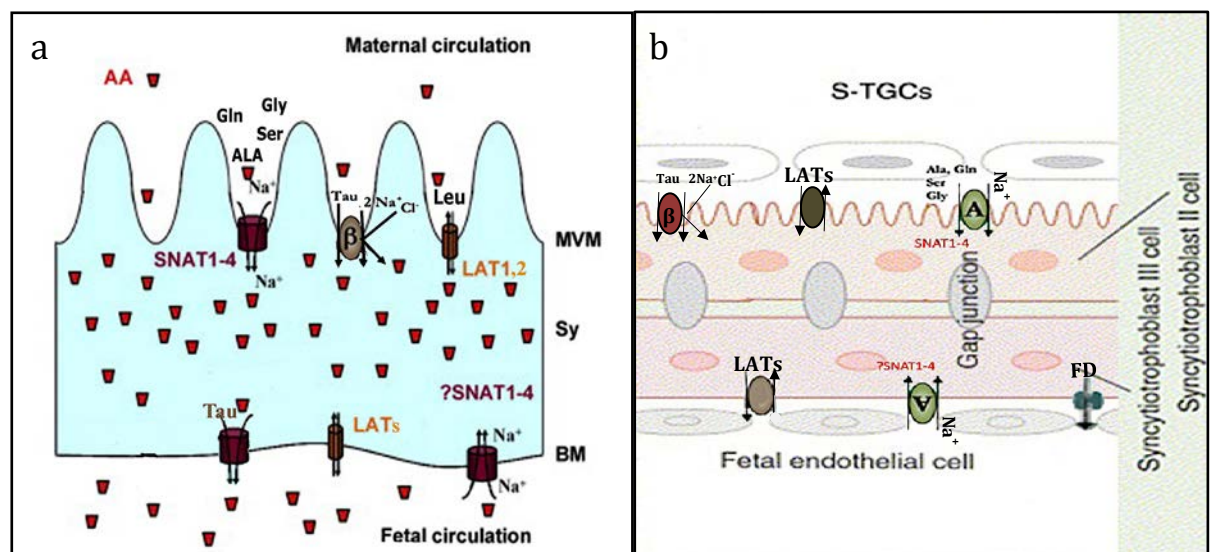
Figure 1.6 Amino acid transport in **a.** human and **b.** rat placentas. **a.** Neutral amino acids are taken up into SynTB by SNAT (system A amino acid transporter) and LAT (system L amino acid transporter) proteins and system β for taurine in MVM. Following translocation across the cytoplasm, the amino acids are effluxed through transporters in BM. **b.** Amino acids pass through the spaces between sinusoidal trophoblast giant cells (S-TGC's) of TB-I and then are taken up into SynTB layer II by similar amino acid transporters to human placenta (a); these pass through gap junctions between SynTB layer II and III and are effluxed across the fetal-facing plasma membrane of SynTB-III by exchangers and/or facilitated transporters. AA, amino acids; MVM, mircovillous plasma membrane; Sy, syncytiotrophoblast; BM, basal plasma membrane; FD, facilitated diffusion; Gln, glutamine; Gly, glycine; Ser, serine; Ala, alanine; Leu, leucine; Tau, taurine. Modified from Sandovici et al. (2012); Terasaki et al. (2003).

This study will focus on the system A amino acid transporter, a transport mechanism that is crucial for fetal growth and which has been identified in the placenta of several species including human (Johnson and Smith, 1988; Mahendran et al., 1993; 1994; Glazier et al., 1997), baboon (Pantham et al., 2015), rat (Glazier et al., 1996; Cramer et al., 2002; Jansson et al., 2006; Rosario et al., 2012) and mouse (Kusinski et al., 2010), with similar transporter rates of activity reported in human and mouse placentas (Kusinski et al., 2010). The importance of placental system A activity for fetal growth is supported by the evidence that a reduction in placental system A transporter activity was found to precede the onset of FGR in rats (Jansson et al., 2006), that the severity of FGR in humans relates to the magnitude of the reduction in system A activity (Glazier et al., 1997) and that blockade of system A activity results in reduced fetal growth (Cramer et al., 2002). Therefore, the characteristics of system A will be outlined in further detail.

1.4.1 System A

1.4.1.1 Human placenta

System A (so called as Alanine-preferring) is a sodium-dependent co-transporter, utilizing the driving force established by the inward sodium gradient maintained by Na^+/K^+ ATPase activity to uptake amino acids into the placenta against a concentration gradient. This system mediates the uptake of short-chain, neutral, aliphatic amino acids such as alanine, serine, methionine, glutamine and glycine (Battaglia and Regnault, 2001). It is a major transporter for both essential and non-essential amino acids, and its accumulative mechanism contributes to the high intracellular concentration of amino acids (Cleal et al., 2011). System L exchanges these intracellular amino acids for extracellular essential amino acids (Bröer, 2002). Hence, the provision of essential amino acids to the fetus is dependent on system A activity. The transport activity of system A is pH-sensitive, being stimulated by alkali pH (McGivan and Pastor-Anglada, 1994). Thus, it displays reduced influx activity with decreased pH over the physiological range (7 - 7.8) (Baird et al., 2006). α- methylaminoisobutyric acid (MeAIB) is a non-metabolized substrate that is uniquely transported by system A and has been widely applied to study system A transporter activity in a range of cells and tissues, including the placenta (Johnson and Smith, 1988; Mahendran et al., 1993; McGivan and Pastor-Anglada, 1994; Glazier et al., 1997).

System A activity is present in both MVM and BM of human syncytiotrophoblast with a higher maximum transport capacity in the MVM compared to the BM (Johnson and Smith, 1988; Jansson et al., 2002b). Three isoforms of system A, sodium-dependent neutral amino acid transporter (SNAT) 1, 2 and 4, are present in the placenta and are encoded by members of solute carrier 38 gene family (*SLC38A*) 1, 2, and 4, respectively (Mackenzie and Erickson,

2004; Hatanaka et al., 2000). SNAT1 and 2 both have activities that are typically characteristic of system A, and they display a higher affinity for neutral amino acids compared to SNAT4. The latter can also interact with cationic amino acids, which is a feature that is atypical for system A (Sugawara et al., 2000). The expression of SNAT1 occurs in various tissues, being highly abundant in the heart and brain as well as placenta. SNAT2 is ubiquitously expressed in mammalian tissues. SNAT4 was initially thought to be expressed in the liver only (Sugawara et al., 2000), but more recent studies have confirmed its expression in the placenta (Desforges et al., 2006).

As fetal growth accelerates while pregnancy advances, the fetal demands for nutrients increases accompanied by an increase in transporter activity. Mahendran et al. (1994) showed a four-fold increase in the system A transport activity in term placental MVM vesicles compared to first trimester MVM. Moreover, an investigation by Desforges et al. (2006) using real-time quantitative PCR (QPCR) revealed no gestational changes in placental *SLC38A1* and *SLC38A2* mRNA expression between first trimester and term, whereas during the first trimester, the expression of *SLC38A4* mRNA was higher. By term, however, using Western blot analysis, SNAT4 protein in placental lysates was found to be more highly expressed. Yet, the expression and activity of SNAT4 in MVM specifically is greater in the first trimester compared to term, suggesting that it may have a role in placental amino acid uptake and amino acid provision to the developing fetus particularly in the first trimester (Desforges et al., 2009). These findings suggest that transporter subtype expression and activity may not be concordant or change in parallel, suggesting in turn that regulation of transporter activity may be occurring at both the post-transcriptional and post-translational levels.

1.4.1.2 Rodent placenta

Rodent placenta expresses all three genes encoding for the SNAT1, 2 and 4 isoforms of system A, with expression increasing towards the final stage of rat gestation (Novak et al., 1996; 2006). This increase is correlated with placental and fetal growth (Novak et al., 1996; 2006). In an early study by Novak et al. (1996), it was established that system A activity in rat placenta is present primarily in the apical membrane of SynTB layer II and basal membrane of layer III, with expression of both SNAT1 and 2 in the labyrinth present from gestational day 14 - 20 (Novak et al., 2006). On day 14, SNAT2 was also observed in parietal trophoblast giant cells and SNAT1 in fetal endothelial cells, with SNAT1 expressed less on day 20 (Novak et al., 2006). Recently, Rosario and coworkers (2011; 2012) confirmed that all three SNAT1, 2 and 4 isoforms of system A are expressed in isolated maternal-facing plasma membranes of rat and mouse placentas, derived from SynTB-II. The distribution of these transporters to the maternal-facing plasma membrane of syncytiotrophoblast layer II of rat (Jansson et al., 2006; Ericsson et al., 2007; Rosario et al., 2011) and mouse placentas (Rosario et al., 2012) are analogous to human MVM distribution (Desforges et al., 2006; 2009; Cleal and Lewis, 2008; Figure 1.6).

Moreover, both rodent species show a similar uptake rate of MeAIB into isolated plasma membrane vesicles with substrate characteristics comparable to the human placental system A transporter (Glazier et al., 1996; Jansson et al., 2006; Ericsson et al., 2007; Kusinski et al., 2010; Rosario et al., 2011).

In addition to system A, the rodent placenta also co-expresses the L-type amino acid transporter (LATs) providing a mechanism for transplacental transfer of a broad array of amino acids including essential amino acids (Figure 1.6). System L transporters are sodium-independent exchangers of neutral amino acids. They allow for the exchange of essential amino acids with aromatic or branched side chains, such as phenylalanine or leucine, for non-essential amino acids that are transported into the placenta by system A, thus enabling transport against their concentration gradient (Prasad et al., 1999; Verrey, 2003; Widdows et al., 2015). LAT1 and LAT2 isoforms are present in MVM of human placenta (Widdows et al., 2015), and analogously, the maternal-facing plasma membrane of SynTB layer II of mouse placenta (Rosario et al., 2012) and rat placenta (Rosario et al., 2011) also have LAT1 and LAT2 distributed to them, as depicted in Figure 1.6. Also, LATs have been shown to be located in the BM of the human placenta and function as facilitative diffusion transporters and exchangers for efflux of amino acids from SynTB (Bodoy et al., 2005; Cleal et al., 2011). Hence, the integrated activities of system A and L together are crucially important for fetal amino acid delivery. Further, data from rodent studies suggest that altered placental system A and system L transporter activities are involved in the pathophysiology of FGR (Ericsson et al., 2007; Rosario et al., 2011; 2012). Consistent with this, system A and L activities are reduced in human placentas from FGR pregnancies (Jansson and Powell, 2007; Sibley, 2009).

1.5 Feto-placental adaptations to maternal undernutrition

Since the placenta is the interface between the maternal and fetal compartments, any perturbations in the maternal environment could affect placental structure or/and function, which may have a major impact on the intrauterine environment triggering fetal adaptive responses (Godfrey, 2002). In normal pregnancy, the increases in fetal growth and nutritional demands, especially in the last half of gestation, are associated with morphological and functional placental adaptation (Fowden et al., 2009). This is met with an increase in placental transport activity, despite the fact that the growth of the placenta is minimal in the later stages of gestation (Fowden et al., 2008). Such an increase in transport is due to a decrease in the thickness of the placental barrier (Mayhew, 2009), an increase in the surface of placental exchange area (Fowden et al., 2008) and up-regulation of transporters in the placenta (Mayhew, 2009). Any changes in intrinsic (e.g. fetal-placental growth genes) or extrinsic (e.g. undernutrition) factors trigger adaptive responses by the placenta to maintain fetal survival (Fowden et al., 2009; Sandovici et al., 2012). These changes are underscored by effects on

morphological and functional capacity of the placenta, as well as an altered placental efficiency (grams fetus produced per gram placenta) (Fowden et al., 2009) as summarized in Table 1.1.

1.5.1 Effects on feto-placental weights

In humans, one of the datasets that has been central in establishing the relationship between maternal undernutrition during pregnancy and effects on feto-placental outcomes has come from the Dutch Hunger Winter. Women subjected to famine during the first trimester of pregnancy had an increased placental weight and placental weight-to-birth weight ratio at term; but no effect was seen on birth weight (Lumey, 1998). However, maternal nutritional restriction in the third trimester led to reduced placental and birth weights, with an unaltered placental weight-to-birth weight ratio compared to control women. These outcomes point towards the ability of the human placenta to evoke adaptive responses to adverse environmental influences during early gestation and to try and compensate for diminished fetal nutrition during the later stages of pregnancy, leading to different placental and birth weight outcomes dependent on when in pregnancy maternal nutrient compromise was experienced.

This concept is consistent with data from animal models, where the effects of maternal nutritional perturbation on placental and fetal growth appear to depend not only on the species studied, but also on the timing, duration, type and severity of nutrient restriction or altered maternal dietary intake (Table 1.1).

Similar to humans, term placental weights were enhanced in ewes that were food-restricted during early to mid-pregnancy with either no effect (Heasman et al., 1998) or increasing fetal weight (Dandrea et al., 2001). In contrast, 50% maternal undernutrition during early pregnancy in mice caused a reduction in placental weights with no effect upon fetal weight. This alteration in placental weight caused by undernutrition in early pregnancy could be reversed by restoration of normal nutrition mid-gestation, which resulted in unaltered placental and fetal weights near term (Harper et al., 2015). Conversely, 50% maternal undernutrition from mid-gestation in mice and rats was associated with reduced placental and fetal weights near term and thus a decrease in placental efficiency and nutrient transport capacity (Ahokas et al., 1981; Belkacemi et al., 2011a, b, c; Ganguly et al., 2012). Similarly, 30% calorific restriction throughout gestation in the baboon decreased placental and fetal weights near term (Schlabritz-Loutsevitch et al., 2007; Pantham et al., 2015). These studies show that at a time when fetal nutrient demand is maximal, placental morphological and functional alterations due to nutrient restriction are insufficient to restore placental and fetal growth trajectories.

Maternal protein restriction, when applied at any time throughout the majority of pregnancy, resulted in a decrease in fetal weight near term in mice and rats. These observations imply that maternal dietary protein intake is important for fetal tissue accretion (Varma and Ramakrishnan, 1991; Malandro et al., 1996; Jansson et al., 2006; Rutland et al., 2007; Rosario

et al., 2011). Low protein diets can have a variety of outcomes on placental weight with either lower, unchanged or higher placental weights reported in rodents near term dependent on the level of protein restriction (Langley-Evans et al., 1996a; Coan et al., 2011; Gao et al., 2012a, b; Table 1.1). Interestingly, a 9%-maternal protein restriction during the pre-implantation period is adequate to modify the allocation of trophectoderm and inner cell mass within the rodent blastocyst, together with trophoblast cell proliferation and differentiation which has an impact on the fetus' and the placenta's subsequent development with later adverse offspring outcomes (Kwong et al., 2000; 2006; Watkins et al., 2008a; 2015), emphasizing that even a relatively short (3.5 d) exposure to altered maternal diet can elicit adaptive responses that have enduring effects for the offspring. Thus, fetal and placental weights during the later stages of pregnancy can be a good indicator that reflects alteration in maternal nutrient balance before and during gestation, starting as early as cell lineage stage through to the morphological and metabolic modifications of the fetus and placenta that occur near term.

A maternal high fat diet intake of approximately three-times the fat content of the control diet consumed before and during pregnancy results in fetal overgrowth, with unaltered placental weight compared to control rodents (Jones et al., 2009b; Gaccioli et al., 2013). When the dietary fat content surpasses this level, fetal weight is often reduced with either decreased or unchanged placental weight (King et al., 2013; Reynolds et al., 2015).

Collectively, these studies show that maternal nutritional challenges at a defined stage of pregnancy can affect the relationship of fetal weight to placental weight, and that the placenta tries to adapt its morphological and functional capacity to meet fetal developmental demand dependent on the prevailing conditions *in utero*.

1.5.2 Effects on placental morphology

Placental transport capacity may be affected by nutrient-induced alterations in barrier thickness, exchange surface area and cell composition of the placenta (Table 1.1).

In rodents and guinea pigs, the junctional and labyrinth zones may respond differentially to maternal nutrient perturbation (Table 1.1). In nutrient restricted mice and guinea pigs, as well as in rats exposed to a high fat diet, the labyrinth zone was preserved at the expense of the junctional zone (Roberts et al., 2001; Coan et al., 2010; Mark et al., 2011; Schulz et al., 2012). This labyrinth zone sparing effect is an adaptive response to preserve the provision of nutrient delivery to the fetus even at the expense of endocrine potential of the placenta, conferred by cells of the junctional zone (Coan et al., 2010).

Maternal nutrient availability may also alter the placental exchange surface area and barrier thickness. Pregnant guinea pigs that were exposed to undernutrition generated placentas in

which the TB-barrier thickness between the maternal and fetal circulations was increased and the exchange surface area was diminished (Roberts et al., 2001). Furthermore, exposure of rodents to protein or caloric restriction reduced placental fetal capillary length and maternal blood spaces and altered the cell composition of the placenta (Rutland et al., 2007; Schulz et al., 2012). Such alterations in the cellular morphology of placenta are likely to impact on the diffusional capacity of the placenta, along with the capacity for substrate transfer and its exchange properties.

1.5.3 Effects on placental amino acid transporter: System A

The activity, affinity, localisation and expression of particular transporters within trophoblast plasma membranes of the placenta, along with the materno-fetal substrate concentration gradients across the placenta, will influence transporter-mediated processes (Jansson and Powell, 2006). Alterations in the placenta which affect these parameters therefore have the potential to affect nutrient acquisition by the fetus and its subsequent growth which can ultimately impact on propensity to disease in adult life (Fowden et al., 2008).

1.5.3.1 Studies in human

Several studies have established an association between altered system A activity and development of FGR. According to Dicke and Henderson (1988) there was a significant reduction in AIB uptake by MVM, the rate-limiting step in transplacental amino acid transfer, in FGR compared to normal pregnancy. Mahendran et al. (1993) demonstrated that pregnancies with FGR (< 3rd percentile) were accompanied by a decrease of 63% in ^{14}C-MeAIB uptake by MVM, as a result of a decrease in the transporter capacity of system A. However, there was no change in the transporter's affinity (Mahendran et al., 1993). This would imply a reduction in either the turnover of system A transporters or the amount of active carrier proteins in the MVM of FGR pregnancies. Furthermore, the reduction of system A activity in MVM has been linked with the degree of severity of FGR, as defined by abnormal pulsatility index in the umbilical artery and abnormal fetal heart rate tracing (Glazier et al., 1997). This was supported by the Jansson et al. (2002b) study, in which system A activity in MVM was significantly reduced in preterm FGR. However, the activity of this transporter was unaltered in BM in either preterm FGR (Jansson et al., 2002b) or small for gestational age (SGA) pregnancies compared with normal pregnancies (Dicke and Verges, 1994). Shibata et al. (2008) also reported that system A activity, as measured in villous explants, was reduced in placentas of SGA pregnancies but was unaltered in SGA babies complicated by preeclampsia. To determine which of the three human placental SNAT isoforms are altered in FGR and contribute to the reduction in system A amino acid transport, it has been reported that the mRNA expression for all three SNAT isoforms were comparable between FGR and normal pregnancies (Malina et al., 2005; Desforges and Sibley, 2010). In contrast, Mandò et al. (2013) showed that *SLC38A2* mRNA

expression and SNAT2 SynTB immunostaining were significantly lower in placentas of FGR pregnancies compared to control, consistent with an essential role of SNAT2 in supporting fetal growth and development.

1.5.3.2 Studies in animals

Studies in animal models indicate that maternal nutritional availability regulates placental nutrient transporter expression and activity (Table 1.1).

In rats that were subjected to 50% undernutrition, placental system A uptake was decreased (Ahokas et al., 1981) with down-regulation of placental SNAT1 and 2 protein expression and up-regulation of placental SNAT4 protein expression (Belkacemi et al., 2011c). In contrast, mice subjected to 20% and 50% undernutrition exhibited reduced placental system A activity near term but enhanced placental *Slc38a1* and *Slc38a2* gene expression (Coan et al., 2010; Ganguly et al., 2012). In baboons, a 30% calorific restriction throughout pregnancy resulted in a down-regulation of system A in isolated plasma membrane vesicles with decreased amino acid concentrations in fetal plasma near term (Pantham et al., 2015).

Protein restriction, from diets containing 4 - 5% protein, in pregnant rats have been shown to decrease the activity of system A near term, in line with decreased protein expression of SNAT1 and 2 amino acid transporters (Rosso, 1975; Varma and Ramakrishnan, 1991; Malandro et al., 1996; Jansson et al., 2006; Rosario et al., 2011). As a consequence of such a functional perturbation, supply of neutral amino acids to the fetus might be expected to be reduced, contributing to the observed FGR. Jansson et al. (2006) also demonstrated that the reduction in system A precedes the onset of FGR in 4% protein restricted rats, suggesting that this might be causally related to FGR development rather than a consequence. Mice appear to respond differentially to protein restriction depending on the degree of severity. In mild protein restriction (16% dietary protein), placental system A transporter activity was diminished near term in association with a decrease in *Slc38a4* gene expression (Coan et al., 2011). However, in more severe protein restriction (8% dietary protein), placental system A capacity was unchanged near term, but accompanied by a reduction in the gene expression of *Slc38a1* and *Slc38a4* (Coan et al., 2011). Therefore, these studies on maternal protein restriction suggest that placental system A transport appears to be regulated differently dependent on the feeding paradigms and severity of protein restriction, timing and duration of the protein restriction and species type (Table 1.1).

High fat diets in mice, on the other hand, resulted in up-regulated placental system A activity in association with elevated SNAT2 protein expression (Jones et al., 2009b). Rats fed a high fat diet also showed a significant increase in *Slc38a2* expression (Reynolds et al., 2015). Since such rodents tend to take in less food as a result of being exposed to a high-energy diet

(Keesey and Hirvonen, 1997), the up-regulation of *Slc38a2* expression can be viewed as an adaptive response to maintain the amino acid supply, which is required for the fetus to grow in an environment of diminished amino acid supply.

Collectively, these observations indicate that the adaptive responses of placental system A transport to environmental cues is specific to the severity and type of nutritional insult and shows temporal plasticity. Importantly, these data also reveal that there is selectivity in the responsiveness of placental genes encoding the SNAT isoforms of system A to a particular nutrient environment, with the directionality of change not always occurring across all *SLC38A* gene subtypes. An initial response to an amino acid-deficient environment may be mediated by an enhancer region of the first intron of the *SLC38A2* gene which contains an amino acid response element sequence, able to stimulate *SLC38A2* gene expression in response to amino acid deficiency (Palii et al., 2006), with such a *SLC38A2* transcriptional responses evident in human TB cells (Jones et al., 2006b).

Table 1.1 Extrinsic factors that affect morphological and functional adaptation of the placenta

Factor	Species	Stage	Fetal Weight	Placental Weight	Placental Morphology	System A	References
Maternal Calorific Restriction							
50%	Rat	GD5-20	↓	↓		↓ placental system A uptake	Ahokas et al., 1981
50%	Rat	GD10-20	↓	↓	↓ LZ & JZ	↓ SNAT1 & SNAT2 ↑ SNAT4	Belkacemi et al., 2011a, b, c
20%	Mouse	GD3-19	↓	↓	↓ LZ, ↓ MBS & FC	↑ system A transport ↑ *Slc38a2* & ↓ *Slc38a4*	Coan et al., 2010
50%	Mouse	GD10-18.5	↓	↓		↑ system A transport (P.A.) ↑ *Slc38a1* & *Slc38a2*	Ganguly et al., 2012
50%	Mouse	GD2-12	GD19 ↔	GD12 ↓ GD19 ↔	GD12 ↑ LZ/JZ & ↓ FC & GlyC GD19 ↔		Schulz et al., 2012 Harper et al., 2015
10-30%	Guinea pig	Before & during pregnancy	↓	↓	↑ BT ↓ ESA		Roberts et al., 2001
30%	Baboon	GD30-165	↓	↓	↑ IVS ↓ VV & ESA	↓ placental system A uptake & SNAT2	Schlabritz-Loutsevitch et al., 2007; Kavitha et al., 2014; Pantham et al., 2015
Maternal Protein Restriction							
6%	Rat	GD1-21	GD14 ↓ GD18 ↓ GD21 ↓	GD14 ↓ GD18 ↓ GD21 ↔	GD14 ↓ LZ & JZ GD18 ↓ LZ, ↔ JZ GD21 ↔ LZ, ↓ JZ		Gao et al., 2012a, b; 2013
5%	Rat	GD1-21	↓	↓		↓ system A transport	Varma and Ramakrishnan, 1991
4%	Rat	GD2-21	GD15-19 ↔ GD21 ↓	GD15-19 ↔ GD21 ↓		GD19 & 21 ↓ system A transport capacity & SNAT2 GD21 ↓ SNAT1 GD19 & 21 ↔ SNAT4	Jansson et al., 2006 Rosario et al., 2011

Factor	Species	Stage	Fetal Weight	Placental Weight	Placental Morphology	System A	References
Maternal Protein Restriction							
5%	Rat	GD6-21	↓	↓		↓ system A transport	Malandro et al., 1996 Rosso, 1975
8%	Mouse	GD1-19	GD15 ↓ GD19 ↓	GD15 ↑ GD19 ↓	GD15 ↔ GD19 ↓ MBS & FC length		Rutland et al., 2007
18%	Mouse	GD1-19	GD16 ↔ GD19 ↔	GD16 ↑ GD19 ↑	GD16 ↓ LZ/JZ GD19 ↓ LZ/JZ	GD16 ↔ system A transport, GD19 ↓ system A transport & *Slc38a4*	Coan et al., 2011
9%			GD16 ↔ GD19 ↓	GD16 ↔ GD19 ↑	GD16 ↔ GD19 ↔	GD16 ↔ system A transport & ↑ *Slc38a2* GD19 ↔ system A transport & ↓ *Slc38a1* & *Slc38a4*	
Maternal High Fat Diet							
2.5x fat	Rat	GD1-21	↓	↔	↓ JZ		Mark et al., 2011
4.5x fat	Rat	Before & during pregnancy	↓	↓	↔ LZ ↓ JZ	↑ *Slc38a2* ↔ *Slc38a4*	Reynolds et al., 2015
5x fat	Rat	Before & during pregnancy	↑	↔		↔ system A transport activity ↓ SNAT1 ↔ SNAT2 & SNAT4	Gaccioli et al., 2013
3x fat	Mouse	Before & during pregnancy	↑	↔		↑ system A transport & *Slc38a2*	Jones et al., 2009b
5x fat	Mouse	Before & during pregnancy	GD15 ↔ GD19 ↓	GD15-19 ↔		GD15 ↑ *Slc38a2* & *Slc38a4* GD19 ↔	King et al., 2013

Increased (↑), decreased (↓), unchanged (↔). BT, barrier thickness; ESA, exchange surface area; FC, fetal capillaries; GD, gestational day; Gly C, glycogen cells; IVS, intervillous space; JZ, junctional zone; LZ, labyrinthin zone; MBS, maternal blood space; P.A., placental accumulation of MeAIB; SNAT, sodium-coupled neutral amino acid transporter; VV, villous volume. Gestational period: mouse ~20 days, rat ~23 days, guinea pigs ~70 days, baboon ~184 days.

1.6 Regulation of system A transport

The regulation of nutrient transport is central to the understanding of normal fetal development as well as the aetiology of complications such as FGR. A number of factors regulating system A transport are summarised in Table 1.2 below.

Table 1.2 Factors regulating system A transport

Factors	Tissue/Cell Types	System A	References
Insulin	Placenta villous fragments	↑ activity at term ↔ activity at 1st trimester	Karl et al., 1992 Jansson et al., 2003 Ericsson et al., 2005
Leptin	Placenta villous fragments	↑ activity	Jansson et al., 2003 von Versen-Höynck et al., 2009
IGF-1	Human placental TB in 2–sided culture	↑ uptake AIB	Bloxam et al., 1994
IL-6 & TNF-α	Cultured human primary TB cells	↑ SNAT 1 & 2 protein exp & *SLC38A2* exp	Jones et al., 2009a
IL-1β	BeWo cell line	↓ *SLC38A1 & 2* exp & activity	Thongsong et al., 2005
Glucose	Materno-fetal clearance in intact placenta	↓ activity	Ericsson et al., 2007
Oxidative stress	MVM & villous fragments	↓ activity	Khullar et al., 2004
mTOR	Human primary TB cells	↑ activity	Roos et al., 2009a Roos et al., 2009b
Hypoxia	Cultured term human TB	↓ *SLC38A1 & 2* exp & activity	Nelson et al., 2003
Adiponectin (globular) (Full length)	Human primary TB cells & TPM vesicles	↑ SNAT 2 protein exp & activity ↓ SNAT 1,2 & 4 protein exp	Jones et al., 2010 Rosario et al., 2012
CRF & urocortin	Placenta villous fragments	↓ activity	Giovannelli et al., 2011
Angiotensin II	Placenta villous fragments	↓ activity	Shibata et al., 2006
Cortisol	BeWo cell line	↑ *SLC38A2* exp & activity	Jones et al., 2006a

Increased (↑), decreased (↓), and unchanged (↔) activity or expression of nutrient transporters. CRF, corticotrophin-releasing factor; Exp, expression; IGF-1, insulin growth factor 1; IL-1β, interleukin1β; IL-6, interleukin 6; mTOR, mechanistic target of rapamycin; MVM, microvillous plasma membrane; TB, trophoblast; TNF-α, tumor necrosis factor-alpha; TPM, trophoblast plasma membrane.

Substrate regulation of the system A transporter is well established in different cell types. Up-regulation in the transporter activity and protein expression due to a lack of amino acids has been observed in hepatocytes (Bracy et al., 1986), adipocytes (Hyde et al., 2001) and TB cells (Jones et al., 2006b). The expression of *SLC38A2* gene in TB cells was found to be increased in cells deprived of amino acids whereas with non-essential amino acid deprivation, *SLC38A1* is down-regulated (Jones et al., 2006b). *In vitro* studies showed that system A activity was down-regulated by both angiotensin II and low oxygen concentrations (Nelson et al., 2003; Shibata et al., 2006). The angiotensin II (Ang II) effect occurs through activation of the angiotensin II type 1 receptor (AT$_1$R), whereas the effect of hypoxia is mediated through decreased *SLC38A1* and *SLC38A2* gene expression. In the endothelial nitric oxide synthase (eNOS) knockout mouse model, which is associated with placental hypoxia, deletion of the eNOS gene was associated with reduced system A activity but with no effect on *Slc38a1*, *Slc38a2* or *Slc38a4* gene expression. This observation suggests that this phenomenon is regulated at the post-transcriptional level, possibly through a hypoxic-related response (Kusinski et al., 2012).

System A transporters are also affected by glucocorticoids; however with variable effects dependent on the length and gestational timing of exposure. In the BeWo cell model, 24 h exposure to cortisol resulted in an increase in *Slc38a2* gene expression and an increase in activity of system A transporters (Jones et al., 2006a). On the other hand, 1 h exposure of primary villous fragments to cortisol had no effect (Jansson et al., 2003; Ericsson et al., 2005). In term placental villous explants treated for 24 h with dexamethasone, a synthetic glucocorticoid, system A activity was stimulated (Audette et al., 2010); however dexamethasone had the opposite effect when administered to mice at mid-gestation (Audette et al., 2011).

Cytokines have also been shown to have an effect on system A transporters. BeWo cells incubated with interleukin-1β (IL-1β) demonstrated decreased *Slc38a1* and *Slc38a2* gene expression and down regulation of system A transporter activity (Thongsong et al., 2005). IL-6 and TNF-α, on the other hand, up-regulated SNAT1 and 2 protein expression and *SLC38A2* gene expression in cultured human primary TB cells (Jones et al., 2009a). The cytokines can partly explain the fetal overgrowth seen in pregnancies complicated by maternal diabetes and obesity through up-regulation of system A transporter and thereby increased placental nutrient transport. Release of free radicals NO and superoxide (O_2^-) stimulated by 3-morpholinosydnonimine (SIN-1) incubation with primary villous fragments inhibited system A activity (Khullar et al., 2004).

Other regulators of system A transporters in the placenta are insulin, leptin and IGF-I (Table 1.2). The insulin effect on system A activity depends on the stage of gestation. In term placenta, increases in the concentration of insulin, for either short or long duration, increase

system A activity (Karl et al., 1992; Jansson et al., 2003). In first trimester placental villous fragments, however, system A activity remains unaffected (Ericsson et al., 2005). Leptin, which is secreted by the human placenta, increases system A activity after 1 h incubation with villous fragments (Jansson et al., 2003). Another study by von Versen-Höynck et al. (2009), where leptin was incubated for 1 h with villous fragments, showed that leptin stimulated system A activity through the JAK-STAT pathway. IGF-1 stimulates system A activity and can initiate signalling through both IGF-1 and insulin receptors. Bloxam et al. (1994) showed that exposure to IGF-1 on the apical side of a two-sided model of human TB cells, resulted in an increase in the accumulation of AIB inside cells, while reducing overall transfer rate. This finding highlights that an increase in uptake does not necessarily cause an increase in net amino acid transport in parallel with SynTB related amino acid accumulation.

Mice lacking IGF-I and II exhibit placental growth restriction as well as FGR. Due to the deployment of alternative *Igf2* gene promoters P0 - P3 in mice, a number of *Igf2* transcripts are expressed. The transcript P0 is unique to placenta and expressed only in the labyrinth TB (Constância et al., 2005). In the P0 *Igf2* mutant mouse, where the P0 transcript has been deleted, a significant increase in *Slc38a4* gene expression was observed at GD 16 with a concomitant increase of materno-fetal clearance of ^{14}C-MeAIB (Constância et al., 2005); this was normalized by GD 19. Kusinski et al. (2011) confirmed that ^{14}C-MeAIB uptakes through the apical membrane of SynTB layer II were up-regulated at GD 16 and then returned to normal by GD 19. This study also concluded that in P0 fetuses, the FGR phenotype occurs predominantly because of a lack of exchange of placental nutrients rather than an alteration in uteroplacental vascular function. Moreover, with *Slc38a4* gene deletion in mice there was a 20% reduction in fetal weight (Angiolini et al., 2009). This observation suggests that expression of SNAT4 influences the regulation of fetal growth of mice (Constância et al., 2005; Angiolini et al., 2009).

Other factors that are reported to regulate system A activity are glucose and mTOR. Brief hyperglycaemia in early rat pregnancy was shown to reduce placental system A activity in late gestation (Ericsson et al., 2007). This was consistent with a study conducted on human primary TB cells, where glucose deprivation up-regulated system A activity by stimulating SNAT2 trafficking to the plasma membrane (Roos et al., 2009b). mTOR is an essential growth regulator whose activity is reduced during FGR (Roos et al., 2007). In cultured TB cells, inhibiting mTOR was associated with reduced system A transporter activity. This reduction in system A transporter activity was not associated with a change in expression of the three SNAT isoforms (Roos et al., 2009a), suggesting that the reduced outcome was caused by post-translational regulation or affecting transporter trafficking to the plasma membrane (Roos et al., 2009a).

Protein restriction in pregnant rats has been found to reduce the expression of activated forms of signalling molecules in the mTOR pathway and has been linked with downregulation of

system A amino acid transport (Rosario et al., 2011). In mice undergoing a calorific restriction of 20%, placental signalling via the mTOR pathway is reduced. Nevertheless, this reduction is associated with an adaptive response through upregulation of both system A activity and *Slc38a2* expression (Sferruzzi-Perri et al., 2011). In combination, these results suggest that placental mTOR affects maternal nutrient availability and fetal growth by regulating amino acid transporters in the SynTB (Roos et al. 2009a). System A transport is therefore regulated by various environmental factors and nutritional cues that modify transporter activity by affecting gene and protein expression, which in turn may impact the development of both the placenta and the fetus.

1.7 Impact of altered nutrition *in utero* on later chronic disease outcomes: cardiovascular, renal and metabolic outcomes

This section considers how prenatal nutrition is a key mechanism in the programming of hypertension, renal and metabolic diseases in later-life.

1.7.1 Hypertension

1.7.1.1 Epidemiological studies in humans

Hypertension is the most prevalent type of cardiovascular disease (Hales et al., 1991; Staessen et al., 2003). The link between birth weight and systolic blood pressure has been studied among diverse races, sexes, and age groups. Barker and Osmond (1988) were the first to report an inverse association between birth weight and hypertension in adult life. Subsequently, this inverse relationship between birth weight and adult hypertension has been described in two large systemic reviews by Law and Shiell (1996), where the authors reviewed 34 studies published between 1956 and 1996, and by Huxley et al. (2000), who reviewed 80 studies published between 1996 and 2000. In all of these studies, a similar pooled blood pressure effect size of around -2 mmHg/kg birth weight was found. However, increased birth weight may also be detrimental: when the risk of hypertension is plotted against birth weight a "U - shaped" relationship is apparent (Curhan et al., 1996). Moreover, babies with a low birth weight which subsequently exhibit catch-up growth during the postnatal period were shown to be at an even greater risk of high blood pressure (Eriksson et al., 2000; Huxley et al., 2000). Besides birth weight, neonatal body measurements have also been used to provide evidence of an association between disproportionate intrauterine growth and cardiovascular risk. Head circumference (in cm) at birth, a marker of fetal growth throughout gestation, was inversely correlated with blood pressure by 0.5 mmHg/cm reduction (Huxley et al., 2000). Reduced abdominal circumference (Martyn et al., 1995) and ponderal index (a weight-height related parameter; Barker et al., 1992) at birth were also associated with increased risk of high blood pressure in adult life.

Studies on twins have attempted to establish the relationship between the intrauterine environment and genetic predisposition on the development of hypertension. For instance, Levine et al. (1994) discovered that the twin who exhibited decreased birth weight showed a more rapid increase in blood pressure during childhood in the first year. Also, Loos et al. (2001) found higher blood pressures in the twin with lower birth weight, but only among females at the ages 18 to 34 years. These findings imply that situational intrauterine components led to the lower birth weight of one twin with a consecutive influence on blood pressure.

Studies by Barker et al. (1990) and Hemachandra et al. (2006) have also linked placental size and placental-to-birth weight ratio to the development of hypertension. In cases where the placenta was disproportionately large in comparison to the size of the baby, an indication of a failure to reach its growth potential, there was a greater risk of developing hypertension at the age of 50 (Barker et al., 1990). Furthermore, the study of Hemachandra et al. (2006) showed that an enhanced placental-to-birth weight ratio was related to an increased risk of higher systolic blood pressure in the offspring at 7 years of age.

The shape of the placenta was also linked to postnatal health outcomes, where a reduction in placental breadth rather than length was associated with higher systolic blood pressure in boys (Winder et al., 2011). On the other hand, girls who developed high systolic pressure had large placental areas in relation to birth weight (Barker et al., 2010b; Winder et al., 2011).

Maternal body composition and nutritional status are also linked to hypertension of the offspring in later life. Studies by Godfrey et al. (1994), Clark et al. (1998) and Adair et al. (2001) showed an association between low maternal weight or low weight gain during pregnancy and raised blood pressure among offspring. Adair et al. (2001) additionally found that maternal triceps skinfold thickness was negatively associated with diastolic blood pressure in adolescents of both sexes and with systolic blood pressure among boys. The study also reported that systolic blood pressure in boys was related to their mother's energy level from dietary protein sources, whereas both systolic and diastolic blood pressure among girls were correlated with maternal calories from dietary fat. These studies illustrate that the mother's past and current nutritional status are important determinants of the offspring developing hypertension in later life. Nevertheless, it is difficult to know if there is a causal relationship in this association.

1.7.1.2 Studies in animals

Well-controlled animal models in multiple species such as rat, mouse, guinea pig and sheep have shown consistently that hypertension arises in offspring subjected to prenatal nutritional manipulations (Table 1.3). With regards to rats, Woodall et al. (1996) and Vickers et al. (2000) showed that 70% nutrient restriction throughout pregnancy resulted in low birth weight and

programmed the offspring for hypertension in adult life. Ellis-Hutchings et al. (2010) evaluated the effect of 50% undernutrition during the first and second half of pregnancy on the development of hypertension in adulthood using two rat strains: Sprague Dawley and Wistar. The results showed that prenatal undernutrition during the second half of pregnancy produced elevations of blood pressure in offspring of both strains. However, exposure to nutritional restriction during the first half of gestation only leads to the development of hypertension in Sprague Dawley rats. In sheep, a modest 15% nutrient restriction in early gestation led to increased mean arterial pressure by approximately 10 mmHg in offspring between 80 and 85 days of age (Hawkins et al., 2000). These studies demonstrate that overall food restriction during some part or all of pregnancy is a contributing factor to a rise in the offspring's blood pressure; however they do not show whether this is due to a reduction in a particular nutrient or due to a general decrease in calorie intake.

One type of nutrient that has been investigated in some detail is protein. A number of studies have been conducted using maternal iso-calorific protein restriction, mostly in rats. The degree of protein restriction imposed ranged between 12% (mild), 9% (modest) and 5% (severe) dietary protein content compared with a normal dietary protein content of 18 - 19%. In a series of studies, several groups demonstrated that exposure of rat offspring to different levels of protein restriction *in utero* resulted in hypertension development in youth (4 weeks of age) and adulthood, with generally greater effects with more severe protein restriction (Langley-Evans and Jackson, 1995; Langley-Evans et al., 1994; 1996b; Woods et al., 2001a; Vehaskari et al., 2001; Sahajpal and Ashton, 2003). Additionally, Watkins et al. (2008a) showed that protein restriction (9%) during the preimplantation period (GD 0 - 3.5) was enough to programme hypertension in mouse offspring later in life.

Overall, different maternal nutritional insults during fetal life are among the drivers of programmed hypertension in later life. The potential mechanisms underlying the fetal programming of hypertension induced by maternal undernutrition will be addressed below.

1.7.1.3 Mechanisms of blood pressure programming

Glucocorticoids, the renin-angiotensin system and the kidney are known to play a critical role in the developmental programming of hypertension.

Alterations in the intrauterine hormonal milieu can lead to long-term effects on fetal outcome and cardiovascular health. Glucocorticoids are among the major hormones whose function has been investigated. For instance, Seckl and Meaney (2004) reported that prenatal glucocorticoid exposure *in utero* leads to a reduction in birth weight. Exposure can be caused by (1) increased maternal glucocorticoids as a result of stress (endogenous) or administration of dexamethasone (exogenous), or (2) activation of the fetal hypothalamic-pituitary-adrenal axis

(HPA) or by increased passage of glucocorticoids across the placenta to the fetus. For example, a 50% calorific restriction during late gestation caused an increase in rat fetal corticosterone concentration (Lesage et al., 2002). In contrast, maternal protein restriction throughout gestation in the rat showed no effect (Fernandez-Twinn, et al., 2003). Nonetheless maternal protein restriction fetuses may be exposed to elevated glucocorticoid concentrations. The fetus is normally protected from exposure to the high maternal concentration of glucocorticoids by the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2). Langley-Evans et al. (1996b) and Bertram et al. (2001) reported that pregnant protein restricted rats have a lower activity of this placental enzyme, which may permit the passage of glucocorticoids from the mother to the fetus. Lindsay et al. (1996) provided further evidence for this notion by showing that the offspring of pregnant rats that were administered carbenoxolone, which inhibits placental 11β-HSD-2 activity, were subsequently programmed for hypertension.

The treatment of pregnant rats or sheep with dexamethasone, a synthetic glucocorticoid used in preterm delivery to accelerate maturation of organs especially the lung, which is not inactivated by the placenta, leads to hypertension in the offspring (Benediktsson et al., 1993; Dodic et al., 1998). The window of dexamethasone exposure to produce adult hypertension differs between species: the last week of gestation is the sensitive window in the rat (Levitt et al., 1996), whereas the earlier stage of pregnancy is a critical time for sheep (Gatford et al., 2000).

The renin angiotensin system (RAS) is a major regulator of blood pressure control and volume homeostasis through both systemic and intrarenal actions (Hall et al., 1990; Carey and Siragy, 2003). Studies in rats reported that protein restriction during pregnancy resulted in low birth weight offspring that become hypertensive in their youth (Langley-Evans and Jackson, 1995) and remain so in adulthood (Manning and Vehaskari, 2001). This hypertension was abolished by a period of angiotensin-converting enzyme (ACE) inhibitor (captopril or enalapril) (Langley-Evans and Jackson, 1995; Sherman and Langley-Evans, 1998; Manning and Vehaskari, 2001) or angiotensin II receptor antagonist (Losartan) (Sherman and Langley-Evans, 2000) treatment. On the other hand, the same study by Sherman and Langley-Evans (2000) showed that treatment with the calcium channel blocker nifedipine between 2 and 4 weeks of age failed to normalise blood pressure of low-protein-exposed offspring, pointing to a RAS-specific mechanism.

The kidney also plays a role in the pathogenesis of hypertension as a result of a reduction in nephron number induced by different models of maternal nutritional insults, as explained in further detail in the section below.

Table 1.3 Effects of intrauterine malnutrition on the glucose tolerance, blood pressure and kidney development and function of offspring rats

Factor	Stage	Pup Weight	Glucose Tolerance	Blood Pressure	Renal morphology and functionality	References
Maternal Calorific Restriction						
50%	1st half, 2nd half & T GD	LBW in RT			At birth: ↓ kid wt in RT, ↓ glomeruli number in all time points. ↔ glomeruli diameter	Lucas et al., 1989; 1991; 1997
		↔ adult			At adult: ↓ kid wt in RT and R1, ↓ glomeruli number in RT and R2, ↑ renal medulla/cortex ratio, glomeruli hypertrophy ↓ GFR, RPF, V (urinary volume) & urinary osmolality (R2 & RT) at 3 months of age	
70%	T GD	LBW		↑ SBP & HR in adult offspring		Woodall et al., 1996
70%	T GD	LBW	Hyperinsulinaemia	↑ SBP and MBP male at PD60, female at PD100	↓ kid/bwt male at PD100	Vickers et al., 2000
50%	GD10 - term	15% LBW			↔ kid wt at PD21 & ↓ at 9 months	Desai et al., 2005a
50%	T GD	LBW		↑ SBP PD90	At adult: ↓ kid wt & glomeruli number ↓ GFR, ↑ serum creatinine & microalbuminurea	Almeida & Mandarim-de-Lacerda, 2005
Maternal Protein Restriction						
12% 9% & 6%	14 PGD & T GD	↔ BW LBW 6%	↔ 9wks ↑ GT 9wks	↑ SBP 9wks female, ↑ ACE activity		Langley & Jackson 1994; Langley et al., 1994
9%	T GD			↑ SBP 13wks Captopril & losartan ↔ SBP	↑ ACE activity 34% (4wks) & 134% (13wks) ↑ Ang II, ↔ renin activities.	Langley-Evans & Jackson 1995; Sherman & Langley-Evans, 2000.

Factor	Stage	Pup Weight	Glucose Tolerance	Blood Pressure	Renal Morphology and Functionality	References
				Maternal Protein Restriction		
9%	14 B GD & T GD	LBW		↑ SBP male & female at 4wks		Langley-Evans et al., 1994.
8%	T GD	LBW	At birth: ↓ β-cell proliferation, islet size, vascularization, & insulin content. At PD70: glucose intolerance & insulin resistance			Snoeck et al., 1990; Dahri et al., 1991
9%	T GD	LBW except GD0-7		↑ SBP male at 4wks, female at 9wks	↓ Kid/bwt male at 4wks & female at19wks ↔ GFR	Langley-Evan et al., 1999; Zimanyi et al., 2000
	GD0-7 GD8-14 GD15-22				↔ GD0-7 ↓ nephron number in GD8-14 & GD15-22	
9%	T GD	↔ BW		At 4wks: ↑ SBP & ↓ HR 20wks: ↑ SBP & ↔ HR	At 4wks: ↓ kid wt: short & wider 20wks: thinner Kid. ↑ urine volume & albuminurea ↔ creatinine clearance	Nwagwu et al., 2000
9%	GD 0-5	Female LBW		Male only had ↑ SBP at 4 & 11wks	↑ kid/bwt male at 12wks	Kwong et al., 2000
6%	GD12-22	LBW		↑ SBP Enalapril ↔ BP	At 8wks: ↓ glomerular volume & ↑ apoptosis ↔ plasma creatinine, ↑ plasma $[Na^+]$ ↓renin activity & proteinuria in males. At 6 months: ↑ plasma renin activity	Manning & Vehaskari, 2001; Vehaskari et al., 2001

Factor	Stage	Pup Weight	Glucose Tolerance	Blood Pressure	Renal Morphology and Functionality	References
Maternal Protein Restriction						
4% & 8%	T GD	LBW		↑ SBP	At birth: ↓ kid/bwt & glomerular number, ↓ renal [renin] & Ang II, ↑ plasma creatinine. Adult: ↔ ERPF, FF & PRA; ↓ GFR/kid wt	Woods et al., 2001a; Courrèges et al., 2002
9%	T GD	LBW		↑ MAP at 4wks	↔ kid wt, ↓ glomerular number ↑ plasma [Na⁺] & Na⁺ excretion & fractional excretion, ↔ renal haemodynamics. ANG II Administration: ↓ GFR.	Sahajpal & Ashton, 2003; 2005; Ashton et al., 2007; Alwasel & Ashton, 2009; 2012; Alwasel et al. 2010b
5%	T GD GD 1-11 GD 11-term	LBW ↔ in GD1-11		↑ MAP; salt sensitive hypertension. ↔ in GD 1-11	↓ kid/bwt, mature:immature glomerular ratio & glomeruli number & volume Adult male: ↓ GFR, ERPF but similar FF.	Woods et al., 2004; Pires et al., 2006
8%	T GD & weaned		At 3 months: ↑ GT & ↑ insulin receptors. ↓ GT & ↑ insulin resistance at 15 months in male & 21 months in female.			Ozanne et al. 1996; Ozanne & Hales, 1999; Ozanne et al. 2003; Fernandez-Twinn et al. 2005
10%	T GD	Female LBW	↑ Fasting glucose in male ↑ insulin & insulin:glucose ratio in both sexes		↓ kid wt	Zambrano et al., 2006

Increased (↑), decreased (↓), unchanged (↔). Ang II, angiotensin II; B, before; ERPF, effective renal plasma flow; FF, filtration fraction; GD, gestational day; GFR, glomerular filtration rate; HR, heart rate; kid/bwt, kidney/bodyweight; kid wt, kidney weight; LBW, low birth weight; MAP, mean arterial pressure; PD, postnatal day; R1, calorific restriction at the first half of pregnancy; R2, restriction at the second half of pregnancy; RPF, renal plasma flow; RT, calorific restriction throughout gestation; SBP, systolic blood pressure; T, throughout; wks, weeks.

1.7.2 Altered renal development and function

1.7.2.1 Studies in humans

The basic functional unit of the kidney is the nephron. In humans, nephron number varies among individuals by up to 13-fold within populations (Puelles et al., 2011), and ranges from approximately 200,000 to over 2,000,000 per kidney (Hughson et al., 2003). The human fetal kidney, known as the metanephros, begins to develop at day 28. Nephrogenesis progresses rapidly in the last trimester to form 60% of nephrons and ceases before birth by week 36 of pregnancy, with the exception in preterm infants where abnormal nephrogenesis continues until day 40 after birth (Rodríguez et al., 2004). After this stage, no new nephrons form; thus a nephron deficit present at birth persists throughout life (Woolf et al., 2003; Table 1.3). As there is rapid growth in the later stage of pregnancy, the kidney is more vulnerable to the adverse effects of sub-optimal maternal nutrition in the third trimester. Consequently, the kidney is a target organ for the effects of programming and therefore is a central organ involved in the onset of hypertension. The association between nephron deficits at birth and the development of hypertension in later life has been illustrated in animal models of nephrectomy. In adult animals, surgical removal of one kidney under varying circumstances, and in different species, does not tend to result in hypertension and renal disease (Kett and Bertram, 2004). Indeed, a decline in nephron number with increasing age in the absence of renal disease is common. Conversely, uni-nephrectomy in fetal sheep or on postnatal day 1 (PD 1) in rats, i.e. manipulated loss of nephrons at a time when nephrogenesis would still be ongoing, does lead to adult hypertension preceding any sign of renal injury (Woods et al., 2001b; Moritz et al., 2002; Singh et al., 2009). These findings show that the age at which nephron loss occurs is critical to the ability of the kidney to adapt and maintain arterial pressure. Progressive loss of nephrons in adulthood can be accommodated, up to a point, whereas loss of nephrons in early life leads to long-term changes in blood pressure regulation.

Several studies have demonstrated a direct linear relationship between birth weight and nephron number. One large study which measured nephron number in normal postnatal human kidneys showed an increase in nephron number of 257,426 nephron per kg increase in birth weight (Hughson et al., 2003). An ultrasound study by Spencer et al. (2001) of Aboriginal children aged between 5 and 18 years showed a positive correlation between low birth weight and kidney volume. Children of lower birth weight had kidneys that were thinner but of normal length, suggesting a decreased nephron endowment. Other histological studies also support the inverse relation between birth weight and nephron numbers. For instance, Hinchliffe et al. (1992) studied the effect of FGR on nephron development and found that growth-restricted infants had a 35% reduction in nephron number compared to the appropriate for gestational age infants. Another study by Mañalich et al. (2000) reported that the kidneys of low birth

weight infants had reduced nephron numbers and increased glomerular size. These studies support the earlier proposal by Brenner et al. (1988) that FGR could be related to an early limitation of the nephron number, resulting in a reduced filtration surface area and hyperfiltration of the remaining nephrons, thus leading to subsequent hypertension and progressive kidney injury (Figure 1.7). An autopsy study by Keller et al. (2003) has supported this proposal, showing that hypertensive German adults had 47% fewer nephrons and 133% greater glomerular volume per kidney with increased glomerulosclerosis compared to matched normotensive control subjects.

Kidney weight, which can only be measured via *ex vivo* autopsy, has been used to measure nephron endowment among humans. Zhang et al. (2008) found a direct association between kidney weight and nephron number in infants aged ≤ 3 months, with a predicted increase of 23,459 nephrons per gram of kidney weight. Similarly, Nyengaard and Bendtsen (1992) found a positive correlation in normal adults.

Studies have also provided evidence of the association between FGR and impairment of renal function. Among Australian Aboriginals, the prevalence of albuminuria was greater in children and adults who had low birth weights (Hoy et al., 1998). Zanardo et al. (2011) also showed that growth-restricted infants at the age of 18 months had significantly greater microalbuminuria compared with normal infants. Furthermore, there seems to be an association between low birth weight and early impairment of kidney function which is consistent with the higher incidence of lower nephron numbers in FGR children. In the study of Schreuder et al. (2009), neonates with low birth weight had a lower renal clearance of amikacin, a marker of neonatal glomerular filtration rate (GFR), on the first day of life in FGR. Moreover, a study conducted on children between the ages 8 and 13 years who were born small for gestational age, demonstrated an inverse association between birth weight and renal function as assessed by levels of cystatin C (Franco et al., 2008).

1.7.2.2 Studies in animals

In rats, sub-optimal intrauterine nutrition is linked to an impairment in glomerulogenesis (Woods et al., 2004), coupled with altered renin-angiotensin-aldosterone system (RAAS) activity (Rasch et al., 2004) and maternal glucocorticoid synthesis (Bertram et al., 2001). Although the rat is not an ideal model for human nephrogenesis, it is widely used to assess how the maternal environment alters kidney function (Benz and Amann, 2010). Rat nephrogenesis, unlike that of humans, starts at day 12 of gestation, with a nephron presence of only 10% at birth, and continues until postnatal day 10 (Table 1.4; Paixão and Alexander, 2013). Various studies have shown that maternal protein restriction causes a significant reduction in nephron number due in part to an increase in apoptosis, which may later contribute to the onset of hypertension (Welham et al., 2002; Sahajpal and Ashton, 2003; Almeida and Mandarim-de-Lacerda, 2005;

Pires et al., 2006; Alwasel et al., 2010b). The severity of the effect depends on the level of protein restriction and the period during gestation (Table 1.3). For example, moderate maternal protein restriction (9% protein) in the last week of gestation caused a 13% deficit in nephron number at birth and was associated with a 13 mmHg elevation in arterial pressure of those offspring (Langley-Evans et al., 1999). Likewise, more severe maternal protein restriction (6% protein) over the last 9 days of pregnancy altered renal development at birth and led to a reduction in nephron numbers with increased apoptosis and systolic blood pressure at eight weeks of age (Vehaskari et al., 2001). These observations highlight that even though the nephrogenesis of rats continues until day 10 after birth, the adverse effect of malnutrition *in utero* persists, and the postnatal development phase does not compensate for the prenatal influence.
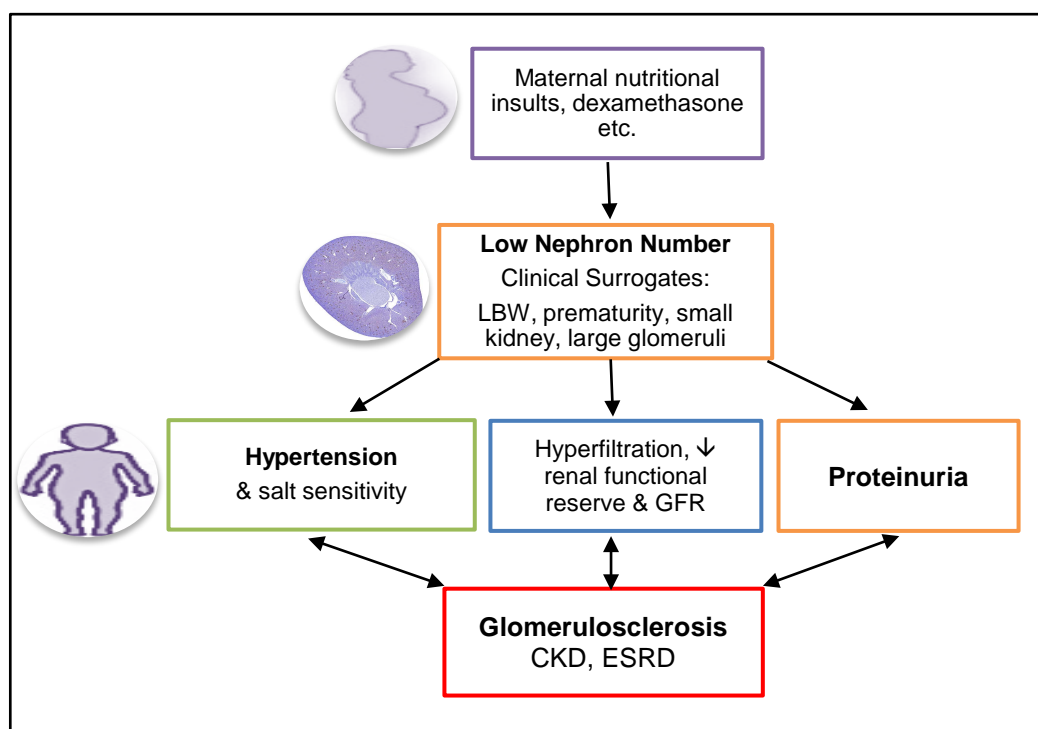
Figure 1.7 Schematic diagram outlining how maternal environmental insults can cause low nephron number and the ensuing consequences. Decreased (↓). CKD, chronic kidney disease; ESRD, end stage renal disease; GFR, glomerular filtration rate; LBW, low birth weight. Modified from Luyckx et al. (2011).

A low nephron number leads to hypertension as a result of hyperfiltration and glomerular hypertrophy, which exacerbate renal impairment and cause hypertension to develop (Benz and Amann, 2010). Nwagwu et al. (2000) showed that offspring exposed *in utero* to protein restriction had increased blood urea, urinary output and urinary albumin excretion, despite a normal GFR (Langley-Evans et al., 1998). In addition, several studies conducted by Lucas et al. (1991; 1997), in which pregnant rats were subjected to 50% food restriction in the first or second half of gestation, or throughout the entire period of pregnancy, showed reduced

glomerular numbers and glomerular hypertrophy in both newborn and 3 month old offspring. The older offspring had impaired renal function irrespective of the time at which the insult was imposed.

Table 1.4 The phase of nephrogenesis in several species

Species	Gestational Period	Nephrogenesis
Human	38 weeks	Week 5 to ~ 36
Rat	23 days	GD 12 to PD 10
Mouse	20 days	GD 11 to PD 7

Adapted from Guron and Friberg (2000). GD, gestational day; PD, postnatal day.

1.7.2.3 Mechanisms of renal programming

The intrarenal RAS plays an important role in normal kidney morphological development and can also contribute to the genesis of hypertension in later life. All of the RAS components, renin, angiotensinogen, ACE and angiotensin II receptors (ATR), are expressed at an early stage of gestation in the rat and during the mesonephros and also the metanephros stage of kidney development in humans (Guron and Friberg, 2000). RAS blockade with Losartan in rats over the first 12 days postnatally caused a reduction in nephron number and hypertension in adulthood (Woods and Rasch, 1998). When exposed to dietary protein restriction, the intrarenal RAS becomes altered. Langley-Evans and Jackson (1995) showed that offspring exposed to a low protein diet during fetal life had significantly increased plasma ACE-1 activity at 4 and 13 weeks of age, and they developed hypertension at 13 weeks of age. The authors demonstrated the ability of the ACE inhibitor, captopril, to normalise blood pressure in those hypertensive offspring relative to their control counterparts. Woods et al. (2001a) found that the kidney/bodyweight ratio, renal renin mRNA and concentration, as well as angiotensin II levels were significantly reduced in newborns who were exposed to moderate protein restriction (8.5% protein) throughout gestation. In their study, mean arterial pressure and glomerular volume were significantly increased, whereas GFR and glomeruli number were lower in 21-weeks old offspring. This indicates that maternal malnutrition promoted the suppression of the newborn's intrarenal RAS, influencing nephrogenesis and resulting in a lower nephron number which could lead to the onset of hypertension in adulthood. Furthermore, other studies on kidneys isolated from protein-restricted rats found that AT_1R and AT_2R protein expression were low from GD 18 to PD 10 (Vehaskari et al., 2004; Alwasel et al., 2010b) and increased compared to the levels of control by PD 28, in association with an elevated AT_1R subtype A mRNA level (Vehaskari et al., 2004) and AT_1R protein expression (Sahajpal and Ashton 2005). In young (4 weeks) protein-restricted offspring, the nephron number was reduced and glomerular sensitivity to angiotensin II (Ang II) *in vivo* was increased with elevated AT_1R expression. This led to a reduction in GFR, and salt and water retention which in turn would

favour the development of hypertension in the offspring (Sahajpal and Ashton, 2003; 2005). Intrarenal renin and Ang II concentrations were not altered at 4 weeks of age (Sahajpal and Ashton, 2005); however Grigore et al. (2007) found that in adult protein-restricted offspring, intrarenal ACE, renin and angiotensinogen mRNA levels were elevated, but no changes in intrarenal Ang II levels were documented. Together these observations suggest that suppression of the intrarenal RAS during development is followed by nephron deficit, renal dysfunction and hypertension in adult life.

1.7.3 Impaired glucose tolerance and insulin resistance

1.7.3.1 Studies in humans

Impaired glucose tolerance (IGT) and increased fasting glucose concentrations are risk factors for non-insulin dependent diabetes; individuals with IGT have an 8% higher risk of developing the disease (Edelstein et al., 1997). Moreover, a cohort study by Tominaga et al. (1999) showed that IGT, but not impaired fasting glucose, was related to an increased risk of cardiovascular disease and mortality in later life. Low birth weight has also been correlated with IGT in adult life, as first reported in two UK cohorts in Hertfordshire (Hales et al., 1991) and Preston (Phipps et al., 1993). In the study in Hertfordshire, the inverted U-shaped relationship between FGR and incidence of IGT and non-insulin dependent diabetes in men aged 64 was interpreted as pancreatic β-cell impairment (Hales et al., 1991). Similarly, in the Preston study individuals who had reduced weight at birth with an increased placental-to-birth weight ratio had increased 2 h plasma glucose concentrations after a glucose tolerance test compared to normal weight at birth individuals (Phipps et al., 1993). Furthermore, there is a twin study which provided strong evidence for the impact of the intrauterine environment on the development of diabetes. Poulsen and Vaag (2001) showed that, among monozygotic twins, the diabetic twin was found to have a lower birth weight compared with the non-diabetic co-twin. Another study by McKeigue et al. (1998) on a Swedish cohort of men at age 70 demonstrated that IGT and insulin resistance are inversely correlated with the ponderal index, and that obesity in adulthood may predict the risk of IGT and non-insulin dependent diabetes.

Insulin resistance is a physiological condition in which cells such as muscle, fat and liver cells are unable to respond to insulin. This resistance causes the plasma glucose concentration to increase. As a consequence, the pancreatic β-cell production of insulin is increased and this leads to hyperinsulinaemia. A large survey of British school children between the ages 10 to 11 years demonstrated reduced fasting insulin and post-load insulin in those who had a lower weight at birth. After adjustment for childhood height and ponderal index, fasting insulin levels decreased by 16.9%, and post-load insulin decreased by approximately 12% for each kg increase in birth weight (Whincup et al., 1997). However, despite being insulin resistant, these children with low birth weight were able to maintain normal plasma glucose concentrations.

Consequently, it was concluded that there is no direct association between birth weight and insulin resistance, but rather that faster growth during childhood is the key factor. Another study in France of adults aged 20 showed that those who had restricted growth *in utero* exhibited reduced height during adulthood and increased concentrations of plasma insulin and proinsulin when fasting and after a glucose challenge (Leger et al., 1997). The Dutch famine cohort showed an increase in 2 h plasma glucose and insulin concentrations in both men and women who were exposed to famine during pregnancy compared to those born before or conceived after the famine (Ravelli et al., 1998).

1.7.3.2 Studies in animals

Many studies in animal dietary manipulation models have established relationships between metabolic disorders (such as type 2 diabetes) and *in utero* growth restriction (Table 1.3). In maternal calorific restriction, 50% of *ad libitum* intake during the last week of pregnancy leads to a decrease in the number of β-cells and islet volume as well as insulin content in the rat neonates (Garofano et al., 1997). Continued calorific restriction during lactation reduced β-cell development by 30% compared to controls and impaired glucose tolerance in the rat offspring at 12 months of age (Garofano et al., 1999). In more severely calorific-restricted offspring (70% *ad libitum*), hypertension, hyperinsulinaemia and hyperphagia were observed (Vickers et al., 2000). Interestingly, the same laboratory was able to show that leptin treatment for the first two weeks postnatally normalised the impacts of such severe calorific restriction (Vickers et al., 2005) and IGF-1 administration could ameliorate these outcomes (Vickers et al., 2001).

Similar phenotypes were observed in protein-restricted rat neonates, in which β-cell proliferation, islet size and vascularisation and insulin content were reduced (Hoet et al., 1992). In protein-restricted adult offspring, however, different phenotypes were observed. At first, the low-protein offspring in early life displayed increased glucose tolerance and insulin sensitivity (Langley et al., 1994; Petry et al., 2000) in muscle (Ozanne et al., 1996) and adipose tissue (Ozanne et al., 1997) even up to 3 months of age (Ozanne and Hales, 1999). After that, and by the age of 15 months, offspring developed impaired glucose tolerance and insulin resistance (Ozanne et al., 2003); eventually by 17 months of age they became diabetic (Petry et al., 2001). Moreover, sexual dimorphism in response to intrauterine protein restriction was reported. Zambrano et al. (2006) showed that male offspring exposed *in utero* to protein restriction (50% protein) had insulin resistance during adult life whereas Berleze et al. (2009) showed that female offspring had greater insulin responsiveness.

Consistent with rat outcomes, mice fed a low-protein diet (6% protein) during pregnancy developed glucose intolerance at 20 weeks of age (Sutton et al., 2010). When fed a high-fat diet from 8 weeks of age for a period of 12 weeks they went on to develop hyperinsulinaemia (Sutton et al., 2010).

Therefore, it can be inferred that pancreatic β-cells are a target tissue for programming in response to fetal insults induced by poor intrauterine nutrition. Alterations in pancreatic β-cell function and insulin action are linked with an increased risk of type 2 diabetes in later life.

1.8 Sex difference

The sex of the fetus is often neglected as a factor in evaluating pregnancy outcomes and deserves greater consideration. In general, female offspring are advantaged over male offspring with regards to pregnancy outcome (Figure 1.8). Several studies show that fetal sex is also a major element in developmental programming (Grigore et al., 2008). Female *in utero* development is more dependent on the lifetime nutrition of the mother (Eriksson et al., 2010), whereas males are more vulnerable to the intrauterine environment, such as its nutritional state. Consequently, males are more prone to developing hypertension and cardiovascular disease later in life (Ozaki et al., 2001; Grigore et al., 2008). Thus, it is important to consider sex differences as a variable in this study.
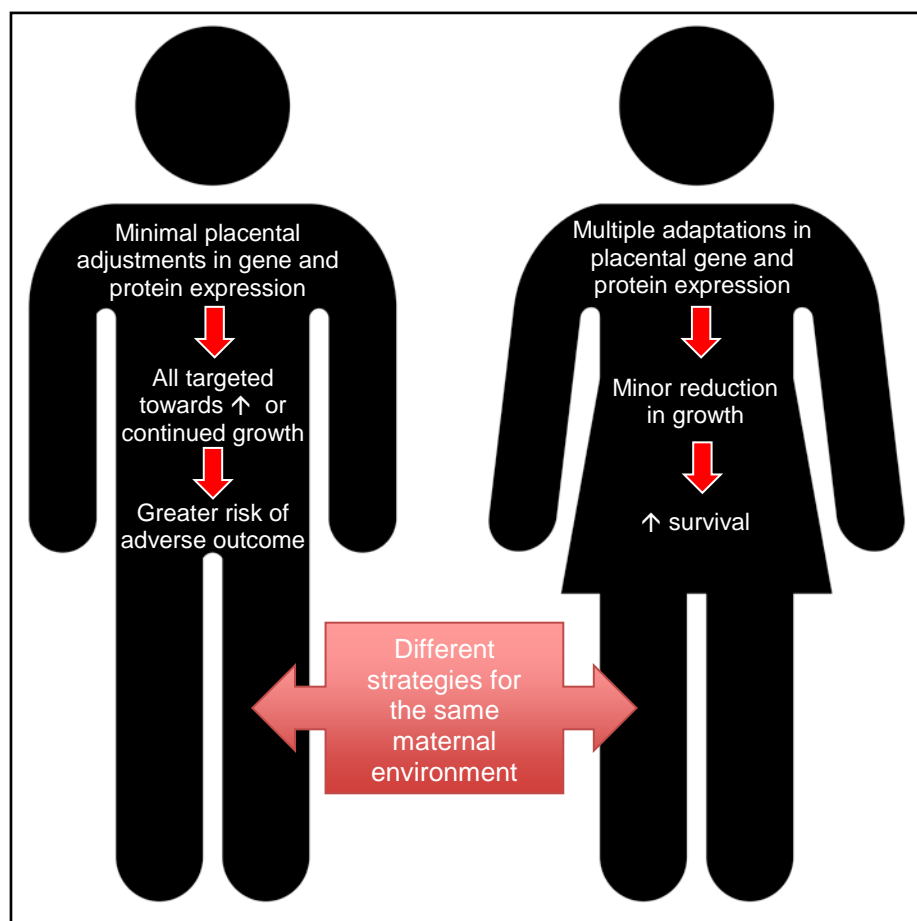
Figure 1.8 Sexual dimorphic responses to the same maternal environment. Increased (↑). Modified from Clifton (2010).

1.8.1 Preterm birth

Human preterm delivery (22 - 32 weeks) occurs mostly (55% - 60%) in male fetuses (Di Renzo et al., 2007). The one-year mortality rate was also found to be higher among male newborns. Male premature infants were found to have a higher rate of pulmonary hypoplasia than female infants, and male newborns of diabetic mothers had higher mortality rates than female newborns (Di Renzo et al., 2007). Male fetuses also bring more disadvantages for the pregnant mother herself. Women carrying males require more emergency Cesarean sections, and are associated more often with diabetes mellitus, fetal macrosomia, failure to progress during the early stages of labour, umbilical cord knots and uterine death (Di Renzo et al., 2007).

1.8.2 FGR

Findings concerning the influence of the fetus' sex on the prevalence of FGR and its complications in infants are contradictory. Miller et al. (2012) reported that FGR produced by synthetic glucocorticoid administration caused a greater weight reduction in female compared to male sheep (38% versus 23%). Engelbregt et al. (2000; 2001) reported that the onset of puberty was delayed in both male and female FGR rats. However, when FGR rats were dietary restricted after birth, only females showed a significant reduction in body mass index (Engelbregt et al., 2001). In addition, a reduction in the levels of other parameters such as food intake, fat percentage and fat mass was observed in food-restricted female rats (Engelbregt et al., 2001; Zambrano et al., 2006). Other researchers concluded that females adapt to poor growth environments with lessened growth, but without developing FGR (Clifton, 2010).

1.8.3 Hypertension and altered kidney function

The relationship between hypertension and placental weight and shape can be sexually dimorphic. In men, a large placental size and small placental diameter have been correlated with lower birth weight and higher risk of hypertension. In women, on the other hand, a risk of hypertension was associated with small placental area and diameters with lower birth weight (Eriksson et al., 2010). In the Dutch Famine Cohort, men who had been exposed to the famine *in utero* developed hypertension and had a large and round-shaped placenta with a large placental breadth (van Abeelen et al., 2011). Women, on the other hand, were found to lack this association between hypertension and placental size and, instead, raised blood pressure was correlated with reduced birth weight (van Abeelen et al., 2011).

In animal experiments, hypertension and nephron deficit are related to the severity and timing of the insult. An increase in blood pressure in male rat offspring was associated with a decrease in nutrition of the mother during the pre-implantation period (Kwong et al., 2000). In

this study, male embryos were more likely to respond to acute changes in the nutritional environment of the mother and were, as a result, more sensitive to particular programming factors. Moderate global dietary restriction (30% food restriction) during the pregnancy period caused sex variations with regards to hypertension in rat offspring, with higher levels of blood pressure and earlier onset of hypertension in males than in females (Ozaki et al., 2001). A mouse study in which a low protein diet (9%) was imposed at the perimplantation period showed that male offspring became hypertensive earlier at 9 weeks postnatally, while female offspring developed hypertension later at 21 weeks of age (Watkins et al., 2008b). Moreover, Woods and coworkers demonstrated that exposure *in utero* to modest protein restriction (8.5% protein) caused a reduction in renin and angiotensin II expression in the kidney of male, but not female, rat offspring at birth (Woods et al., 2001a; 2005). Thus, the variation in intrarenal RAS expression between the sexes during development provides evidence that there are sex-specific adult outcomes associated with nephron number and blood pressure which have their origins during fetal life. Moderate maternal protein restriction caused a reduction in the nephron number in adult male offspring but not in female offspring (Woods et al., 2005). However, only severe levels of protein restriction (5% protein) resulted in hypertension and alterations in the structure of the kidney in both female and male offspring (Woods et al., 2004). Joles et al. (2010) noted that exposure of the offspring to protein restriction *in utero* increased the kidney injury in aging male offspring. In contrast, female offspring that had been subjected to moderate protein restriction during pregnancy were protected against the accelerated development of renal injury (Pijacka et al., 2015).

Consistent with these observations, variations in sodium intake during pregnancy and lactation produced a similar pattern of response (Vehaskari and Woods, 2005). Moderate maternal sodium intake programmed a significant increase in blood pressure in male, but not female offspring; whereas high sodium during the same window of development leads to hypertension in both male and female offspring (Vehaskari and Woods, 2005). Also, exposure *in utero* to high dietary fat is associated with the development of hypertension only in female offspring (Khan et al., 2003).

From the above mentioned epidemiologic and animal studies, it is clear that intrauterine nutritional perturbation is one of the main causes of altered placental morphology and functionality, and thereby induces functional and structural adaptations in fetal development. Despite the divergence in maternal undernutrition models (calorific restriction, low protein diet or high fat diet), all of these models converge into similar outcomes of altered fetal growth and birth weight. These responses persist into adulthood, affecting later development of hypertension, renal disease, and metabolic disorders such as glucose intolerance and insulin resistance (Figure 1.1). Furthermore, several studies indicate that male offspring are more sensitive to nutritional insults during development, and that the female offspring are protected.

Against this background detailing the influence of altered maternal nutritional intake on fetal growth and development, linked to ensuing changes in the propensity to metabolic disease in the offspring, the rationale of this study was to investigate the impact of Islamic Ramadan fasting during pregnancy and subsequent effects elicited in the offspring. An overview of Ramadan fasting in Islamic culture is outlined in the next section.

1.9 Ramadan fasting as a model of prolonged intermittent fasting

Intermittent fasting (IF) is practiced widely across the world either as a diet for health benefits or for religious or spiritual purposes. There are three main types of intermittent fasting: calorific restriction, skipping one or more meals per day and alternate day fasting (Trepanowski and Bloomer, 2010).

With regards to calorific restriction, health improvement and lifespan extension have been observed in different species including studies on rodents (Spindler, 2010). Varady (2011) and Harvie et al. (2011) have reported that IF and daily calorific restriction achieve weight loss in overweight and obese individuals. Additionally, calorific restriction appears to improve cardiovascular health through lower blood pressure, increases in heart rate variability and improvement in left ventricular function (Mattson and Wan, 2005). Furthermore, calorific restriction reduces fasting glucose and insulin concentrations and increases insulin sensitivity (Masoro, 2005).

Alternate day fasting is a form of fasting in which food is consumed normally for 24 h and then restricted for 24 h in a 2 day cycle, with water consumption *ad libitum* at all times. In the study of Heilbronn et al. (2005), eight healthy males and females engaged in alternate day fasting for 21 days, and as a result they exhibited a 2.5 % reduction in their bodyweight and 4% in their body fat mass. Fasting blood glucose and ghrelin (an appetite hormone) concentrations remained similar before and after the intervention, whereas fasting insulin concentrations decreased suggesting greater insulin sensitivity (Heilbronn et al., 2005).

Religious fasting also has a positive impact on human health. Greek Orthodox Christians fast for roughly 180 - 200 days per year in total, during which they abstain from animal-derived foods (Trepanowski and Bloomer, 2010). Thus, the fasting periods are a variation of vegetarianism as well as a form of diet restriction. Among the positive effects that are derived from these fasts are decreases in body mass, LDL-C (low-density lipoprotein cholesterol), and the LDL-C/HDL-C ratio (low density versus high-density lipoprotein cholesterol ratio) (Trepanowski and Bloomer, 2010).

Another Christian fast is the bible-themed Daniel Fast, which is based on a diet of vegetables. It excludes the consumption of any products of animals, as well as alcohol, caffeine, refined

carbohydrates or any artificial additives. This fast can be anything between 10 and 40 days long, although it is mostly observed for a period of 21 days. According to Trepanowski and Bloomer (2010), the Daniel Fast brings a number of positive health effects, such as positive results for biomarkers of oxidative stress, insulin sensitivity, blood lipids and blood pressure.

1.9.1 Islamic Ramadan fasting

Ramadan fasting is one of the five pillars of Islam and as such it is prescribed for all Muslims. It is the abstinence from food and fluid practiced between dawn and sunset for the period of the holy month of Ramadan. The Islamic calendar consists of twelve lunar months, and therefore each year Ramadan begins 11 days prior to the previous year of the Gregorian calendar. The daytime hours during which fasting is practiced can vary from between 9 and 19 h per day, contingent upon the season of the year and whether the month of Ramadan falls nearer the shortest or longest day of the year. Therefore fasting over Ramadan can be considered as an example of prolonged intermittent fasting (i.e. approaching global nutrient restriction). During these daytime hours the consumption of oral medicine, smoking and sexual relations are also prohibited (Bragazzi, 2014).

According to the Islamic religion, a number of people are excused from fasting during Ramadan. Those who are exempt include children, the elderly, sick people and also women under various circumstances, such as during their period of menstruation, pregnancy and periods of breastfeeding (Bragazzi, 2014). In many cases, these individuals have to compensate for the fasting days that they have missed by fasting after Ramadan when their condition has changed back to normal, which in the case of pregnant women is after they have delivered. However, despite the fact that pregnant women are excused from Ramadan fasting, it is very common for Muslim women to fast during their pregnancy in order to join in with the traditions and celebrations of their families, instead of fasting alone at a later time (Robinson and Raisler, 2005; Mirghani et al., 2003). The general misconception of pregnant women who observe this practice is that Ramadan fasting is healthy, safe and without any relevant negative side effects on them or their offspring; moreover, to them, fasting during that month means following a divine ordinance (van Ewijk, 2011; Mubeen et al., 2012). However, contrary to these popular beliefs among pregnant Muslim women, it has been shown that skipping meals during pregnancy is one of a number of maternal behaviours which potentially could have detrimental effects on their health and that of their offspring. Furthermore, these effects can endure into the adulthood of the offspring leading to a long-term impact on health and disease (Meis et al., 1984; Almond and Mazumder, 2011; van Ewijk, 2011).

1.9.1.1 Effect of Ramadan fasting on maternal physiology

According to the literature, pregnant Muslim women who observed Ramadan fasting suffered adverse effects on their health status in a number of different ways. Rabinerson et al. (2000) documented increases in the incidence of hyperemesis gravidarum (extreme nausea and excessive vomiting) during the first trimester of pregnant women who fasted. Malhotra et al. (1989) reported changes in the metabolic measurements of fasting pregnant women, such as reductions in the concentrations of glucose, insulin, lactate and carnitine accompanied by increases in 3-hydroxybutyrate, triglyceride and non-esterified fatty acids. These changes indicated that the women underwent an accelerated starvation phenomenon due to the 17 h period of fasting. Contrary to this, ketosis and ketonuria did not develop in 20 week pregnant women who fasted for 13 to 14 h/d (Dikensoy et al., 2008). This discrepancy between the two studies can be attributed to differences in the duration of fasting and to the fact that the fasting women in the Dikensoy study were encouraged to drink two litres of water daily prior to fasting to prevent dehydration. In addition, Dikensoy et al. (2009) reported a significant rise in the maternal serum cortisol level of 36 fasting women at 20 weeks of pregnancy. This can be ascribed to changes in the eating and sleeping patterns during Ramadan, which affects the circadian rhythm of cortisol secretion.

The types of food consumed once the fast is broken tend to differ from a normal diet, and there are regional differences in tradition. Nonetheless, there is evidence that pregnant women who fast during Ramadan consume fewer calories. Arab (2004) reported that when pregnant women broke their fast in the evening they usually consumed large amounts of food including many sweets, but they still had a calorie deficiency of more than 500 kcal.

1.9.1.2 Effect of Ramadan fasting on fetal physiology

With the increased awareness of fetal programming and how different maternal nutritional insults can alter fetal growth and development, researchers have focused on fetal responses to Ramadan fasting by women during pregnancy. Evidence is building that exposure to maternal fasting during Ramadan may have adverse impacts on fetal health. Mirghani et al. (2005) observed fewer heart rate accelerations in the fetuses of fasting pregnant women compared with non-fasting women. This was found regardless of the existence of maternal normoglycaemia or the duration of the fast. In another study by Mirghani et al. (2004) fetal breathing movements were measured twice a day: once during the fasting period and a second time two hours after breaking the fast. They found a negative correlation between fasting and the occurrence and frequency of fetal breathing movements, which was linked to hypoglycaemia. However, overall fetal body movement and tone were unaffected, indicating that the vulnerability of fetal breathing movement was unlikely to be directly linked to changes in glucose concentration. The fetal biophysical profile, amniotic fluid volume (Mirghani et al.,

2003; Kamyabi and Naderi, 2004), fetal bladder volume and umbilical artery Doppler flow were also evaluated and no significant changes were observed in fasting pregnant women (Mirghani et al., 2003). In addition, uterine artery blood flow was not affected by short diurnal fasts (Mirghani et al., 2007). However, more recent studies conducted during the summer of 2013 in Turkey, in which the temperature was between 36 °C and 43 °C and the fasting duration more than 17 h/d have shown reductions in the amniotic fluid index of the pregnant women (Seckin et al., 2014; Sakar et al., 2015). Reductions in fetal head circumference and fetal femur length (Sakar et al., 2015) as well as lower birth weight were observed (Savitri et al., 2014). These conflicting results show that the season in which Ramadan fasting falls and consequently the length of the daily fasting period can influence health outcomes differently.

1.9.1.3 Effect of Ramadan fasting on placenta

The placenta has the plasticity to adapt to nutritional changes to protect fetal development. This was demonstrated in a large study of 7083 babies born in Saudi Arabia who were exposed to fasting in the second and third trimester of pregnancy. Although the placental weight and the placental weight-to-birth weight ratio were reduced, the babies' weights remained normal. The study concluded that, although the placental weight was reduced, its efficiency increased by up-regulating the transport activity to maintain an adequate materno-fetal nutrient transfer (Alwasel et al., 2010a). However, it is important to note that a change in placental size, either above or below normal, is associated with chronic disease later in the offspring's life (Barker et al., 1990; Godfrey, 2002).

1.9.1.4 Effect of Ramadan fasting on offspring

A number of studies have reported the effects of Ramadan fasting on the health of the offspring. One important indicator of offspring health is birth weight. A retrospective cohort study in Tehran found that neonatal birth weight, height and maturity were unaffected by Ramadan fasting (Kavehmanesh and Abolghasemi, 2004). Contrary to this, a large study by Almond and Mazumder (2011) found that prenatal exposure to Ramadan fasting negatively affected birth weight and caused an increase in the impairment of mental development and learning abilities of school children. Moreover, the exposure to Ramadan fasting *in utero* was associated with symptoms of cardiovascular problems, type 2 diabetes and anaemia among elderly people (van Ewijk, 2011). Fasting during Ramadan while pregnant may therefore negatively affect developmental programming of the offspring's metabolic phenotype later in life.

1.10 SUMMARY

Overall, maternal nutritional status and intake during pregnancy are critical for feto-placental development. Evidence has revealed that influences on fetal development *in utero* can impact on human health in adult life. Therefore, maternal sub-optimal nutrition will not only have a short-term impact with the alteration of the neonatal body composition and increased morbidity and mortality, but it will be associated with a greater risk of chronic disease later in life. FGR is associated with alterations in placental transport mechanisms, an outcome related to nutrient deprivation. One of the transport mechanisms demonstrated to be pivotal for normal fetal development is the system A amino acid transporter, whose activity is diminished in FGR and is related to FGR severity.

Ramadan fasting during the annual holy festive month of Ramadan may be considered a form of global nutritional restriction. Both the pregnant women and their fetuses are exposed to periods of repeated food and water restriction for a significant proportion of the day over a month's time. This has been reported to have a detrimental impact on fetal and placental development and this could lead to developmental programming of the offspring and the genesis of adverse health outcomes later on in life.

1.11 HYPOTHESIS

This study tested two hypotheses. The first is that exposure to maternal intermittent fasting *in utero* results in adverse effects on maternal physiology leading to fetal growth restriction with impairment of placental development and transport function at a late stage of gestation, and that these responses exhibit fetal sex dependency. The second hypothesis is that prenatal intermittent fasting results in low birth weight and leads to long-term impacts on renal function and the risk of developing metabolic disease and hypertension among adult offspring. These long-term outcomes in offspring can exhibit sex-dependent specificity.

1.12 AIMS AND OBJECTIVES

The overall aim of this study was to investigate how intermittent maternal food restriction, as a model of Ramadan fasting, affects placental development and nutrient transport function, fetal growth and development of the offspring, particularly with respect to kidney function and the genesis of hypertension and insulin resistance, and how fetal/offspring sex influences these outcomes. In addition the impact of maternal food restriction on maternal metabolic status and how this relates to fetal growth were examined.

In the rat model applied here, maternal food intake was restricted intermittently for a period of 16 h each day over the dams' active phase, corresponding to the maximum duration of human

fasting during Ramadan. Intermittent maternal food restriction of this kind in rats represents a unique model, designed to evaluate the outcome of significant changes in the pattern of food intake during pregnancy and in relation to sex of the fetus/offspring. It differs from previous models which have been more commonly studied such as global food restriction (typically 70% of *ad libitum*), in that the fetus is exposed to periods of maternal calorific deficit followed by periods of *ad libitum* intake. Thus, the nature of the nutritional challenge faced by the IF fetus differs from that experienced by a chronically under-nourished fetus, but mimics the repeated cyclical 'fasting-feeding' eating patterns of Ramadan fasting more closely.

The objectives for this study were:

Chapter 2: Effects of maternal intermittent fasting on fetal development and placental function in the rat

(1) To examine the effect of maternal IF on maternal physiology in terms of daily food and water intakes and weight gain, as well as the maternal metabolic response to IF, by measuring plasma glucose, insulin and glucagon concentrations.

(2) To determine the effect of maternal IF on fetal growth outcomes at GD 21, assessed by changes in fetal weight and anthropometric measurements, and fetal organ weights and to determine whether fetal sex affects the fetal response to maternal IF.

(3) To analyse maternal and fetal amino acid concentrations providing an insight into how maternal IF regimen alters amino acid balance.

(4) To assess placental development by wet weight measurements and analysis of placental morphology using standard histological techniques in which placental junctional and labyrinth zone areas were recorded.

(5) To investigate the effect of maternal IF on placental nutrient transport capacity in particular system A transport, using *in vivo* and *in vitro* techniques and whether alterations of this transport system contribute to FGR. This transport system has been chosen, as it is particularly important for fetal growth and has been shown to be down-regulated with nutritional insults and when FGR is apparent (Jansson et al., 2006; Rosario et al., 2011; Glazier et al. 1997).

(6) To underpin the studies examining the activity of system A: placental mRNA expression for SNAT subtypes 1, 2 and 4 (*Slc38a1, Slc38a2 and Slc38a4* respectively) were measured using qRT-PCR. Western blotting was also performed using isolated, maternal-facing SynTB-II plasma membrane vesicles with antibodies to determine expression of SNAT1 and 2 within this trophoblast plasma membrane.

Chapter 3: Effects on cardio-renal function in offspring exposed to intermittent fasting *in utero*

(1) To characterise the effect of prenatal IF on litter size, birth weight and postnatal growth until 14 weeks of age.

(2) To explore kidney function in offspring at 14 weeks of age.

(3) To determine the effect of maternal IF on: (i) offspring blood pressure using tail cuff plethysmography and (ii) metabolic function in offspring through glucose and insulin tolerance tests.

(4) To determine the sex-dependency of responses evoked in the offspring.

Chapter 4: The impact of dietary salt challenge on cardio-renal function

(1) To challenge the IF offspring kidney with a high-salt diet in order to identify potential deficiencies in renal excretory capacity.

(2) To determine whether this challenge will induce hypertension in IF offspring.

(3) To determine the effect of IF on saline preference and saline aversion.

(4) To determine the sex-dependency of responses evoked in the offspring.

CHAPTER 2

EFFECT OF MATERNAL INTERMITTENT FASTING ON FETAL DEVELOPMENT AND PLACENTAL FUNCTION IN THE RAT

2.1 INTRODUCTION

Maternal undernutrition during pregnancy has become a global health concern that carries the risk of fetal growth restriction (FGR), leading to a higher incidence of infant mortality and morbidity (Brodsky and Christou, 2004). Impaired fetal growth *in utero* is associated with an increased risk for the development of metabolic and cardiovascular disease in the offspring's later life (Langley-Evans, 2009). Maternal undernutrition is very common in developing countries and remains a major factor underlying FGR, as indicated by evidence from famine periods such as the Dutch Famine during World War II and the Great Chinese Famine (Ravelli et al., 1999; Brodsky and Christou, 2004; Li et al., 2010). Pregnant women from affluent cultures may also engage in voluntary calorific restriction in an attempt to retain a positive body image (Davies and Wardle, 1994). Therefore, maternal undernutritional status, or sub-optimal nutrient intake, during pregnancy is an important risk factor for an altered fetal growth trajectory and a predisposition to adverse health outcomes later in life.

Animal models of prenatal undernutrition programming, such as restriction of protein or calorific intake, have demonstrated FGR and alterations in placental development, morphology and function that predisposed the offspring to diseases in adulthood (Kwong et al., 2000; Fowden et al., 2008; Watkins et al., 2008b; Coan et al., 2010; Sandovici et al., 2012). In addition, maternal and fetal metabolic changes have been reported (Jansson et al., 2006; Coan et al., 2010; Belkacemi et al., 2011c). One of the underlying causes of FGR is the decreased expression and activity of placental amino acid transporters (Glazier et al., 1997; Jansson and Powell, 2006; 2007; Sibley, 2009). The activity of the system A amino acid transporter has been shown to be altered prior to the onset of FGR in rats (Jansson et al., 2006) and the magnitude of the reduction in system A activity contributes to the severity of FGR in humans (Glazier et al., 1997). These observations, together with the evidence that inhibition of system A activity during rat pregnancy leads to diminished fetal weight (Cramer et al. 2002), argues for the integral involvement of system A as a crucial amino acid transporter necessary for normal fetal growth.

In Islamic culture, the majority of pregnant Muslim women choose to fast voluntarily during the month of Ramadan by abstaining from food and drink during daytime hours from sunrise to sunset. Although their religion does not require them to do so during pregnancy, this practice is frequently adopted. By abstaining from food and drink during the daytime and only eating at night, these pregnant women expose their unborn babies to repeated periods of intermittent fasting, which can last between 8 to 18 hours a day, depending on the month in which Ramadan falls, which varies every year. As a consequence, both the pregnant women and their fetuses experience periods of repeated food and water restriction for a significant proportion of the day over a month's duration (Mirghani et al., 2003; Robinson and Raisler, 2005; Bragazzi, 2014).

Bearing in mind the importance of adequate maternal nutrition during pregnancy, it is perhaps not surprising that the practice of Ramadan fasting by pregnant women has been reported to have a detrimental effect on maternal and fetal health. Pregnant fasting women displayed higher cortisol concentrations (Dikensoy et al., 2009), hypoglycaemia (Malhotra et al., 1989), a reduction in calorie intake (Arab, 2004) and diminished weight gain (Kiziltan et al., 2005). With regards to fetal health, the studies have shown reductions in amniotic fluid index (Seckin et al., 2014; Sakar et al., 2015), fetal anthropometric measurements (Sakar et al., 2015) and birth and placental weights (Alwasel et al., 2010a). Further, sex-specific differences in birth size in response to Ramadan fasting were also reported (Alwasel et al., 2011). However, the broader impact of intermittent fasting on fetal development and placental function is not yet fully understood. Therefore, to investigate these aspects more fully, a rat model has been developed to mimic aspects of Ramadan fasting in humans.

The objectives of this chapter were to determine the effects of maternal intermittent fasting in pregnant rat dams on the outcomes listed below, by measurement of the indicated variables, and to examine the sex-dependent specificity of responses:

- Maternal physiology - daily food and water intakes and weight gain.
- Fetal development - fetal weight, fetal anthropometric measurements and fetal organ weights.
- Maternal and fetal homeostasis - plasma amino acid and glucose concentrations, plasma concentration of insulin and glucagon and fetal liver glycogen content.
- Placental growth and development - placental wet weight, placental junctional and labyrinth zone areas and placental glycogen content.
- Placental metabolism - placental metabolomic analysis.
- Placental nutrient transport capacity - placental expression of *Slc38a1, Slc38a2 and Slc38a4* mRNA encoding SNAT subtypes 1, 2 and 4 respectively, placental SNAT1 and SNAT2 protein expression and measurement of system A transporter activity using *in vivo* and *in vitro* techniques.

2.2 MATERIALS AND METHODS

All experiments involving animals were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986; all work was carried out under a Home Office Project Licence granted to Dr Nick Ashton (PPL number 40/3646).

2.2.1 A model of maternal intermittent fasting

To mimic the pattern of repeated daily intermittent fasting adopted by humans during Ramadan fasting, a novel rat model was developed during this study. As discussed previously, pregnant women observe Ramadan fasting by abstaining from food and drink intake during their active phase over daylight hours. In the rat model, daily intermittent fasting (IF) was imposed on a group of pregnant dams, with access to food withdrawn over 16 h per day between 5:00 pm and 9:00 am; denoted as the IF group. This timeframe corresponds to the rat's active phase, as rats are nocturnal feeders. The period of daily food withdrawal in the rat model equates to the maximum duration of human fasting during Ramadan. During the day (9:00 am until 5:00 pm), the IF group had free access to standard rat chow (BK001 (E) SDS Rodent Breeder and Grower, LBS Biotech, Redhill, UK; Table 2.2.1). Hence a daily regime of maternal intermittent fasting and a repeated pattern of a 'fast-feed' cycle over 24 h was imposed. All rats had free access to water at all times.

2.2.2 Animals and husbandry

A total of 72 virgin female Wistar rats weighing 250 - 275 g and 25 male Wistar rats weighing ~350 g (10 - 12 weeks of age) were obtained from Charles River Laboratories (Oxford, UK). They were allowed to become acclimatised for 1 week upon arrival at the Biological Services Facility (BSF), supplied with nesting material, and maintained under a constant 12:12 h dark/light cycle at 21 - 23 °C with 65% humidity. Then, the baseline bodyweight of each female was recorded before being paired with a male Wistar rat in individual breeding cages for mating. The day a vaginal plug was found on the cage floor was denoted as gestational day 1 (GD 1) (term is 23 days). Pregnant dams were then housed individually in ventilated cages and randomly allocated to one of two groups (N = 36 / group where N represents number of dams/litters): either IF (Section 2.2.1) or control. The control group (C) was fed standard rat chow (Table 2.2.1) *ad libitum*. Both C and IF groups had free access to water throughout. For both dietary groups, the dam's food and water intake and bodyweight were recorded daily at approximately the same time (5:00 pm), before food withdrawal from the IF group. Pregnant rat dams in C and IF groups were maintained on their respective dietary regimes from GD 1 until GD 21, when the experiment was terminated.

Table 2.2.1 Composition of rodent diet (g/kg diet)

Ingredient	Fat	Protein	Vitamins	Minerals & Trace elements	Energy (MJ/kg)
	32.7	193.7	3	49.2	55.8

A number of animals were excluded from the study for one of the following reasons:

1. Pregnant dams failed to mate such that this could be timed accurately, with the absence of a vaginal plug (N = 6).

2. IF dams were withdrawn from the intermittent fasting regime when more than 15% weight loss was encountered (N = 3).

3. Failure of the conducted experiment (N = 2, Section 2.2.9.1).

2.2.3 Collection of blood and tissue samples

At GD 21 between 9:00 and 10:00 am, the pregnant dams were weighed and then anaesthetised with isoflurane by inhalation (4% in oxygen at 2 L/min). Maternal blood was obtained by cardiac puncture using a heparinised (1000 IU/mL) syringe. The blood was collected into heparinised tubes, and centrifuged at 14000 xg for 2 min (Sigma 1-14K, Germany) at room temperature. The plasma was aliquoted, flash frozen and stored at -80 °C for later analysis. Under anaesthesia, the dams were euthanized following surgical laparotomy and cutting of the diaphragm. Then the dams' livers, kidneys and hearts were harvested, frozen and stored at -80 °C for further studies.

The uterine horns were exposed; fetuses were identified according to their position in each uterine horn, noting any resorptions. Fetuses and placentas were then removed, blotted and weighed. The order of removal of fetuses from the two uterine horns was alternated between experiments. Placentas were stored in various formats for future analyses, as detailed subsequently. Fetal blood was collected individually in heparinised capillary tubes (Fisherbrand™ Microhematocrit capillary tubes, Loughborough, UK) from an axillary incision (Figure 2.2.1) and centrifuged at 15290 xg for 1 min (haematocrit Centrifugette 4203, ALC International Srl., Italy). Following centrifugation the plasma was removed, frozen in eppendorf tubes and stored at -80 °C for subsequent analyses. The fetal organs (11 litters / group), including brain, heart, liver and kidney were dissected, weighed and frozen rapidly for further analysis. All fetuses/placentas were chosen at random when allocated for later analyses. Fetal tail tips were also collected for determination of fetal sex and stored at -20 °C prior to analysis.
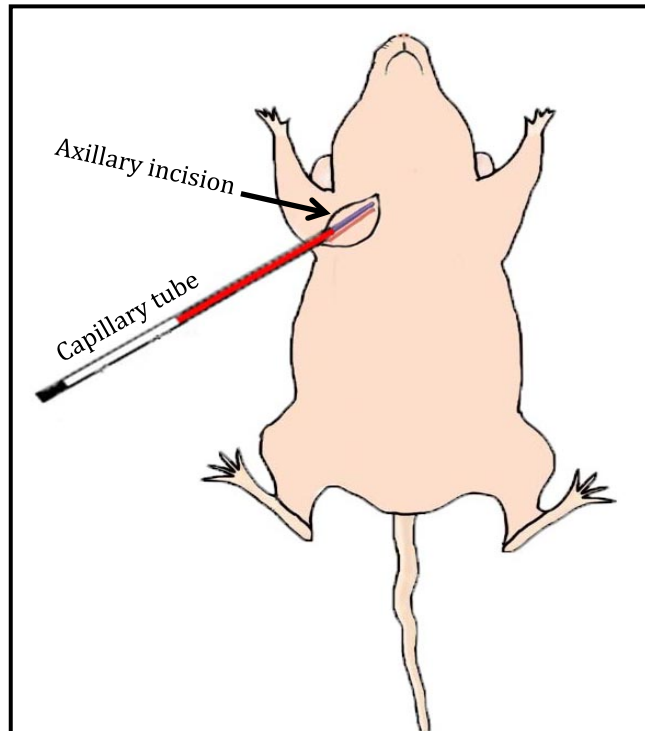
Figure 2.2.1 Fetal blood collection. Fetal blood was collected into a capillary tube from an axillary incision. Modified from Ciulla et al. (2013).

2.2.4 Fetal anthropometric measurements

In a set of 5 litters per group (n = 82 - 85 fetuses / group where n represents the number of individual fetuses), fetal anthropometric measurements were performed using a cotton thread in a standardized manner, as previously described (Kusinski et al., 2012). The following body proportions were measured (Figure 2.2.2):

- o Head circumference (above the eyes with head tilted 45 degrees)
- o Abdominal circumference (at the umbilicus)
- o Crown-rump length (from the tail following the curve of the spine to the top of the head)
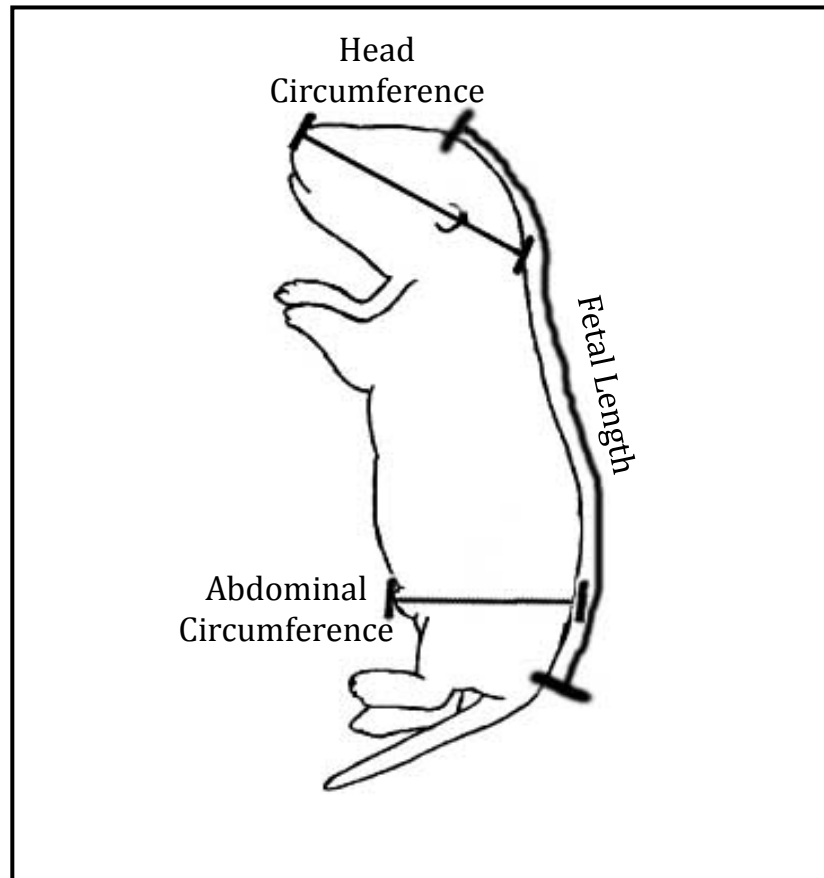
Figure 2.2.2 Fetal anthropometric measurements were performed as illustrated.

2.2.5 Determination of fetal sex

2.2.5.1 Genomic DNA extraction

Fetal sexes were determined using genomic DNA (gDNA), extracted from fetal tail tips using a DNeasy extraction kit (Qiagen, Manchester, UK) according to the manufacturer's protocol. Fetal tail tips were lysed in 180 µL Buffer ATL and 20 µL Proteinase K and incubated at 55 °C for 2 h in an oven (Hybaid, VWR, UK), on a shaking platform at speed 40. The lysates were vortexed and centrifuged at 15115 xg for 1 min to remove debris. The supernatant was transferred into a clean 1.5 mL eppendorf tube. The second step was DNA binding, in which 400 µL Buffer AL-ethanol mixture (mixed 1:1 v/v) was added to the supernatant and vortexed. The mixture was loaded onto the DNeasy mini-column in a 2 mL collection tube and centrifuged at 5724 xg for 1 min at room temperature. The DNA was bound to the DNeasy mini-column matrix and the flow-through was discarded. This was followed by washing the DNA twice in two different buffers: (i) 500 µL Buffer AW1 and centrifuged for 1 min at 5724 xg; and (ii) 500 µL Buffer AW2 and centrifuged for 3 min at 15115 xg. The flow-through was discarded after each spin. Finally for DNA elution, the DNeasy mini-column was placed in a 1.5 mL eppendorf tube and 200 µL Buffer AE was added and incubated at room temperature for 1

min, then centrifuged for 1 min at 5724 xg. The eluted gDNA was then stored at -20 °C overnight, prior to the fetal sex determination polymerase chain reaction (PCR).

2.2.5.2 PCR for fetal sex determination

The gene encoding the sex determining region Y (*Sry*) protein, located on the short arm of the Y chromosome, was used to positively identify male fetuses. Primer sequences for rat *Sry* gene were taken from An et al. (1997; Table 2.2.2). Lack of *Sry* gene amplification was taken to infer female sex.

Table 2.2.2 Primer sequences for determination of fetal sex using the *Sry* gene (sequences 5'→3')

Gene	Accession No	Forward Sequence	Reverse Sequence
Sry	NM_012772	CATCGAAGGGTTAAAGTGCCA	ATAGTGTGTAGGTTGTTGTCC

PCR was performed in a total volume 20 µL containing 2 µL 10x PCR buffer, 2 µL 2 µM dNTP's 12.1 µL PCR water, 1.2 µL 1.5 mM MgCl$_2$, 0.8 µL 0.2 µM forward and reverse primers and 0.1 µL 5 U/µL Taq DNA polymerase (all from Invitrogen Life Sciences, Paisley, UK) with 1 µL gDNA. PCR was carried out in a DNA Thermal Cycler (Perkin Elmer, UK), starting with a single denaturing step for 5 min at 94 °C. Amplification was performed for 30 cycles, each cycle consisting of 3 steps for 30 s each: denaturation at 94 °C, annealing at 60 °C and extension at 72 °C. An additional extension step was carried out after 30 cycles at 72 °C for 5 min.

2.2.5.3 Agarose gel electrophoresis

PCR products (18 µL) were added to 2 µL XC loading buffer (1.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in water) and separated by electrophoresis through a 2.5% (w/v) agarose gel (100 mL in 1 x Tris-Acetate-EDTA (TAE) buffer) containing 10 µL Gel Red stain (10,000 x stock; Biotium, California, USA). A 50 bp ladder (Bioline, London, UK) was run in parallel to allow amplicon size determination. Electrophoresis was performed at 120 V for 50 min. A UV transilluminator (Gel Doc 2000 system, BioRad, SnapGene software, UK) was used to visualise PCR products. The visualisation of a single *Sry* amplicon of the correct size (104 bp) was used to identify male gender whilst absence of amplicon was used to infer female gender (Figure 2.2.3).
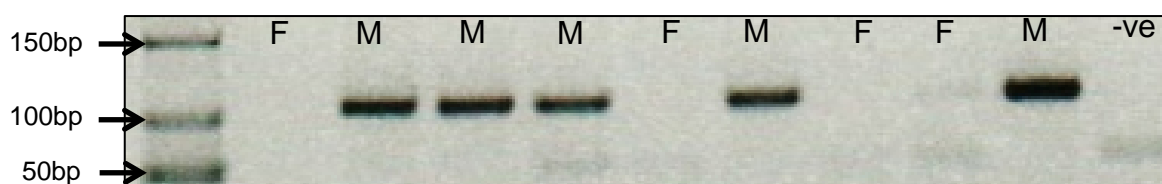
Figure 2.2.3 PCR for fetal sex determination using the male-specific *Sry* gene. Abbreviations: F, female; M, male; -ve, control (water).

2.2.6 Metabolic analysis

All maternal and fetal plasma samples were defrosted on ice and analysed for glucose, insulin, glucagon and branched chain amino acids (BCAA) using commercial kits. All assays were performed according to the manufacturer's instructions and standards and experimental samples were run in duplicates. In order to assess assay precision, the intra-assay co-efficient of variation (CV) was measured by running one sample three to ten times in the same assay.

2.2.6.1 Glucose

Glucose concentration in maternal (C, N = 11; IF, N = 15) and fetal (N = 5 litters / group with n = 10 male and female fetuses / group) plasma was determined using a glucose hexokinase (HK) assay kit (Sigma Aldrich; GAHK-20, Dorset, UK). In this enzymatic method, glucose is catalysed by hexokinase and glucose-6-phosphate dehydrogenase to form NADH. The concentration of NADH is directly proportional to the sample glucose concentration and determined by measuring the absorbance at 340 nm. Briefly, maternal and fetal plasma samples were diluted to 1:5 and 1:7 respectively. 12.5 µL glucose standard or plasma were added to the appropriate wells of a 96 well microplate. 250 µL HK reagent was added to each well and mixed thoroughly by pipetting up and down for 40 s. The mixture was incubated at room temperature for 15 min protected from light. Absorbance was then measured at 340 nm (FLUOstar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Buckinghamshire, UK). A standard curve of glucose (µg) versus absorbance at 340 nm was plotted using a linear regression plot (Prism® software, Graphpad, UK) and used to interpolate sample glucose concentration. Interpolated values were multiplied by the appropriate dilution factor to obtain concentration (mg/mL) and then divided by glucose MW (180.2 mg/mmol) to give glucose concentration (mM) in maternal and fetal plasma. The intra-assay CV was 2.82% (n = 10 replicates).

2.2.6.2 Insulin

Insulin concentration was measured in maternal (N = 11 / group) and fetal (N = 5 litters / group with n = 10 male and female fetuses / group) plasma by the Ultrasensitive Rat Insulin ELISA kit

(Mercodia; 10-1251-01, Uppsala, Sweden). Fetal plasma samples only were diluted 1:4; maternal samples were used undiluted. 25 µL insulin standard or plasma were added carefully into an antibody-bound 96 well plate. 100 µL enzyme conjugate 1x solution was added to each well and incubated at room temperature whilst being agitated for 2 h. 350 µL wash solution was added to each well and washed 6 times. After wash, the plate was inverted and tapped firmly against absorbent paper to ensure that all buffer was removed. 200 µL substrate TMB was then added to each well and incubated at room temperature for 15 min. This was followed by the addition of 50 µL stop solution to each well and mixed thoroughly by pipetting up and down for 5 s. Absorbance was then measured at 450 nm (FLUOstar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Buckinghamshire, UK). Insulin concentrations (µg/L) were determined following nonlinear regression 2nd order polynomial analysis of the standard curve, corrected for dilution as appropriate. The sensitivity of the kit as stated by the manufacture was ≤ 0.020 µg/L. Intra-assay CV was 7% (n = 3 replicates).

2.2.6.3 Glucagon

Maternal (N = 6 / group) and fetal (N = 6 litters / group with n = 12 male and female fetuses / group) plasma glucagon concentration was determined by Glucagon ELISA kit (Mercodia; 10-1271-01, Uppsala, Sweden). 25 µL calibrators or undiluted plasma were added to wells of an antibody-bound 96 well plate, followed by the addition of 200 µL enzyme conjugate 1x solution into each well. The plate was incubated at 4 °C whilst being agitated over night and then washed 6 times with 350 µL wash solution per well. After wash, the plate was inverted and tapped firmly against absorbent paper to ensure all buffer was removed. 200 µL substrate TMB was then added to each well and incubated for 15 min at room temperature. Following this, 50 µL stop solution was added to each well and mixed thoroughly by pipetting up and down for 5 s. Absorbance was then measured at 450 nm (FLUOstar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Buckinghamshire, UK). Glucagon concentrations (pmol/L) were determined following nonlinear regression 2nd order polynomial analysis of the standard curve. The sensitivity of the kit as stated by the manufacture was 1 pM. As stated in this assay kit, the intra-assay coefficient of variance was 4%.

2.2.6.4 Branched chain amino acids (BCAA)

The concentration of BCAA (leucine, isoleucine and valine) was measured in both maternal (N = 9 / group) and fetal (N = 4 litters / group with n = 8 male and female fetuses / group) plasma using a coupled-enzyme colorimetric assay (Sigma Aldrich; MAK003, Dorset, UK). Maternal and fetal plasma samples were diluted 1:4 and 1:8, respectively. 50 µL BCAA standard or diluted plasma were added into each well of a 96 well microplate. 50 µL reaction master mix (46 µL BCCA Assay Buffer + 2 µL BCCA Enzyme Mix + 2 µL WST Substrate Mix) was added to each well, mixed gently to avoid foaming and incubated for 30 min at room temperature

protected from light. Absorbance was then measured at 450 nm (FLUOstar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Buckinghamshire, UK). A standard curve of BCAA (nmol) versus absorbance at 450 nm was plotted (Prism® software, Graphpad, UK) and linear regression was used to interpolate sample BCAA concentration. Interpolated values were multiplied by x 20 and the appropriate dilution factor to obtain BCAA concentration (nmol/mL) in maternal and fetal plasma. The intra-assay CV was 1.7% (n = 4 replicates).

2.2.7 Amino acid concentrations

Determination of amino acid concentration profiles in maternal (N = 6 / group) and fetal (N = 7 per sex / group) plasma was kindly conducted by Dr Franchesca Houghton (University of Southampton). The concentration of amino acids was measured by reverse-phase HPLC (Agilent 1100 Series, Waldbronn, Germany). First, plasma samples were deproteinised; 50 µL maternal or 10 µL fetal plasma was precipitated with 50 µL or 10 µL 10% trichloroacetic acid (TCA) respectively. 2 µL TCA-precipitated supernatant was then diluted with 23 µL HPLC-grade water. For the amino acid standards, 2 µL 50 µM amino acid mixture was diluted with 23 µL HPLC-grade water. Diluted standards or samples were loaded onto the HPLC column. Pre-column derivatisation was achieved by the automated reaction of the 25 µL diluted sample with an equal volume of o-phthaldialdehyde (Sigma Aldrich, Dorset, UK) reagent containing 0.2% β-mercaptoethanol (Sigma Aldrich, Dorset, UK). Amino acids were eluted using an elution gradient. Buffer A consisted of 15 mL tetrahydrofuran (Fisher Scientific, Loughborough, UK), 800 mL sodium acetate (83 mM, pH 5.9) and 200 mL HPLC-grade methanol (Fisher Scientific, Loughborough, UK) and buffer B 800 mL HPLC-grade methanol and 200 mL sodium acetate (83 mM, pH 5.9). In this method, 18 amino acids, including alanine, arginine, asparagine, aspartate, glutamine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine, were separated and analysed. Proline and cysteine were undetected by the analytical method.

2.2.8 Placental histology

Placentas harvested at GD 21 (N = 4 litters / group) were fixed in 10% neutral buffered formalin pH 7 (100 mL 37% formaldehyde, 900 mL dH_2O, 4 g monobasic NaH_2PO_4 and 6.5 g dibasic Na_2HPO_4) for histological studies. Samples were left overnight at 4 °C to fix. The next day, the placentas were washed three times in phosphate buffered saline (PBS tablets, Oxoid, Loughborough, UK) for 15 min each and placed in 70% ethanol at 4 °C. The tissues were processed using a Microm STP 120 Spin Tissue Processor (Thermo Scientific, Loughborough, UK) and embedded in paraffin wax. Sections of 5 µm thickness were prepared using a microtome (Leica EG1150 Modular Tissue Embedding Centre, Leica Biosystems, UK). Sections were then mounted on poly-L-lysine coated slides (Fisher Scientific, Loughborough, UK) and left overnight in a drying oven at 40 °C prior to histological studies.

2.2.8.1 Haematoxylin and Eosin Staining

Placental sections were stained with Haematoxylin and Eosin (H&E) using standard procedures (H&E staining of placental sections was carried out by myself and Hager Kowash). Sections were placed in the oven at 60 °C for 2 h prior to staining to melt the wax and then at room temperature for 3 h to cool. The paraffin-embedded tissues were then de-waxed in Histo-Clear (National Diagnostics, Leicestershire, UK), three changes for 10 min and the sections were then rehydrated through a series of alcohols with gentle agitation: 100% ethanol, two changes for 3 min each, and 70% ethanol, one change for 3 min. This was followed by tap water for 5 min. The slides were then stained with Harris' Haematoxylin Solution (Sigma Aldrich, Dorset, UK) for 1.5 min. The slides were placed in running tap water for 3 min followed by three 1 s dips in acid-alcohol (70% ethanol with 1% concentrated HCl) for differentiation and washed again in running tap water for 3 min. The slides were then stained with Eosin Y alcoholic solution (Sigma Aldrich, Dorset, UK), diluted to 5% for 30 s followed by placing in tap water for 3 min. Finally, the slides were dehydrated through a series of graded alcohols: 70% and 95%, two changes for 3 min and three changes of 100% ethanol for 3 min each and cleared in Histo-Clear two changes for 5 min and one more change for 20 min before being mounted with DPX mounting medium. The slides were scanned by the Bioimaging Facility at the University of Manchester and the images were processed by Panoramic Viewer software (3DHISTECH Ltd, Budapest, Hungary). Placental zone area quantification was performed using ImageJ software analysis at magnification (1x) from 2 placental sections of 5 placentas per 3 control litters/sex and 3 IF litters/sex.

2.2.9 Tissue glycogen content

Glycogen content in the junctional zone of placentas (n = 12 placentas / N = 3 litters per dietary group) and the fetal liver (n = 10 livers / N = 5 litters per dietary group) was measured using a commercial kit (Sigma Aldrich, Dorset, UK), according to the manufacturer's instructions. Briefly, the placental junctional zone (containing glycogen cells) and fetal liver weighing ~100 mg and 260 mg were homogenized (kept on ice) in 1000 μL and 2600 μL dH$_2$O respectively. The homogenates were boiled at 100 °C for 5 min to inactivate enzymes and centrifuged at 13,000 xg for 5 min to remove insoluble material. The junctional zone and fetal liver supernatants were then diluted 1:2 and 1:40 in Hydrolysis Assay Buffer respectively. Glycogen standard (2 mg/mL) was also diluted 1:10 in dH$_2$O and a standard curve was prepared according to the manufacturer's instructions. 10 μL standards or diluted samples were added to the wells of a 96 well microplate in duplicate. 40 μL Hydrolysis Assay Buffer was then added to each well. Following this, 2 μL Hydrolysis Enzyme Mix was added to standards and samples, mixed thoroughly and incubated at room temperature for 30 min. 50 μL reaction master mix (46 μL Development Buffer + 2 μL Development Enzyme Mix + 2 μL Fluorescent Peroxidase

Substrate) was then added to each well, mixed by pipetting and incubated at room temperature for 30 min protected from light. Absorbance was then measured at 570 nm (FLUOstar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Buckinghamshire, UK). From the plotted standard curve, the interpolated glycogen content (µg) was multiplied appropriately, taking account of appropriate dilution factors, to generate tissue supernatant concentration (µg/mL) which was then multiplied by total homogenate volume of the supernatant (assuming 1 g tissue weight = 1 mL). This was then divided by tissue wet weight (mg) to give relative glycogen content (µg glycogen / mg tissue). The intra-assay CV was 2.22% (n = 3 replicates).

2.2.10 Placental metabolomics

Control and IF pregnant dams (N = 3/ group) at GD 21 were killed by cervical dislocation. Following surgical laparotomy, placentas were removed as quickly as possible, rinsed thoroughly in sterile phosphate buffer saline (PBS), blotted gently on filter paper to remove excess fluid, and were cut into quarters using a sterile blade. A placental-quarter was flash-frozen immediately in a dry shipper containing liquid nitrogen. Samples were then stored at -80 °C until shipping to the Mass Spectrometry, Phenome Centre at the University of Birmingham. At the same time, fetal tail tips were retained for determination of fetal sex. Six placental samples from each group, from fetuses of each gender (24 samples in all) were dispatched on dry ice to Dr Warwick Dunn at the University of Birmingham for metabolomic analysis.

2.2.10.1 Sample preparation

In advance of sample extraction, an extraction solvent mixture consisting of HPLC grade methanol, water and chloroform (J.T. Baker, Scientific and Chemical Supplies Ltd. UK), was prepared in a ratio of 2.5:1:1, in solvent cleaned glass-wear, and placed at -20 °C for a minimum of 12 h. The placental tissues were weighed and transferred to Precellys ceramic bead soft tissue homogenisation tubes (Bertin Technologies, Stretton Scientific UK). Sample weights ranged from 63 - 135 mg. An additional homogenisation tube, without placental tissue, was employed to generate a blank control sample. The samples were extracted in a randomised order in a single extraction batch. To each Precellys tube, extraction solvent was added at 11.2 µL per mg tissue; for the blank control sample the volume of extraction solvent employed was the average volume applied to the placental tissue samples. After addition of extraction solvent, the tubes were placed in a Precellys 24 homogeniser (Bertin Technologies, Stretton Scientific UK), prior to applying the following homogenisation cycle: 6400 rpm 15 s, 0 rpm 15 s, 6400 rpm 15 s. The tubes were then centrifuged at 2 °C and 10,000 xg for 15 min to pellet the placental tissue. Finally, 600 µL extracted supernatant was transferred to a 2 mL safe-lock microcentrifuge tube (Eppendorf, UK). Once all the samples had been extracted, equal volumes of the remaining supernatants were mixed thoroughly to generate a Quality Control (QC) sample (Dunn et al., 2011; 2012b), of which 600 µL aliquots were transferred to 2

mL safe-lock microcentrifuge tubes. Finally all samples were dried under a nitrogen gas stream prior to storage at -80 °C until the day of LC-MS analysis, performed by Dr Warwick Dunn and Dr Will Allwood.

2.2.10.2 Ultra Performance Liquid Chromatography Mass Spectrometry Analysis (UPLC-MS)

For UPLC-MS analysis, the samples were reconstituted in 60 µL HPLC grade methanol:water 1:1, vortex mixed and centrifuged for 15 min at 10,000 xg. The supernatants were transferred to analytical vials with 200 µL fixed inserts (Thermo-Fisher Ltd. UK) and capped (Thermo-Fisher Ltd. UK). The samples were stored in the autosampler at 5 °C and analysed within 72 h of reconstitution in both negative and positive electrospray ionisation (ESI) modes. Ultra High Performance (UHP)LC separations were performed essentially according to the method of Dunn et al. (2008); however the gradient was reduced to 15 min and applied to an alternative UHPLC system, the Dionex U3000 (Thermo-Fisher Ltd. U.K., UHPLC 3000 pump model DGP3600RS, column oven model FLM3100, autosampler model WPS3000TFC). The UHPLC was operated at a flow rate of 400 µL/min, the column (Hypersil Gold C18, 100 x 2.1 mm, 1.9 µm particle size; Thermo-Scientific Ltd. UK) was maintained at a temperature of 40 °C. Solvent A, HPLC grade water, and solvent B, HPLC grade methanol (J.T. Baker, Scientific and Chemical Supplies Ltd. UK) were acidified with 0.1% formic acid (Aristar grade, VWR Ltd. UK). The gradient programme was as follows: hold 100% A 0 - 1.5 min, 100% A - 100% B 1.5 - 6 min curve 3, hold 100% B 6 - 12 min, 100% B - 100% A 12 - 13 min curve 3, hold 100% A 13 - 15 min. Prior to sample analysis a new LC column was conditioned with solvents A and B for a minimum of 40 min at a flow rate of 400 µL/min. A sample injection volume of 5 µL was employed in partial-loop mode. After each sample analysis, the UHPLC system was equilibrated with the initial gradient solvent conditions prior to the analysis of the next sample. Autosampler syringe and line washes were performed with 80% HPLC grade methanol. The Thermo LTQ-FT-MS Ultra system was operated under Xcalibur software (Thermo-Fisher Ltd. UK), in full scan mode (m/z 100 - 1000) at a mass resolution of 50,000 (FWHM defined at m/z 400). A scan speed of 0.1 s and 0.4 s were applied in the LTQ and FT-MS respectively. The Automatic Gain Control was set to 1x105 and 1x106 for the LTQ and FT-MS respectively. Prior to the analytical run, the LTQ and FT-MS were tuned to optimise conditions for the detection of ions in the mid detection range of m/z 100 - 1000, as well as being calibrated with the manufacturer's recommended calibration mixture. The ESI conditions were optimised to allow efficient ionisation and ion transmission without causing insource fragmentation. The following settings were applied to ESI: Spray voltage -4.5 kV (ESI-) and +5 kV (ESI+); Sheath gas 30; Aux gas 15; Capillary voltage 35 V; Tube lens voltage -100 V (ESI-) and +90 V (ESI+); Capillary temperature 280 °C; ESI heater temperature (300 °C). The samples were analysed in a completely randomised order. Initially 10 injections of QC sample were performed for column conditioning, after which six injections of experimental samples were made and followed by a

QC injection. This was repeated until all samples were analysed, finally three QC injections were made at the end of the analytical block. The control blank sample was analysed at the start and end of the run, thus providing a measure of the sample background and also a measure of compound carry over resulting from dirtying of the ESI source.

2.2.10.3 Raw data processing

UPLC-MS raw data profiles were first converted into a NetCDF format within the Xcalibur software File Converter program. Each NetCDF based three-dimensional data matrix (intensity x m/z x time - one per sample) was converted (or deconvolved) into a vector of peak responses, where a peak response is defined as the sum of intensities over a window of specified mass and time range (e.g. m/z = 102.1 +/- 0.01 and time = 130 +/- 10 s). In this experiment the deconvolution was performed using the freely available XCMS software (Smith et al., 2006; Dunn et al., 2008). Data were exported from XCMS as a .csv file for further data analysis. The quality of data was assessed applying QC data as previously described (Dunn et al., 2011) with all metabolite features with a relative standard deviation (RSD) > 20% for QC samples being removed from the data set prior to data analysis. The data for each sample was normalised (as a percentage) to the total peak area for all metabolites in the sample. Metabolite annotation was performed applying the PUTMEDID_LCMS workflow as previously described (Brown et al., 2011). All metabolite annotations are reported at level 2 (putatively annotated compounds) according to MSI reporting standards (Sumner et al., 2007). In cases where a single metabolite is detected as multiple metabolite features (as described previously by Brown et al., 2009), only a single feature is reported chosen as having a critical p-value (= q value) nearest to 0.05.

2.2.11 Gene expression of system A transporter isoforms

To measure mRNA expression of *Slc38a1, Slc38a2* and *Slc38a4* genes encoding the SNAT1, SNAT2 and SNAT4 isoforms of system A in control and IF placentas, the following steps were performed.

2.2.11.1 RNA extraction

Placentas were harvested at GD 21 as previously described in Section 2.2.3 and were either flash frozen or placed in 2 mL RNA*later*® (Sigma Aldrich, Dorset, UK) and stored at -80 °C until RNA extraction. Frozen placentas (C, n = 2 of each sex; IF, n = 1 of each sex) were placed in 5 mL RNA*later*®-ICE Frozen Tissue Transition Solution (Thermo Fisher Scientific, Leicestershire, UK) at -20 °C overnight prior to RNA extraction. For RNA extraction, placentas (1 placenta of each sex from 8 litters) were thawed on ice and RNA isolation was performed using the Qiagen RNeasy mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. The

samples were lysed in 3 mL RLT buffer containing 30 µL β-mercaptoethanol (Sigma Aldrich, Dorset, UK) and homogenized in a glass Dounce homogenizer mortar. The lysates were aliquoted into two 1.5 mL eppendorf tubes and centrifuged at 14,000 ×g for 3 min. 700 µL supernatant was carefully transferred to three gDNA elimination spin columns and centrifuged at 10,000 ×g for 30 s. The flow-through elute was retained, to which 700 µL 70% ethanol was added and mixed by pipetting up and down. 700 µL alcohol-washed mixture was then transferred to a RNeasy spin column in a 2 mL collection tube, centrifuged at 10,000 ×g for 30 s, and the flow-through was discarded. This step was repeated with the rest of the alcohol-washed mixture. Following this, the RNAeasy spin column was washed with 700 µL RW1 buffer and centrifuged at 10,000 ×g for 30 s. 500 µL RPE buffer was added twice to the spin column and centrifuged at 10,000 ×g for 30 s for the first wash and 2 min for the second wash. The flow-through was discarded after each spin. The column was then placed in a fresh 1.5 mL sterile eppendorf tube and 50 µL RNase-free water was added to the column. The column was incubated for 1 min at room temperature, followed by centrifugation at 10,000 ×g for 1 min, and the flow-through containing the eluted RNA was retained. RNA eluates derived from the same placenta were pooled together and placed on ice for RNA concentration quantification.

2.2.11.2 RNA concentration quantification using NanoDrop spectrophotometer

RNA concentration was measured in 1 µL eluted RNA by measuring spectrophotometric absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific Inc., Wilmington, UK). RNA purity was assessed by measuring the absorbance ratio at 260:280 nm. RNA was considered to be of suitable purity for further analysis where the A_{260}/A_{280} ratio ≥ 1.8, and all samples met this requirement (Table 2.2.3). Following quantification, RNA was stored at -80 °C.

2.2.11.3 Agarose gel electrophoresis of RNA integrity

The integrity of extracted RNA was assessed using non-denaturing gel electrophoresis (1.5% (w/v) agarose gel; 100 mL containing 10 µL Gel Red Stain in 1 × TAE buffer). RNA was diluted to 100 ng/µL. 8 µL diluted RNA (800 ng) was added to 2 µL 5X DNA loading buffer (Bioline, London, UK) and separated by electrophoresis at 120 V for 55 min. A UV transilluminator (Gel Doc 2000 system, BioRad, SnapGene software, UK) was used to visualise the gel. The visualisation of two discrete 28S and 18S ribosomal RNA (rRNA) bands was taken to infer good RNA integrity (Figure 2.2.4).

Table 2.2.3 RNA concentration and purity ratios in placental samples

			Placentas Placed in RNA*later*®					Placentas Placed in Tissue Transition Solution	
			Concentration (µg/mL)	A_{260}/A_{280}				Concentration (µg/mL)	A_{260}/A_{280}
Control	N = 6	Male	867 ± 75	2.08 - 2.11	Control	N = 2	Male	363 ± 89	2.07 - 2.08
		Female	1093 ± 71	2.07 - 2.13			Female	417 ± 98	2.04 - 2.08
IF	N = 7	Male	1054 ± 97	2.07 - 2.1	IF	N = 1	Male	359	2.08
		Female	955 ± 87	2.06 - 2.1			Female	309	2.07

Concentration values are mean ± SEM, A_{260}/A_{280} ratios are value ranges
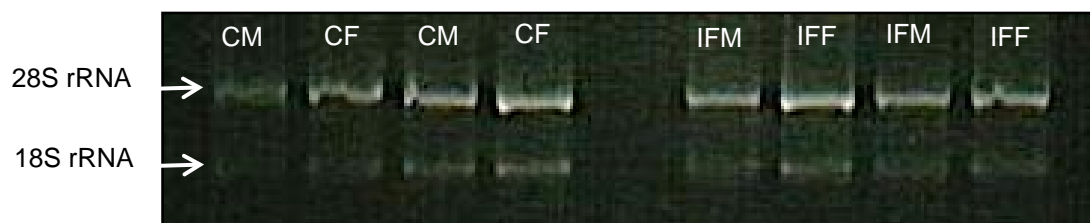
Figure 2.2.4 RNA integrity of rat placenta samples. Abbreviations: CF, control female; CM, control male; IFF, intermittent fasted female; IFM, intermittent fasted male.

2.2.11.4 Reverse transcription of RNA

Reverse transcription of RNA to produce complementary DNA (cDNA) was performed using a Quantitect Reverse Transcription Kit with random primers (Qiagen, Manchester, UK), which includes a gDNA elimination treatment, according to the manufacturer's instructions. All RNA samples were reverse transcribed concurrently, to ensure comparable reverse transcription efficiency. From each sample, 2 µg RNA was mixed with variable volumes of RNase-free (RF) water (dependent on RNA concentration) to give a total volume of 24 µL. All samples were run in duplicate to ensure adequate cDNA stock for further analysis. Two negative controls were also included; minus reverse transcriptase (-RT; RT replacement with water) and no template control (NTC; RNA replacement with water). 24 µL RNA samples and negative controls were added to a 8 well PCR strip, followed by the addition of 4 µL gDNA wipeout buffer (7x). This was then mixed thoroughly and pulsed down in a picofuge and incubated for 2 min at 42 °C in a Stratagene MX3000P quantitative PCR machine (Agilent Technologies, UK) for the gDNA digestion step. A master mix (12 µL per sample comprising 8 µL Quantiscript reverse transcription buffer, 2 µL reverse transcription primer mix and 2 µL Quantiscript reverse transcriptase) was added to all samples except for -RT (where water replaced RT) and was incubated at 42 °C for 15 min followed by 95 °C for 3 min. The generated cDNA and negative controls were stored at -20 °C until qPCR was performed.

2.2.11.5 Quantitative Real-Time PCR (qPCR)

To measure the mRNA expression of *Slc38a1, Slc38a2* and *Slc38a4* genes, real time qPCR assays were performed using gene-specific primers (Quantitect Primer Assays, Qiagen; Table 2.2.4) with QuantiFast SYBR Green PCR Master Mix (Qiagen) according to the manufacturer's recommendations. All primers were exon-exon spanning (Table 2.2.4), ensuring amplification only from RNA-generated cDNA.

Table 2.2.4 Primers for real-time qPCR

Gene Name	RGD ID*	Quantitect Primer Assay	Genbank Accession	Amplified Exons	Product Size (bp)
Slc38a1	69645	QT00187586	NM_138832	4/5	97
Slc38a2	69420	QT00186116	NM_181090	12/13	86
Slc38a4	621836	QT00187943	NM_130748	4/5	89
Ywhaz	3980	QT02382184	NM_013011	2/3	99

*RGD ID gives Rat Genome Database reference number

A standard curve was created from pooled placental cDNA (10 µL), prepared as shown in Figure 2.2.5. The standards were then further diluted 1:10 by adding 90 µL molecular grade water (Sigma Aldrich) creating a two-fold serial dilution from 50 ng (Standard 1) to 0.391 ng (Standard 8). Placental cDNA and negative controls were diluted 1:100 for all genes of interest, as at this cDNA dilution, all samples fell within the range of cycle threshold (Ct) values generated by the standard curve. 10 µL standards, diluted samples and negative controls were run in duplicate in 8 well strips. Duplicates of PCR blanks, in which cDNA was replaced with 10 µL molecular grade water, were also included. 15 µL qPCR master mix (12.5 µL Quantifast SYBR Green PCR Master Mix + 2.5 µL QuantiTect Primer Assay) was then added to all standards, diluted samples and negative controls and mixed thoroughly. qPCR conditions were as follows:

Thermal Profile	
Segment 1	95 °C for 1 min
Segment 2	95 °C for 10 s
35 cycles	60 °C for 30 s
Dissociation Curve	
	95 °C for 1 min
Segment 3	55 °C for 30 s ramping to 95 °C at 0.2 °C increments per second
	95 °C for 30 s
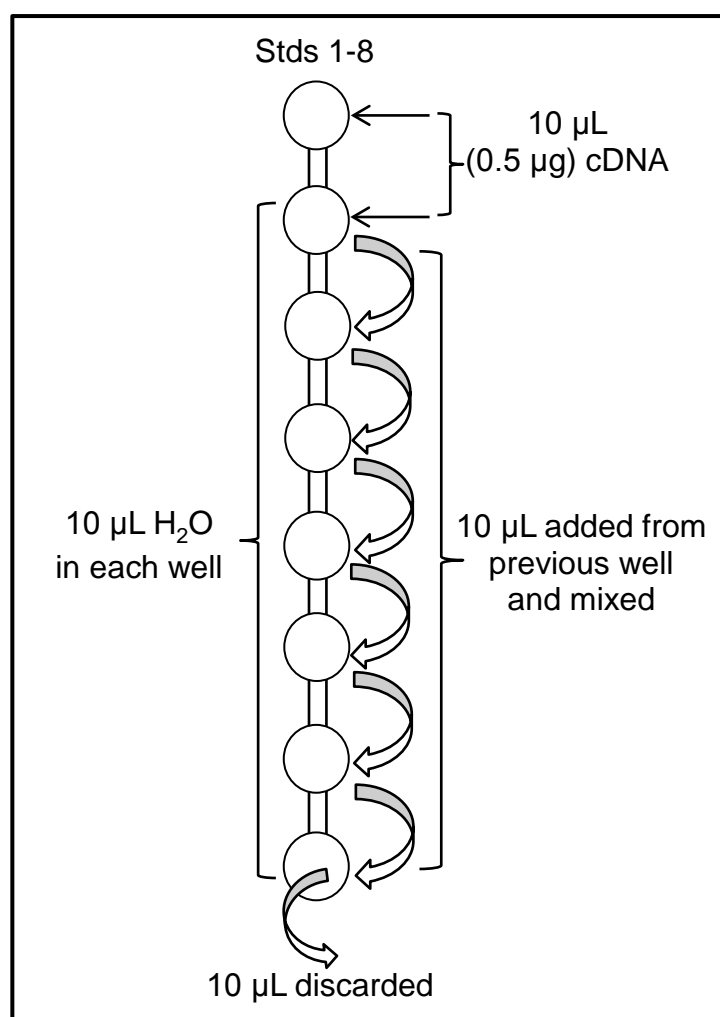
Figure 2.2.5 Schematic diagram of qPCR standard curve preparation. Stds, standards.

Sample mRNA expression was interpolated from the linear standard curve constructed by software within the qPCR MX3005P machine where the input cDNA (ng) was plotted against Ct value. The relative expression of each gene expression was expressed normalised to *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) as a reference gene; *Ywhaz* mRNA expression was found to be stable across diets and fetal sex (Figure 2.3.22).

2.2.11.6 Gel electrophoresis of qPCR products

Agarose electrophoresis was performed to visualize PCR products of each gene of interest to determine whether PCR was successful and the resulting amplification products were of the predicted size (Table 2.2.4). 18 µL qPCR product was mixed with 2 µL XC loading dye and separated by agarose gel electrophoresis (2.75% (w/v) agarose gel, 100 mL in 1 x TAE buffer containing 10 µL Gel Red stain). A 25 bp ladder (Bioline, London, UK) was run in parallel to

allow amplicon size determination. Electrophoresis was performed at 120 V for 55 min. For the visualization of PCR product, the gel was exposed to a UV transilluminator (Gel Doc 2000 system, BioRad, SnapGene software, UK) as shown in Figure 2.2.6.
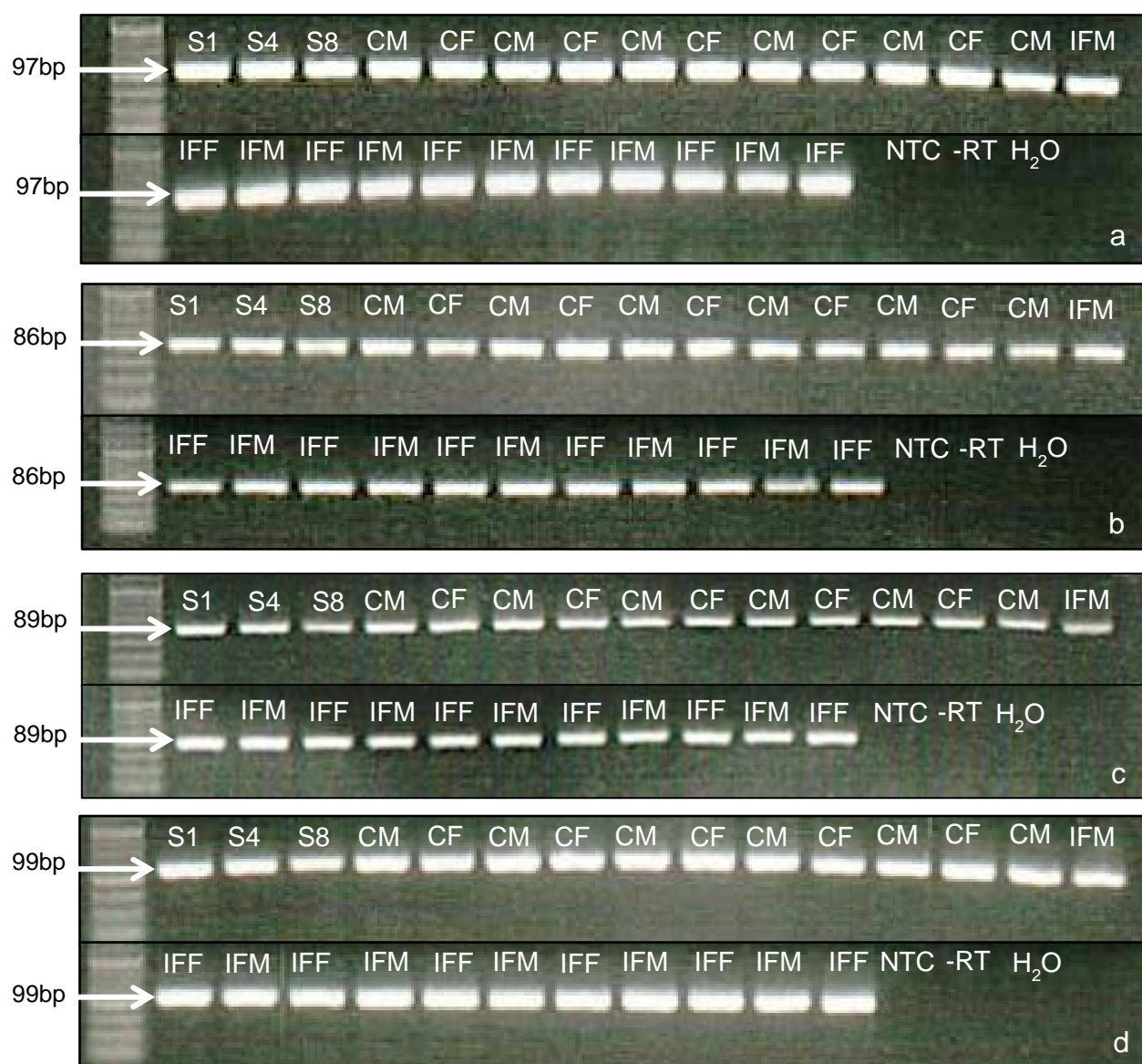
Figure 2.2.6 Agarose gel electrophoresis of qPCR products for **a.** *Slc38a1*, **b.** *Slc38a2*, **c.** *Slc38a4* and **d.** *Ywhaz*. Amplicons of the genes of interest were of predicted size with no amplification products seen in the negative controls (NTC, -RT and H$_2$O). Abbreviations: S1, S4 and S8, Standards; CM, control male; CF, control female; IFM, intermittent fasted male; IFF, intermittent fasted female.

2.2.12 Western blot analysis

Western blotting was performed to detect protein expression of SNAT1 and SNAT2 in rat placental membrane vesicles. Previous investigations in this laboratory had failed to find a reliable commercial antibody to detect SNAT proteins, so donated antibodies were employed. Rabbit affinity-purified anti-SNAT1 (1.5 mg/mL) and anti-SNAT2 (1.58 mg/mL) antibodies were kindly provided by Dr Jeffrey D. Erickson, Louisiana State University, along with blocking peptides to confirm antibody specificity. Several prior optimization steps were performed to determine optimal antibody binding conditions, as detailed in the Appendix (Chapter 7).

2.2.12.1 Sample preparation

Rat placental membrane vesicles were isolated from the placentas of control and IF groups at GD 21, as described below (Section 2.2.13.2.1). Protein concentration was determined by the Lowry assay (Section 2.2.13.2.2).

Each sample was diluted in dH_2O to a concentration of 1 mg protein/mL. For SNAT1, samples were added to the boil reducing buffer (Table 2.2.5) in a 1:1 ratio, heated at 60 °C for 5 min and then chilled on ice for 5 min. For SNAT2, samples were added to a non-boil reducing buffer (Table 2.2.5) in a 2:1 ratio. 20 µL (20 µg protein) was loaded per lane.

2.2.12.2 Gel preparation and electrophoresis

2.2.12.2.1 Gel preparation

4 - 20% Mini-PROTEAN ® TGX™ precast protein gels (Bio-Rad, UK) were used for Li-COR detection. Alternatively, for some preliminary optimizations described in the Appendix, 10% polyacrylamide resolving gels were prepared between two glass plates (mini-PROTEAN® 3 cell, Bio-Rad Laboratories Ltd, Watford, UK). In this instance, gel mixture (Table 2.2.5) was poured to a level of 1 cm below the top of the front plate and allowed to set for 30 min. 100% ethanol was placed on top of the gel to prevent gel dehydration and to ensure a uniform gel level. After that, the ethanol was removed, and 3% stacking gel (Table 2.2.5) was poured to the top of the glass plate. A 10-well comb was then inserted, and the gel was left to polymerise for 15 min.

2.2.12.2.2 Electrophoresis

Both gel types were transferred to an electrophoresis unit (Bio-Rad Laboratories Ltd). The comb was removed, and the wells were washed with 1x electrode buffer (Table 2.2.5). Molecular weight markers (5 µL; Precision Plus Protein ™ All Blue Prestained Protein, Bio-Rad) and samples (20 µg protein/20 µL) were loaded into the wells (except where other protein

loadings were trialled as indicated in Figure legend). The unit was placed in an electrophoresis tank, which was filled with 1X electrode buffer. Electrophoresis was performed at 200 V for ~30 min until the tracker dye had reached the bottom of the gel. The gel was removed carefully from the plates, the bottom left-hand corner was cut for gel orientation, and the stacking gel was carefully excised where present. The gel was washed 3 x 5 min with transfer buffer (Table 2.2.5).

2.2.12.3 Electro-blotting and transfer of protein to membrane.

Following electrophoresis, proteins were blotted from the gel onto a membrane. The gel was sandwiched between Immobilon®-FL PVDF membrane (Merck Millipore, pre-wet with methanol and rinsed in dH_2O) for Li-COR detection or HyBond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) for optimizations performed by ECL detection (Pierce ECL, ThermoFisher Scientific, UK; see Appendix). Membranes were pre-equilibrated with transfer buffer (Table 2.2.5). A layer of filter paper pads (Sigma) and sponges, which had been pre-soaked in transfer buffer, were assembled in a cassette as shown in Figure 2.2.7.

The sandwich cassettes were placed into a transfer unit with an ice block to maximize heat dissipation during the blotting process. The transfer unit was then filled with transfer buffer and transferred at 100 V for 30 min (PVDF membranes) or 120 V for 70 min (nitrocellulose membranes). The membrane was removed and the migration of the molecular weight markers was recorded. Blots were stored in PBS or TBS overnight at 4 °C prior to probing or taken immediately for probing.
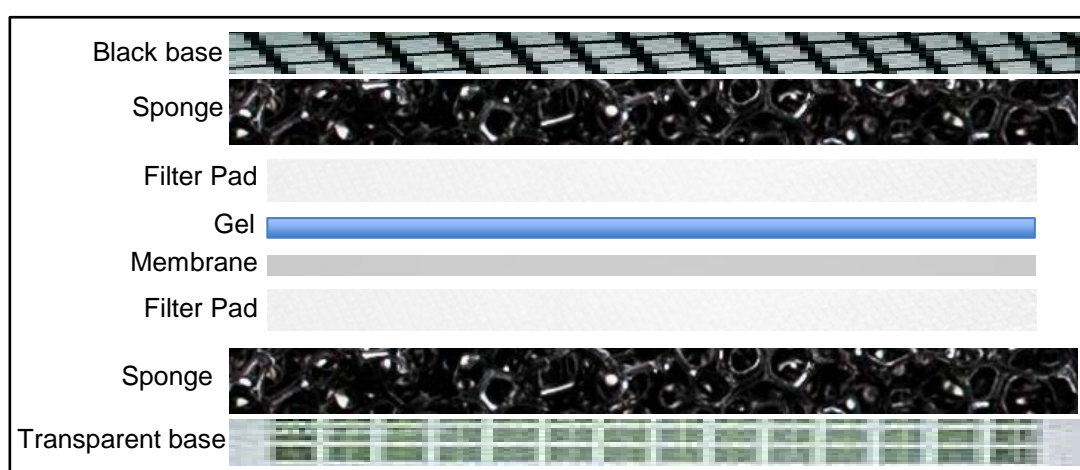
Figure 2.2.7 Schematic diagram of Western blot sandwich cassette.

2.2.12.4 Probing the membrane with SNAT antibodies

The PVDF membrane was blocked with 15 mL Odyssey Blocking Buffer for 1 h. The membranes were then incubated with 5 mL SNAT1 (1:1000) or SNAT2 (1:1000) antibodies diluted in Odyssey Blocking Buffer with 0.2% Tween®20 overnight at 4 °C with gentle agitation. Following a further 2 h antibody incubation at room temperature, the membranes were washed with PBS (Table 2.2.5) with 0.1% Tween (PBS-T) for 3 x 5 min. The membranes were then incubated for 1 h, protected from light, with anti-rabbit secondary antibody (IRDye® 800CW, green, Li-COR Biotechnology, UK) diluted 1:20,000 in Odyssey Blocking Buffer with 0.2% Tween and 0.01% SDS. The membranes were then washed with 0.1% PBS-T for 3 x 5 min and then rinsed with 1x PBS for 5 min. All the membranes were probed with β-actin antibody (1:2000; Sigma Aldrich, UK) and anti-mouse IRDye® 680LT secondary antibody (red, 1:40,000; Li-COR Biotechnology, UK) to confirm equal protein loading. Negative control blots were also included, where primary antibody was omitted.

2.2.12.5 Data analysis

For Li-COR detection, antibody-protein complexes were visualised using Li-COR's Odyssey imaging machine and quantification conducted with ImageStudio Lite software (Li-COR Biotechnology, UK). To interpolate the size of the immunoreactive species, a non-linear plot of log marker size (kDa) against the distance migrated (cm) was used (Prism® software, Graphpad, UK, $r^2 = 0.98$). For ECL detection in optimization studies, the distance from the top of the film to the visualised immunoreactive species was recorded and a curvilinear plot of marker size (kDa) against distance migrated (cm) used to interpolate size of immunoreactive complexes.

Table 2.2.5 Western blotting reagents

Reagent	Composition
Laemmli reducing boil sample buffer (2x)	0.125 M Tris-HCl, pH 6.8, 4% SDS, 0.004% Bromophenol Blue, 20% glycerol, 10% β-mercaptoethanol (added fresh on day of use)
Non-boil reducing sample buffer	8 M urea, 5% SDS, 455 mM DTT, 0.4% bromophenol blue in 50 mM Tris-HCl, pH 6.9
Solution A	Ultrapure Protogel (National Diagnostics)
Resolving gel buffer (Solution B)	22.7% Tris, 0.5% SDS, pH 8.8 with concentrated HCl
Stacking gel buffer (Solution C)	6.1% Tris, 0.4%SDS, pH 6.8 with concentrated HCl
10% resolving gel (2 gels)	30 mg ammonium persulphate, 6.6 mL solution A, 4 mL solution B, 9.4 mL dH$_2$O, 20 µL TEMED (added last to induce gel polymerisation)
3% stacking gel (2 gels)	20 mg ammonium persulphate, 2 mL solution A, 5 mL solution C, 13 mL dH$_2$O, 30 µL TEMED (added last to induce gel polymerisation)
Phosphate buffered saline (BPS, BR0014, Dulbecco A Tablets)	10 tablets in 1 L dH$_2$O
Tris-buffered saline (TBS)	150 mM NaCl, 10 mM Tris-HCl, pH 8 with concentrated HCl
Electrode buffer (5x)	250 mM Tris, 34.7 mM SDS, 1.92 M glycine
Transfer buffer	25 mM Tris, 192 mM glycine, 20% methanol

2.2.13 Placental system A amino acid transporter activity

2.2.13.1 *In vivo*: unidirectional maternofetal clearance of ^{14}C-MeAIB

To measure placental system A amino acid transporter activity *in vivo*, an adaptation of the method of Flexner and Pohl (1941) was used to measure maternofetal clearance of ^{14}C-α-methylaminoisobutyric acid (^{14}C-MeAIB; $^{MeAIB}K_{mf}$), a non-metabolisable substrate for system A, as described previously (Kusinski et al., 2012). System A amino acid transporter activity was selected for study based on its crucial importance for fetal growth (Glazier et al., 1997; Jansson et al., 2006). Pregnant rats at GD 21 (C, N = 6; IF, N = 5) were anaesthetised with isoflurane by inhalation (4% in oxygen at 2 L/min), and then with an intraperitoneal injection of sodium thiobutabarbital (Inactin® hydrate T133-1G, Sigma Aldrich, Dorset, UK) at a dose of 100 mg/kg bodyweight. The anaesthetised rat was then placed on a heated surgical table to maintain body

temperature at 37 °C. After a small incision in the neck, cannulae (polythene tubing ID 0.58 mm, OD 0.96 mm; length of 6 cm, Portex Ltd, Nottingham, UK) were placed into the maternal external jugular vein for the administration of radioactive tracer and the carotid artery was also cannulated to allow maternal blood sampling (Figure 2.2.8). A bolus of ~220 µL 0.9% saline (0.154 M NaCl) containing 0.37 MBq (10 µCi) ^{14}C-MeAIB (PerkinElmer, London, UK) was injected at time zero into the maternal jugular vein. This was equivalent to an injection dose of ~0.814 - 1.073 MBq/kg maternal weight (22 - 29 µCi/kg maternal weight). The isotope syringe was weighed before (with isotope) and after injection to determine the exact volume of isotope injected into each rat. This was followed by measurement of isotope disappearance in maternal blood through sequential sampling of maternal blood. Approximately 100 µL blood was taken from the carotid artery into non-heparinised 1.5 mL eppendorf tubes. Samples were taken every 20 s over 2 min and then at every min until 5 min after injection. Blood samples were centrifuged immediately at 14000 xg for 1 min and the plasma recovered for radioactivity analysis. At 5 min, the dam was sacrificed by cervical dislocation; fetuses and their corresponding placentas were removed, trimmed free of cord and fetal membranes, and carefully blotted and weighed. Fetal tail tips were removed for fetal sex determination. Individual placentas and fetuses were placed in vials to measure accumulation of ^{14}C-MeAIB radioactivity.

2.2.13.1.1 Sample preparation and isotope counting

For radioactivity analysis, 5 µL each of maternal plasma and isotope stock were taken and 1.865 mL dH$_2$O was added, followed by the addition of 16 mL liquid scintillation fluid (Optiphase Hisafe II, Fisher Scientific Ltd, Leicestershire, UK). Fetuses and placentas were minced with scissors, and 10 mL and 3 mL 3% KOH added respectively to solubilise tissues. The tissues were solubilised overnight at 55 °C. 1 mL solubilised tissue lysate was taken and 0.865 mL 0.535 M HCl was added for pH neutralisation and reduction of inherent chemiluminescence. The vials were then counted in a liquid scintillation counter (Packard 2000CA) using standard windows for ^{14}C with water blanks (for correction of background count), until all counts had stabilised from chemiluminescence. All sample counts were adjusted for background counts. Maternal plasma counts were divided by 5 and expressed as dpm/µL plasma. Placental and fetal counts (dpm) were multiplied by 3 and 10 to give total radioactivity in each placenta and fetus respectively.
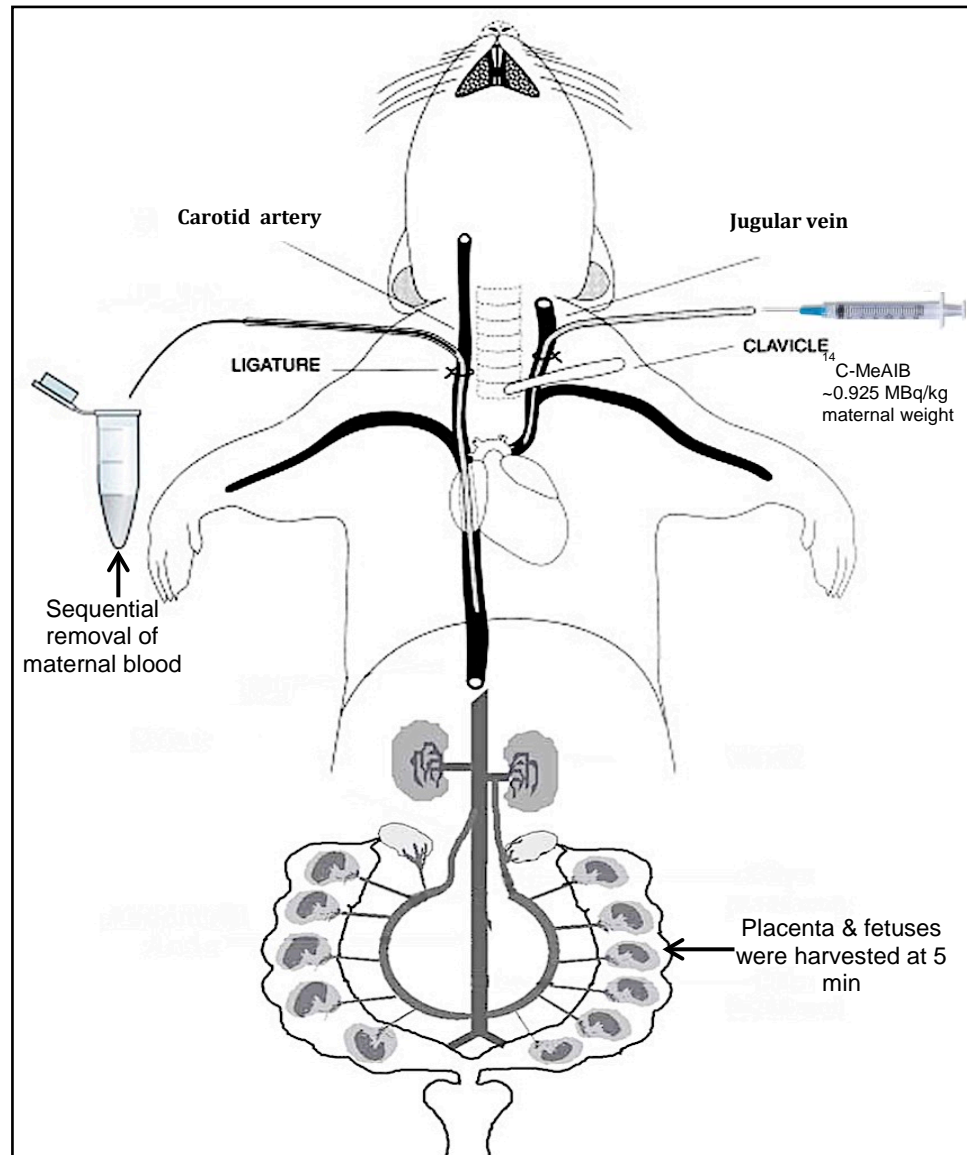
Figure 2.2.8 Schematic illustrating the procedure for *in vivo* measurement of unidirectional maternofetal clearance across the placenta. Modified from Granger et al. (2006).

2.2.13.1.2 Maternal plasma ^{14}C-MeAIB disappearance curve

A maternal plasma ^{14}C-MeAIB disappearance curve was constructed from dams of both groups (6 C and 5 IF), and fitted to a one-phase exponential decay model (Prism® software, Graphpad, UK). As ^{14}C-MeAIB disappearance curves were similar between control and IF dams, the data were pooled to generate a single maternal plasma ^{14}C-MeAIB disappearance curve. Each time point in the curve corresponded to a sequential blood sample taken from the carotid artery over 5 min following the bolus injection of ^{14}C-MeAIB.

2.2.13.1.3 Calculation of unidirectional maternofetal clearance

Unidirectional maternofetal clearance of ^{14}C-MeAIB, $^{\text{MeAIB}}K_{\text{mf}}$ (µL/min/g placenta), across the intact placenta was calculated as:

$$^{\text{MeAIB}}K_{\text{mf}} = \frac{Nx}{W \int_0^x Cm\,(t)dt}$$

Where, Nx is the total radiolabel accumulation (dpm) by the fetus at x min after injection of radiolabel into the maternal vein, W is the placental wet weight (g) and $\int_0^x Cm\,(t)dt$ is the time integral of radioisotope concentration in maternal plasma (dpm·min/µL) from 0 to x min (taken from the area under the curve of the maternal plasma ^{14}C-MeAIB disappearance), where $x = 5$.

2.2.13.2 *In vitro*: system A activity in isolated rat placental vesicles

2.2.13.2.1 Isolation of rat placental membrane vesicles

The method used for the isolation of the maternal-facing plasma membrane of SynTB layer II is based on a single Mg^{2+}-precipitation step and differential centrifugation, as described recently for mouse placenta (Kusinski et al., 2010) and modified from that described previously for rat placenta (Glazier et al., 1990). Placentas were harvested from each litter (C, N = 13, male n = 92 and female n = 111; IF, N = 15, male n = 111 and female n = 104) at GD 21 and stored individually in 3 mL Belzer's tissue preserving medium (of the following composition: 50 g/L pentafraction, 35.83 g/L lactobionic acid, 3.4 g/L potassium phosphate monobasic, 1.23 g/L magnesium sulfate heptahydrate, 17.83 g/L raffinose pentahydrate, 1.24 g/L adenosine, 0.136 g/L allopurinol, 0.922 g/L glutathione, 5.61 g/L potassium hydroxide and water with pH 7.4; Bridge to Life Ltd, London, UK). The placentas were stored in this medium at 4 °C to maintain tissue preservation whilst determination of fetal sex was conducted. Male and female placentas were then pooled from an individual litter (ranging from 3 to 12 placentas for each fetal sex) and placed on ice. The placentas were weighed and homogenised in 4 vol (w/v) ice-cold mannitol buffer (300 mM mannitol, 10 mM Hepes-Tris, 1 mM MgSO$_4$, pH 7.4) using a polytron homogeniser. A sample of the homogenate (10 µL) was retained for subsequent protein assay and alkaline phosphatase activity analysis and 12 mM MgCl$_2$ was added to the remaining homogenate and stirred on ice for 10 min. The Mg^{2+} treatment served to aggregate plasma membranes other than the maternal-facing plasma membrane of SynTB layer II (Glazier et al., 1990). The homogenate was then centrifuged at a low-speed of 2300 xg for 15 min to remove the Mg^{2+}-aggregated contaminating membranes. The pellet was discarded and the resultant supernatant was re-centrifuged at 23,500 xg for 40 min to pellet down the plasma membranes. All centrifugation steps were conducted at 4 °C. After discarding the supernatant, the pellet was drained and weighed and re-suspended in 2.8 vol intravesicular buffer (290 mM sucrose, 5 mM

Tris, 5 mM Hepes, pH 7.4) by sucking up and down gently through a pipette tip. Then the vesicle suspension was passed 15 times through a 25-gauge syringe needle to vesiculate the membranes whilst on ice. A sample (5 μL) was retained for protein assay and enzyme marker (alkaline phosphatase activity) analysis. The vesicles were snap-frozen in liquid nitrogen and stored at -80 °C until use (the activity of system A amino acid transporter was found to be unaffected by freezing in preliminary studies; also shown in previous studies by Malandro et al., 1996).

2.2.13.2.2 Protein assay

The protein contentration of homogenate and vesicle fractions was determined using the Lowry protein assay (Lowry et al., 1951). Standards of 0, 10, 20, 50, 100, 150 and 200 μg bovine serum albumin (BSA, Sigma A2153, Dorset, UK) were prepared from a 2 mg/mL BSA stock. Homogenate and vesicles were diluted 1:20 and 1:50 respectively. 100 μL standard or samples was added to the wells of a 96 well microplate in triplicate and were solubilized following addition of 100 μL 2 M NaOH for 15 min at room temperature. Then, 20 μL solubilised standard or sample was added to separate wells of another microplate followed by the addition of 20 μL dH_2O. 200 μL reagent C (50 mL 2% Na_2CO_3 + 500 μL 1% $CuSO_4$ + 500 μL 2% Na/K tartrate) was added to each well in a timed manner and mixed thoroughly by pipetting up and down, with 40 s intervals between lanes. After 10 min incubation, 20 μL Folin reagent (2 N Folin Ciocalteau reagent, diluted 1:1 with dH_2O, Sigma UK) was added to each well with 40 s intervals between lanes and mixed thoroughly. The mixture was allowed to stand at room temperature for 30 min for blue colour development. Absorbance was measured at 750 nm (Versamax microplate reader, Berkshire, UK). A standard curve of protein (μg) versus absorbance at 750 nm was plotted to a 2nd order polynomial curve (Prism® software, Graphpad, UK) and used to interpolate sample protein content. Interpolated values were multiplied by 2000 and 5000 to give protein concentration (mg/mL) in homogenate suspension and vesicle respectively.

2.2.13.2.3 Alkaline phosphatase activity

Alkaline phosphatase is distributed to the maternal-facing plasma membrane of SynTB layer II in both rats (Glazier et al., 1990) and mice (Kusinski et al., 2010) and can be used as a marker for this plasma membrane to determine vesicle purity. Alkaline phosphatase was measured by the method of McComb and Bowers (McComb and Bowers, 1972). 5 μL vesicles (1:100 dilution) and homogenate (1:20 dilution) were added to the wells of a 96 well microplate in triplicate, followed by the addition of 250 μL diethanolamine (DEA; Sigma, Dorset UK) buffer (1M DEA + 0.5 mM $MgCl_2$; pH 9.8 with HCl) to each well. Then 25 μL Comix (3 tablets p-nitrophenylphosphate (Sigma, Dorset, UK) in 4 mL DEA buffer) was added as substrate to initiate the enzymatic reaction. Absorbance at 410 nm (A410) was measured at time zero and

then at 2 min. The difference between the two values (t = 2 min and t = 0 s) provided the activity of alkaline phosphatase over 2 min (ΔA410/2 min). The ratio of alkaline phosphatase activity, normalised to protein content, in vesicles to that of placental homogenate (ΔA410/2 min/µg protein) was then used to calculate the alkaline phosphatase enrichment factor in the rat placental vesicles relative to the initial homogenate.

2.2.13.2.4 System A activity in rat placental vesicles

To measure system A activity in rat placental vesicles, ^{14}C-MeAIB was used as a non-metabolisable substrate specific for the system A amino acid transporter. Since system A is a Na$^+$-dependent transporter, ^{14}C-MeAIB uptake into the vesicles was measured in the presence of an inwardly directed Na$^+$ gradient (Na$^+$ contained in the extravesicular buffer) and in the absence of Na$^+$ (by K$^+$ replacement) at room temperature (21 - 25 °C). ^{14}C-MeAIB uptake was initiated by the addition of 20 µL vesicle suspension (~8 - 10 mg/mL protein) to 20 µL extravesicular buffer (5 mM Tris, 5 mM HEPES, 145 mM NaCl or KCl, with 0.33 mM ^{14}C-MeAIB, specific activity 2197.8 MBq/mmol (59.4 mCi/mmol)). Vesicles were also incubated with 0.2% Triton for 60 s to disrupt vesicle integrity, to confirm uptake of tracer was into an intravesicular space. At time intervals over 1 min (15, 30, 45 and 60 s), uptake was stopped by the addition of 2 mL ice-cold Krebs Ringer Phosphate buffer (KRP; 130 mM NaCl, 10 mM Na$_2$HPO$_4$, 4.2 mM KCl, 1.2 mM MgSO$_4$ and 0.75 mM CaCl$_2$, pH 7.4). An aliquot (2 mL) of the resultant solution was applied to a pre-soaked 0.45 µm HAWP filter (Millipore, Hertfordshire, UK) under vacuum and washed with 10 mL KRP. The filters were then dissolved in 2 mL 2-ethoxyethanol, and 12 mL scintillation fluid (Optiphase Hisafe II, Fisher Scientific Ltd, Leicestershire, UK) was added.

Samples were counted in a liquid scintillation counter (Packard 2000CA) on standard windows for ^{14}C. Counts (dpm) were corrected for radiolabel retained by the filters in the absence of vesicle protein (no-protein controls) whereby 20 µL intravesicular buffer replaced vesicle suspension. Standards (5 µL in triplicate) were counted in order to determine counts relative to substrate content. Blanks (2 mL dH$_2$O) were also mixed with 12 mL scintillant (Optiphase Hisafe II, Fisher Scientific Ltd, Leicestershire, UK) and counted in order to measure background radioactivity. All sample counts (dpm) were adjusted for background and no-protein control counts. The counts (dpm) from the 5 µL standards were averaged and ^{14}C-MeAIB content (pmol) in 5 µL standard was divided by mean standard dpm (pmol/dpm) and multiplied by the corrected sample dpm, divided by the protein applied to the filter (µg) and multiplied by 1000 to calculate ^{14}C-MeAIB uptake (pmol/mg protein). Na$^+$-dependent uptake of ^{14}C-MeAIB was calculated by subtracting uptake in the absence of Na$^+$ from that in the presence of Na$^+$.

2.2.14 Statistical analysis

Data were analysed using the IBM SPSS statistical package (version 22, New York, US) and GraphPad Prism® software (La Jolla, USA). After applying a Shapiro-Wilks normality distribution test, the data are presented as mean ± SEM and parametric analyses were used. Where data did not conform to a normal distribution, data are expressed as box and whisker plots unless stated otherwise and non-parametric analyses were used. The boxes mark the interval between the 25th and 75th percentile, the line inside the box represent the median, and the whiskers denote the interval between the 5th and 95th percentile. For all analyses, N = number of litters and n = number of individual fetuses. $P < 0.05$ was considered statistically significant.

2.2.14.1 Maternal daily measurement and organ weights

Maternal daily food and water intake and weight gain were assessed by repeated-measures two-way analysis of variance (ANOVA) with Tukey's post hoc test. Maternal organ weights were analysed using an unpaired t-test.

2.2.14.2 Fetal and placental weights, fetal:placental ratio, anthropometric measurements and fetal organ weights

Data were analysed by two-way ANOVA with Tukey's post hoc test, the litter sizes were taken into account as a co-variant in all the analyses. For placental weights, $\log_{10}$ transformation was performed prior to data analysis. Fetal and placental weight frequency distribution curves were constructed using a non-linear regression (Gaussian distribution). The 5th percentile weight was calculated as: [(-Z score x SD) + mean] where Z score = 1.645 and SD = standard deviation (Dilworth et al., 2011). Litter size was analysed by Fisher's exact test. Fetal organ weights were expressed as absolute weights or relative to fetal weight. For fetal anthropometric measurements, Kruskal-Wallis test followed by Dunn's multiple comparisons test were used

2.2.14.3 Maternal and fetal metabolic analysis and amino acid measurements

Maternal and fetal metabolic analyses were compared using a unpaired t-test. Maternal amino acid concentrations were analysed using an unpaired t-test with some data requiring log transformation, except for Glu, Gln, Gly, Thr, Tyr, Val, Phe and Ile for which Mann-Whitney test was used. Fetal amino acid concentrations were assessed by two-way ANOVA with Tukey's post hoc test, except for Asn, Ser, Gln, Phe and Lys for which a Kruskal-Wallis test with Dunn's post hoc test was used. For fetal/maternal ratio for plasma amino acid concentrations, unpaired t-test were used except for Met, where a Mann-Whitney test was used.

2.2.14.4 Tissue glycogen content

Placental glycogen content was analysed using Kruskal-Wallis test followed by Dunn's multiple comparisons test whereas for fetal hepatic glycogen content two-way ANOVA followed by Tukey's post hoc test.

2.2.14.5 Placental area analysis

The percentage ratio of the junctional area to total placental area and the percentage ratio of the labyrinth area were analysed using two-way ANOVA followed by Tukey's post hoc test.

2.2.14.6 Placental metabolomics

The processed data were analysed in R applying the unsupervised multivariate principal components analysis (PCA) and a univariate one-way ANOVA test was applied with correction for multiple tests applied (Benjami-Hochberg test). Pathway analysis was performed in MetaboAnalyst v3.

2.2.14.7 Measurement of system A amino acid transporter activity

A linear regression and repeated-measures two-way ANOVA followed by Tukey's post hoc test was performed on the data for ^{14}C-MeAIB vesicle uptake in the presence and absence of Na$^+$ and Na$^+$-dependent amino acid uptake over time. Data from unidirectional maternofetal clearance across rat placenta were analysed by two-way ANOVA with Tukey's post hoc test.

2.2.14.8 Gene expression in rat placenta and quantification of protein in rat placental vesicles

The qPCR data for each gene of interest was normalized to the housekeeping gene (*Ywhaz*) and the normalised data were analysed by using two-way ANOVA with Tukey's post hoc test. Protein bands were visualised with ImageStudio Lite software (Li-COR Biotechnology, UK). Quantification was performed after subtracting the background from a comparable area. All proteins were normalised to β-actin and the normalised data were analysed by by using two-way ANOVA with Tukey's post hoc test.

2.3 Results

2.3.1 Maternal food intake

The food intake of both IF and control dams was monitored on a daily basis; while the control dams had free access to food, the IF group had access restricted to 8 h per day throughout gestation. In both dietary groups, the consumption of food by pregnant dams increased considerably as pregnancy progressed ($P < 0.0001$), rising daily in both groups (Figure 2.3.1 a). The total food consumption by control and IF dams over the course of pregnancy was around 500 ± 5 g and 350 ± 5 g respectively. IF pregnant dams consumed $30 \pm 1\%$ less food at all stages of pregnancy compared to the control group (Figure 2.3.1 a). When maternal food intake was normalised per 100 g dam bodyweight, the data showed that IF dams still ate less food than controls throughout pregnancy (Figure 2.3.1 b).

2.3.2 Maternal water intake

Both IF and control dams had unrestricted access to water throughout pregnancy. The water intake gradually increased in both groups as pregnancy advanced ($P < 0.0001$, Figure 2.3.2 a). The water intake was initially very similar: both groups consumed 36 ± 2 mL of water per day. From GD 4 onwards, water intake was found to be $11 \pm 1\%$ lower in IF dams compared to control ($P < 0.001$, Figure 2.3.2 a). This reflects the effect of a reduction in food intake. The difference was normalised at GD 20. However, when maternal water intake was adjusted relative to 100 g bodyweight there was no difference in water intake between the two dietary groups (Figure 2.3.2 b).

2.3.3 Maternal weight gain

Pregnant dams in both groups started with equivalent bodyweights (~250 g) and gained weight steadily throughout pregnancy ($P < 0.0001$, Figure 2.3.3). The increase in bodyweight was greatest during the final part of gestation (GD 18 - 21) when compared with early- and mid-gestation. Maternal total weight gain during 20 days of gestation relative to weight before pregnancy was 134 ± 3 g and 95 ± 3 g for control and IF dams respectively. Therefore, the lower food intake by the pregnant IF dams had a negative impact on maternal weight gain, which was lowered from GD 8 onwards compared to control dams ($P < 0.0001$, Figure 2.3.3). At GD 21, rats were weighed just prior to sacrifice. There was a mean weight difference of 19 ± 1 g in IF rats compared to control rats, which could be ascribed to the overnight food restriction prior to sacrifice, as this weight difference corresponded to the amount of food (~20 g) that the IF dams would have normally consumed following food replacement after the overnight fast.
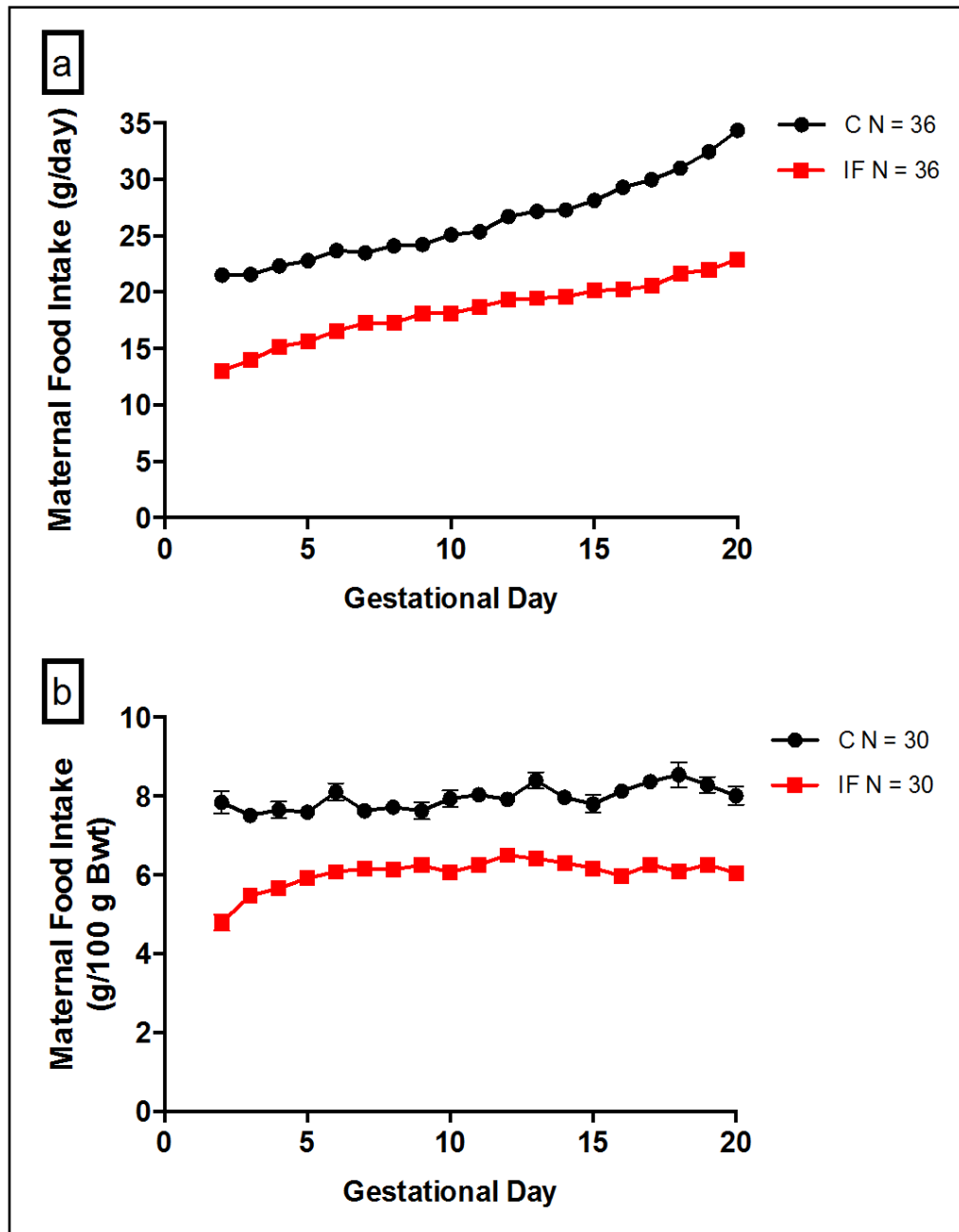
Figure 2.3.1 Daily maternal food intake of pregnant dams throughout gestation. **a.** Daily food consumption of pregnant dams. **b.** Daily maternal food intake normalised to the pregnant dam's bodyweight (Bwt). Control dams (●) had free access to food throughout pregnancy. IF dams (■) had food restricted for 16 h per day from 5:00 pm to 9:00 am daily throughout pregnancy. No data are shown for day 1, as this did not reflect a complete 24 h period. Daily maternal food intake and maternal food intake normalised to dam bodyweight was significantly lower in IF dams compared to controls throughout pregnancy. Data are presented as mean ± SEM. $P_{Day} <$ 0.0001, $P_{Diet} < 0.0001$ (repeated-measures two-way ANOVA followed by Tukey's post hoc test).
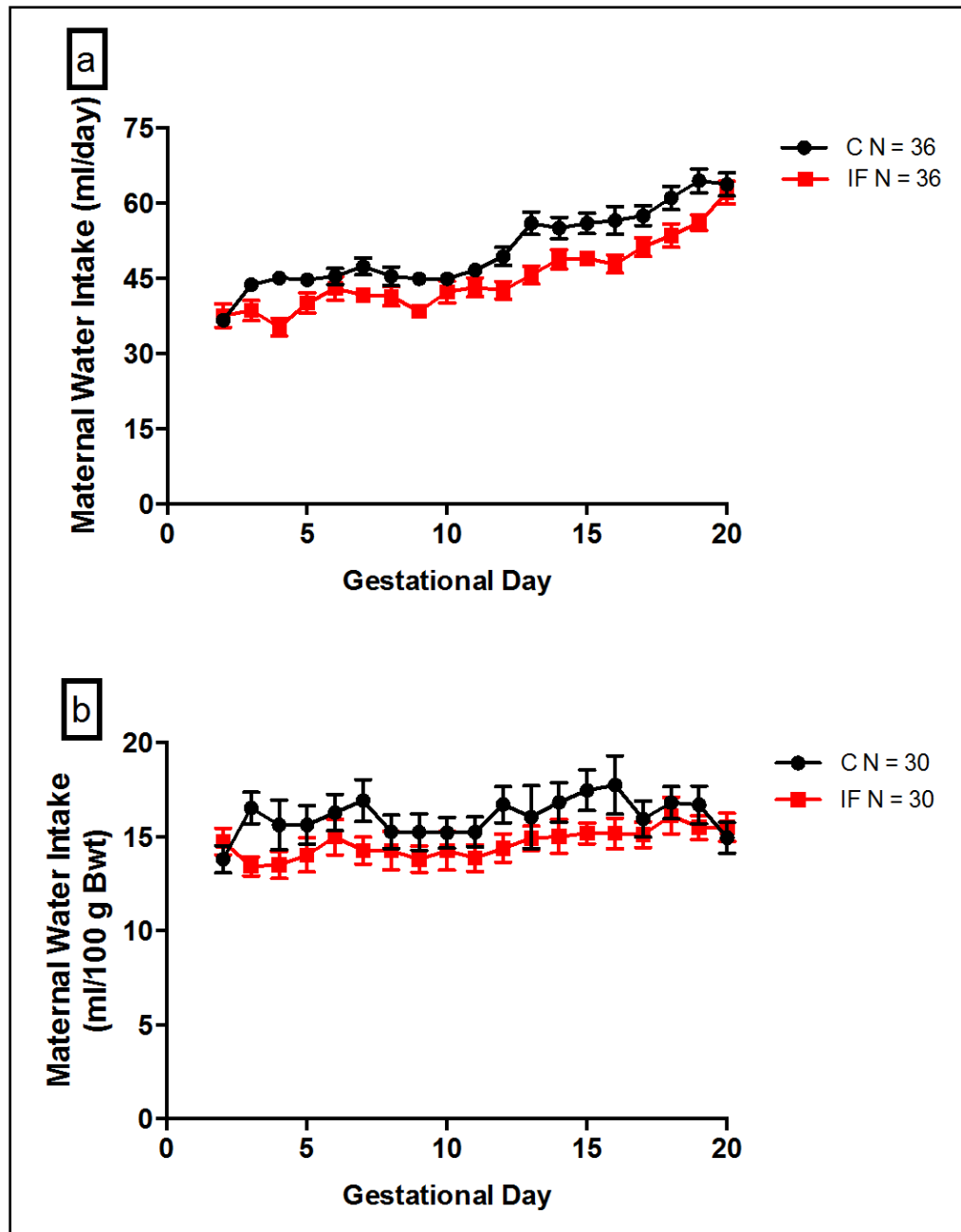
Figure 2.3.2 Daily maternal water intake by pregnant dams throughout gestation. **a.** Daily water consumption by pregnant dams. **b.** Daily water intake normalised for the pregnant dam's bodyweight (Bwt). Control (●) and IF (■) dams had unrestricted access to water throughout pregnancy. No data are shown for day 1, as this did not reflect a full 24 h period. IF dams drank less water than control dams from GD 4 onwards; however, when normalized to dam bodyweight, the differences were abolished. Data are presented as mean ± SEM. Water intake increased as pregnancy progressed in both groups $P_{Day} < 0.0001$. **a.** $P_{Diet} = 0.29$ at GD 2, 3 and 20, $P_{Diet} < 0.001$ from GD 4 to 19. **b.** $P > 0.05$. Statistical analysis was carried out using a repeated-measures two-way ANOVA followed by Tukey's post hoc test.
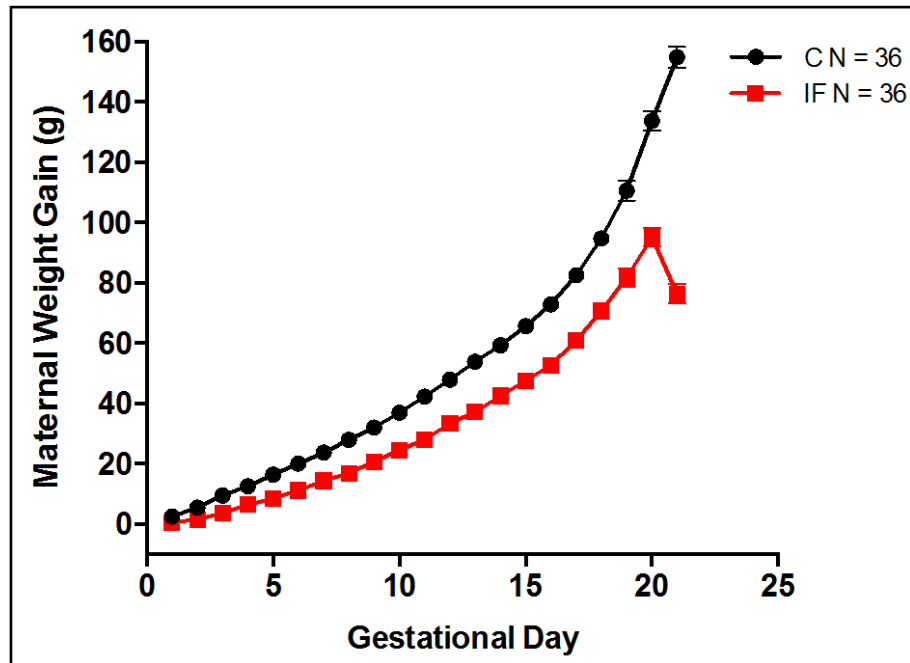
Figure 2.3.3 Daily bodyweight gain in pregnant dams during gestation. Weight gain was calculated as increase in weight per day minus baseline (pre-pregnancy) weight. IF dams (■) gained less weight than control dams (●) from GD 8 onward. At GD 21, as IF dams were weighed following an overnight fast before sacrifice, IF dams exhibited a 19 ± 1 g drop in their daily weight gain. Data are presented as mean ± SEM. $P_{Day} < 0.0001$, $P_{Diet} < 0.0001$ from GD 8 onwards. Statistical analysis was carried out using repeated-measures two-way ANOVA followed by Tukey's post hoc test.

2.3.4 Maternal organ weights

The wet weights of maternal organs (kidney, heart and liver) harvested at GD 21 were significantly lower in dams of the IF group compared to the control (heart, $P < 0.0001$, Figure 2.3.4 a; liver, $P < 0.0001$, Figure 2.3.4 b; kidney, $P < 0.05$, Figure 2.3.4 c). However, when considering maternal organ weight relative to dam weight after subtracting the weight of uterine contents, there were no differences in heart weight between the two dietary groups (Figure 2.3.5 a). IF liver weight, on the other hand, was significantly reduced ($P < 0.01$, Figure 2.3.5 b) whilst kidney weight was significantly increased compared to the control dams ($P < 0.05$, Figure 2.3.5 c). This suggests that the different maternal organs appear to respond differently to maternal intermittent fasting.
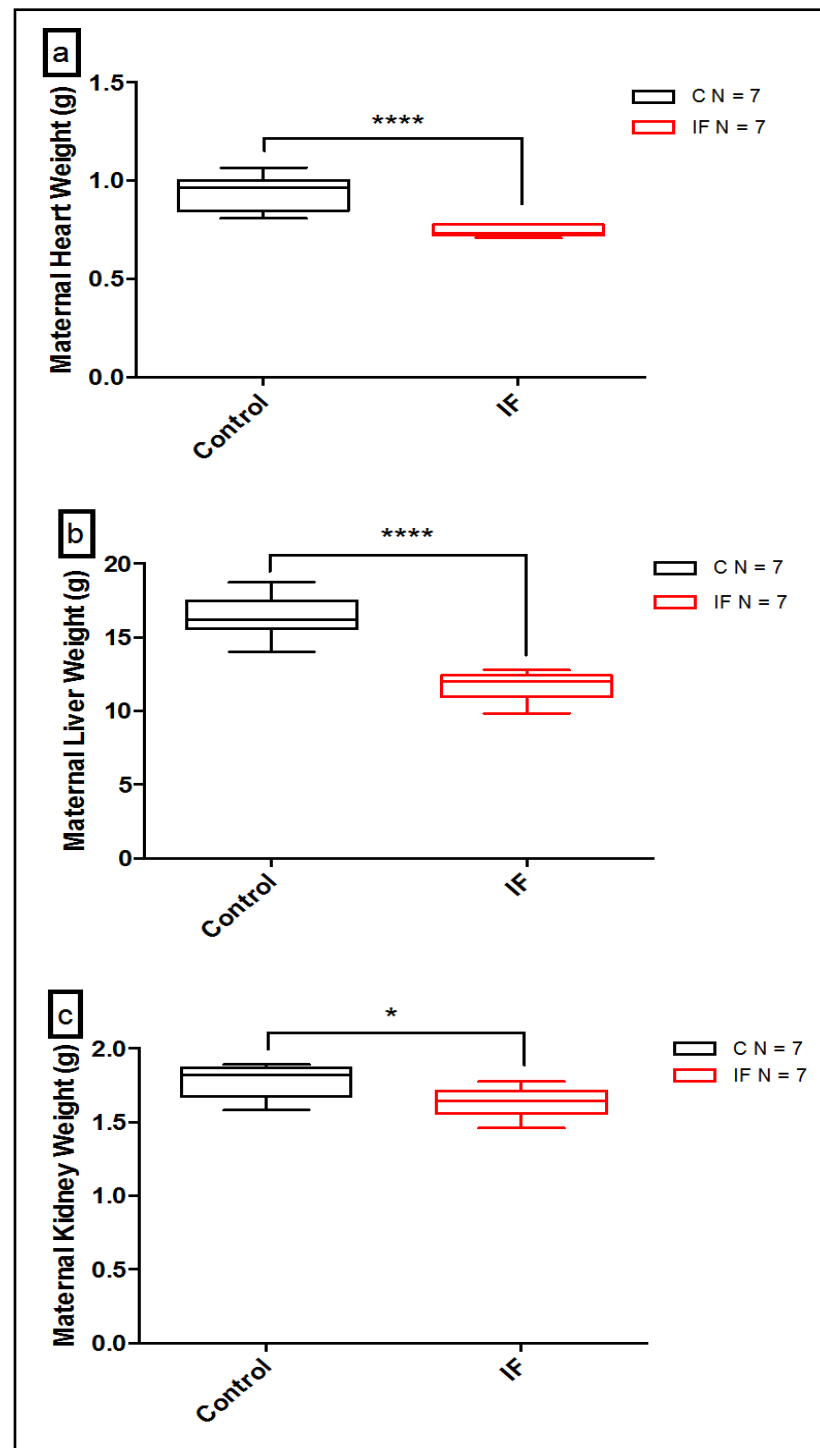
Figure 2.3.4 The effect of maternal intermittent fasting on maternal organ weights at GD 21. Maternal organs **a.** heart, **b.** liver and **c.** kidney. IF (red) dams had significantly reduced organ weights compared to control (black) dams. Data are expressed as box and whisker plots, with the lines inside the box representing the median, the boxes mark the interval between the 25th and 75th percentile, and the whiskers denote the interval between the 5th and 95th percentile. * $P < 0.05$, **** $P < 0.0001$ IF versus control dams (unpaired t-test).
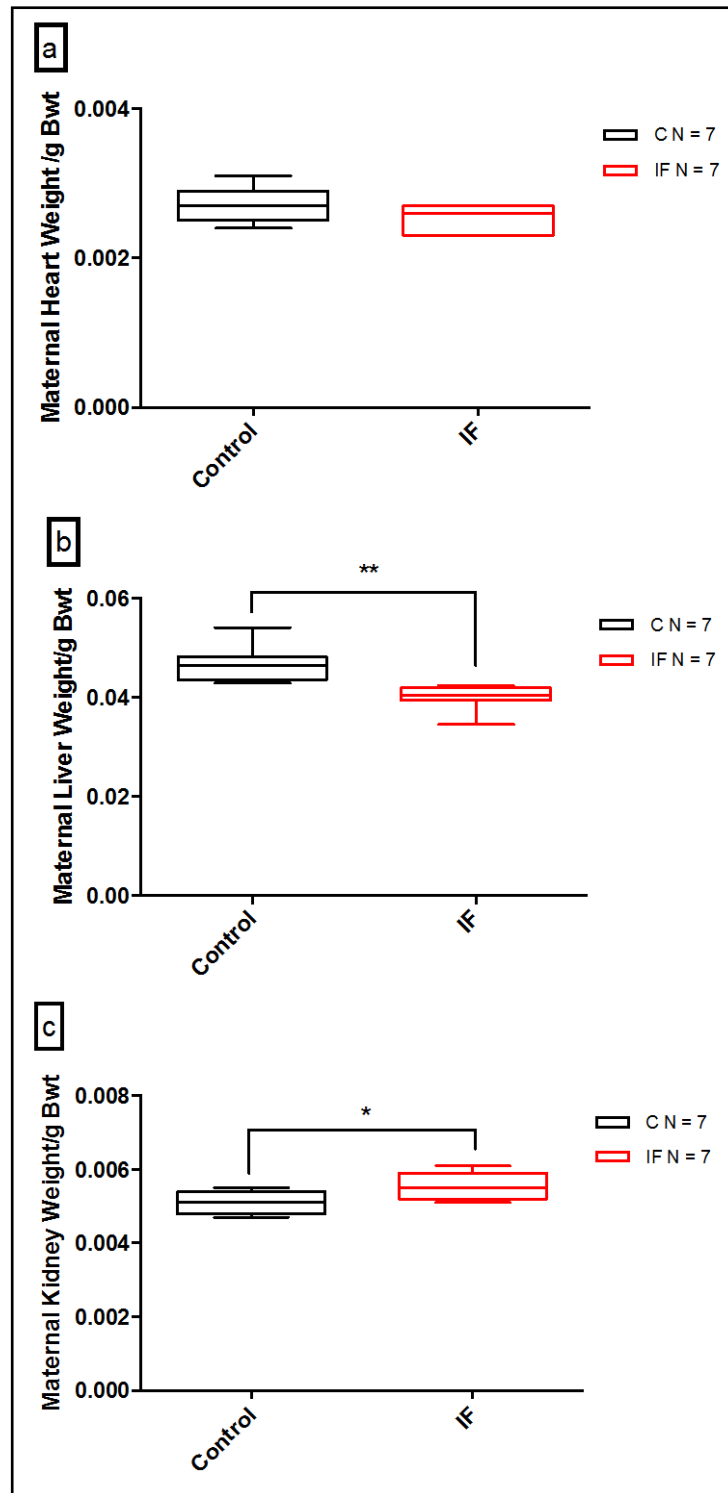
Figure 2.3.5 Maternal organ weights relative to pregnant dam bodyweight at GD 21. Maternal organs **a.** heart, **b.** liver and **c.** kidney were normalised to maternal bodyweight after subtracting uterine contents. The relative liver and kidney weights were significantly different between the two dietary groups. In contrast, relative heart weight in dams did not differ between groups. Data are expressed as box and whisker plots. * $P < 0.05$, ** $P < 0.01$ IF versus control dams (unpaired t-test).

2.3.5 Fetal and placental weights

Maternal intermittent fasting diet did not alter litter size (P = 0.42, Table 2.3.1) or the number of resorption sites per litter between the two groups; invariably no resorption sites were visible or only one. Fetal sex was also equally distributed amongst the dietary groups (Table 2.3.1).

Table 2.3.1 Litter size for control and IF dams

Experimental groups	Control (N = 35)		IF (N = 35)	
	Male	Female	Male	Female
Litter size	8 ± 1	8 ± 1	7 ± 1	7 ± 1

Values are mean ± SEM with P > 0.05 (Fisher's exact test)

To investigate the effects of intermittent fasting on fetal and placental growth, the fetal and placental wet weights were recorded at GD 21. The mean litter weights were significantly reduced in the IF group by 15 ± 1% in comparison to the control group (P < 0.0001, Figure 2.3.6 a), with female fetuses being lighter than male fetuses in the control group (P < 0.05, Figure 2.3.6 a). However, the weights of male and female IF fetuses were not statistically different. Plotting fetal weight distribution curves revealed that the weight of 61 - 67% IF fetuses fell below the 5th centile of the control population (taken as a clinical indicator of fetal growth restriction (FGR; Dilworth et al., 2011)), indicating that a majority of fetuses exhibited a FGR phenotype (Figures 2.3.6 b, c and d).

In contrast, there was no statistically significant difference in placental weights either between fetal sexes or between dietary groups (Figure 2.3.7 a), indicating a lack of effect of maternal intermittent fasting on placental weight, as a proxy of placental growth. However, a frequency distribution curve of placental weights from the control and IF groups reveals that 4% of IF male and 15% of IF female placental weights fell below the 5th centile of the control population (~0.32 g), suggesting a greater tendency towards a lower placental weight in female fetuses of the IF group (Figure 2.3.7 b).

Calculating the fetal/placental weight ratio as a proxy of placental transport efficiency revealed a significant decrease in the ratio in the IF group of both sexes compared with control (P_{Female} < 0.05; P_{Male} < 0.01, Figure 2.3.8), suggesting an adverse effect of intermittent fasting on placental transport function.

2.3.6 Fetal anthropometric measurements

The effect of maternal intermittent fasting on the fetal anthropometric measurements revealed a diminished crown:rump length (P < 0.01, Figure 2.3.9 a) and a smaller head circumference (P < 0.05, Figure 2.3.9 b) for the fetuses of both sexes in the IF group compared to control. Maternal

intermittent fasting, however, failed to alter the abdominal circumference of IF fetuses (Figure 2.3.9 c).

2.3.7 Fetal organ weights

To augment the findings of fetal anthropometric measurements, fetal organs (brain, heart, liver and kidney) were harvested. In both IF sexes, the weights of fetal brain ($P_{Male} < 0.05$, $P_{Female} < 0.01$, Figure 2.3.10 a) and kidney ($P < 0.05$, Figure 2.3.10 b) as well as fetal liver of IF female fetuses ($P < 0.05$, Figure 2.3.10 c) were diminished by maternal intermittent fasting. Yet for fetal liver and heart, there were no differences for either fetal sex between groups (Figures 2.3.10 c and d). However, when the fetal tissue weights were expressed relative to fetal bodyweight, there were no significant differences between dietary group comparisons for any tissue (Figures 2.3.11 a, b, c and d). This indicated that for organs that were lighter, the reduction in organ weight was proportionally related to the diminished fetal weight in the IF group.

The fetal brain/liver weight ratio was also analysed, as this is commonly used as an index to distinguish between different FGR phenotypes (Cox and Marton, 2009). This ratio was significantly elevated in both sexes of IF fetuses compared to controls ($P < 0.01$, Figure 2.3.12). This indicates that maternal intermittent fasting is associated with fetal brain sparing and asymmetrical FGR, where fetal brain development is protected at the expense of other visceral organs such as the liver at GD 21.

It is worth noting that several malformations were observed in IF fetuses. Defects in cranium (exencephaly) and vertebrae, snout face deformity, shortening of the upper limb and death *in utero* were observed at GD 21 with a predominance in female fetuses (Figure 7.6, Appendix).
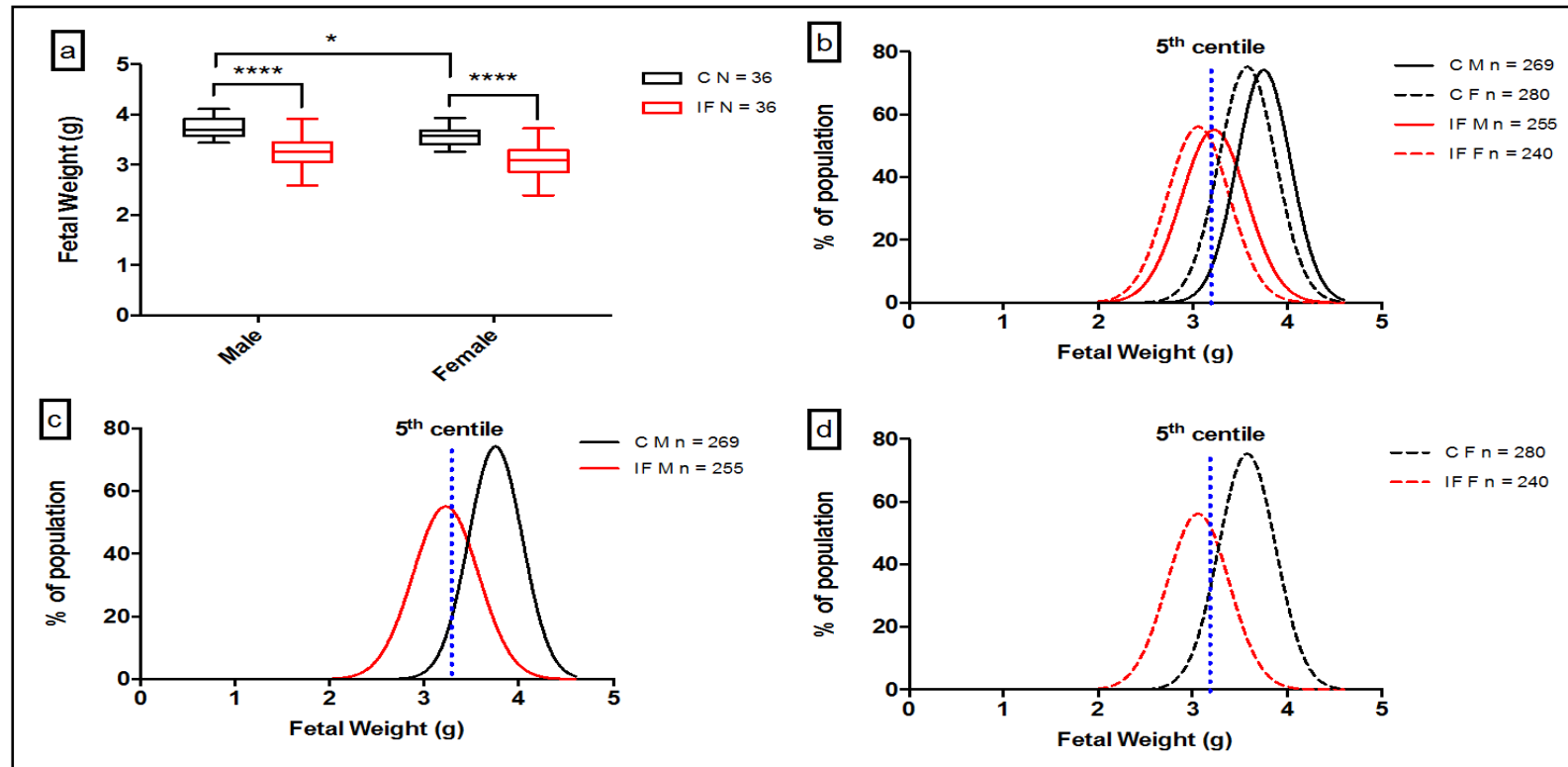
Figure 2.3.6 The effect of maternal intermittent fasting on fetal weight at GD 21. **a.** Mean fetal weight was taken for each sex/litter. **b.** Fetal weight distribution curve for all fetuses, and split into **c.** male and **d.** female fetuses. **a.** Fetal weight was lower in males compared to females in the control group and reduced by IF diet for both sexes. **a.** Data for litter mean fetal weight are expressed as box and whisker plots and were analysed using two-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, C female fetuses versus C males, **** $P < 0.0001$, IF fetuses versus control for both sexes. **b., c. and d.** Data for fetal weight distributions were analysed using the Gaussian non-linear regression function and demonstrate a left-shift to a predominantly lower weight for both sexes. The vertical dashed line represents the 5th centile of the control population, revealing that for both sexes a majority of IF fetuses fall below this point and exhibit FGR.
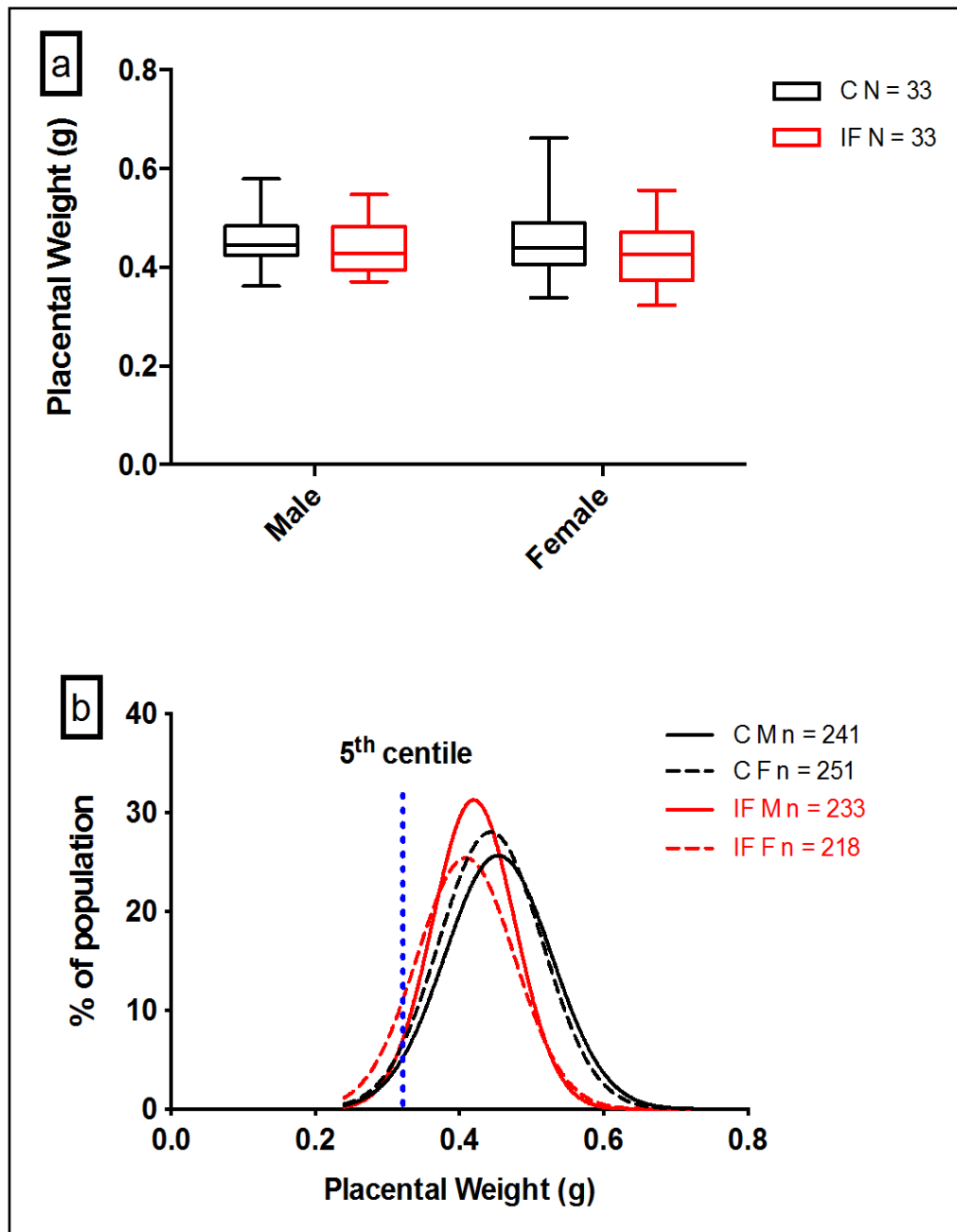
Figure 2.3.7 The effect of maternal intermittent fasting on placental weight at GD 21. **a.** Mean placental weight was taken for each sex/litter. **b.** Placental weight distribution curve for both fetal sexes in C and IF groups. There were no statistically significant differences in placental weight between control (black) and IF (red) groups or between fetal sexes. **a.** Data are expressed as box and whisker plots and were analysed using two-way ANOVA followed by Tukey's post hoc test, with $P > 0.05$ for diet and sex comparisons. **b.** Data for placental weight distribution curves was analysed using the Gaussian non-linear regression function. The vertical dashed line denotes the 5th centile for placental weight of the control population, with 4% and 15% placentas of IF males and IF females respectively falling below the 5th centile.
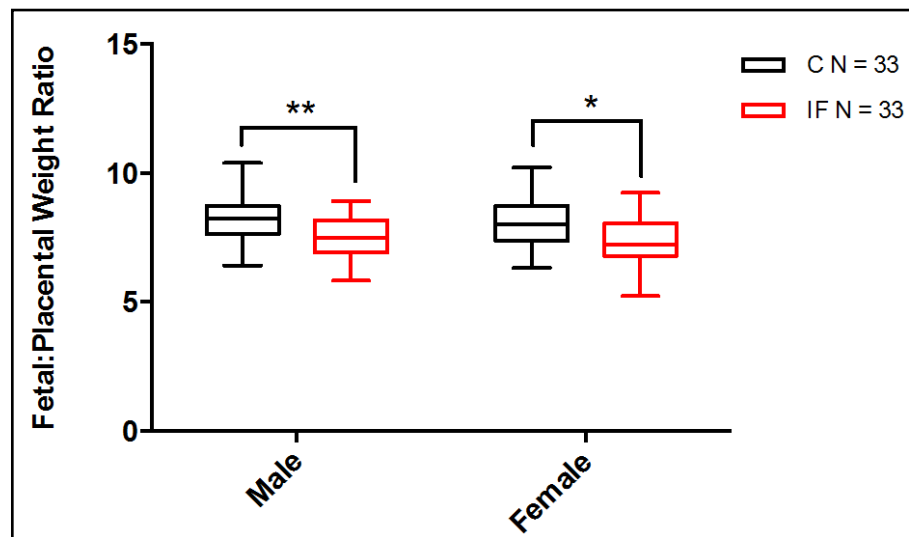
Figure 2.3.8 The effect of maternal intermittent fasting on fetal:placental weight ratio at GD 21. The fetal/placental weight ratio (a proxy of placental transport efficiency) was reduced in both sexes of the IF (red) group compared to controls (black). Data were analysed as mean fetal/placental weight taken for each sex/litter and expressed as box and whisker plots. ** P_{Male} < 0.01, * P_{Female} < 0.05 IF fetuses versus control for both sexes (two-way ANOVA followed by Tukey's post hoc test).
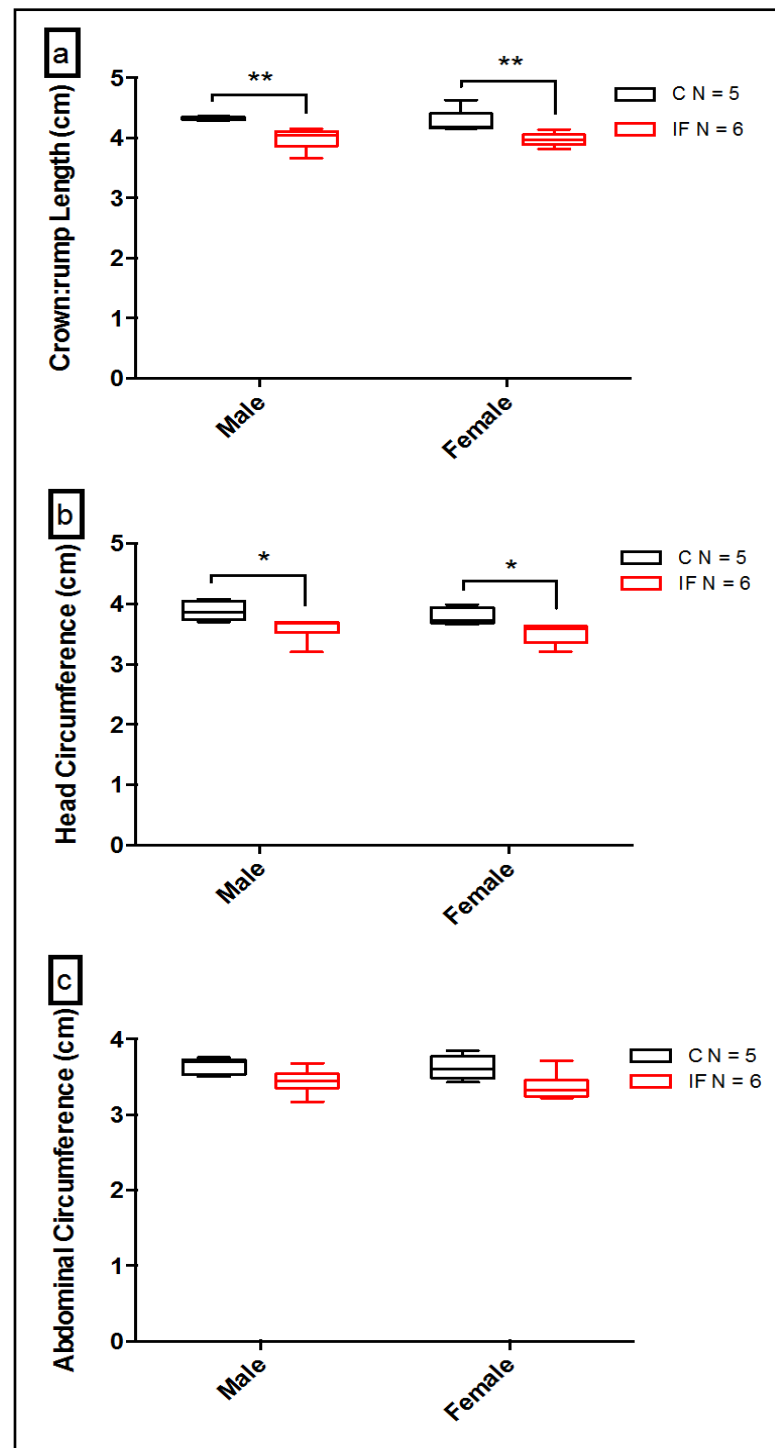
Figure 2.3.9 The effect of maternal intermittent fasting on fetal anthropometric measurements at GD 21. **a.** crown:rump length **b.** head circumference and **c.** abdominal circumference. Fetuses in the IF group (red) were shorter with a smaller head circumference than control fetuses (black). The abdominal circumference, however, did not differ significantly between dietary groups. Data were analysed as mean/litter and expressed as box and whisker plots. * $P < 0.05$, ** $P < 0.001$, IF fetuses versus control (Kruskal-Wallis test followed by Dunn's multiple comparisons test).
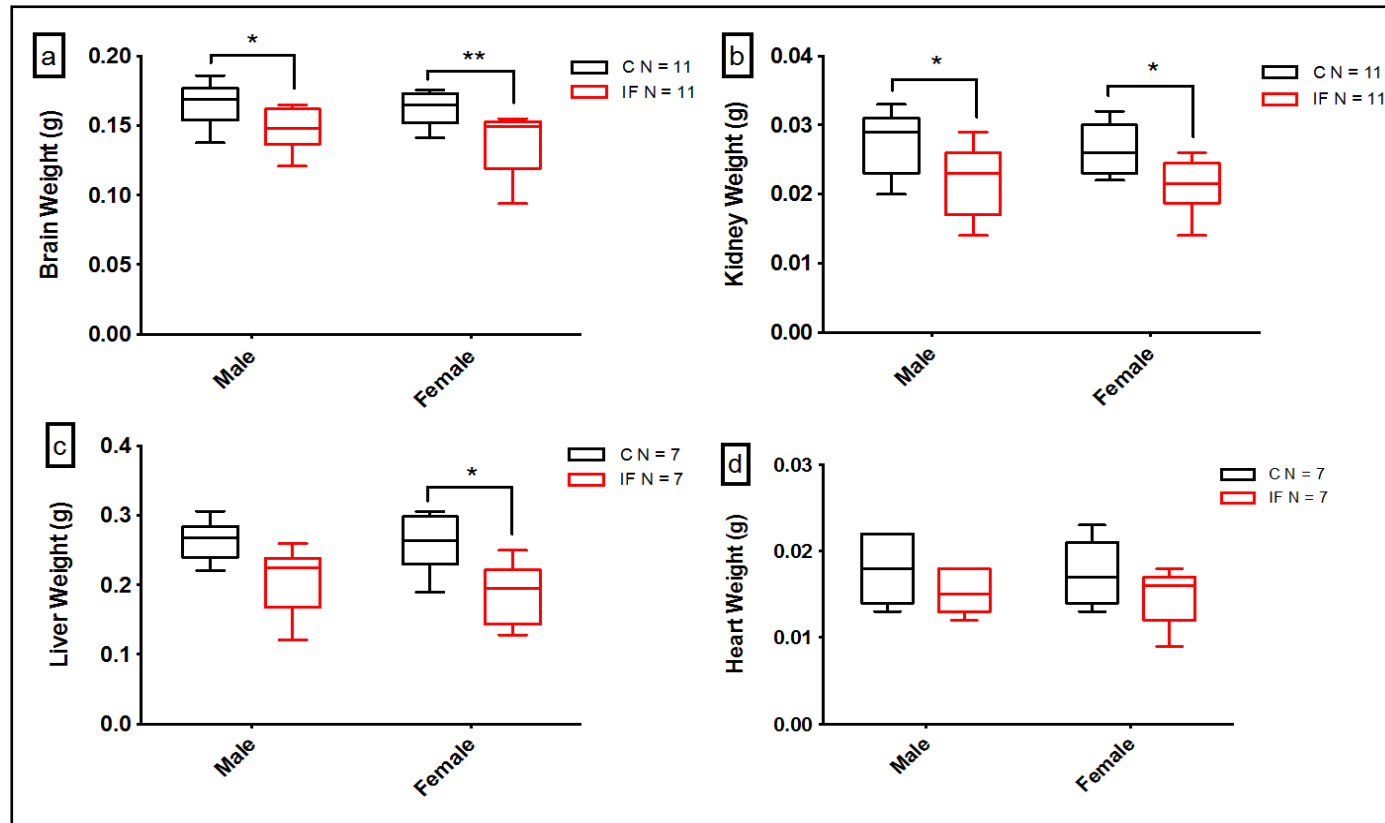
Figure 2.3.10 The effect of maternal intermittent fasting on fetal organ weights at GD 21. **a.** brain, **b.** kidney, **c.** liver and **d.** heart. Control (black) and IF (red) groups. There was a significant decrease in brain and kidney weights of both sexes in the IF group and a significant decrease in liver weight for IF female fetuses only. Heart weight was comparable between both dietary groups for both fetal sexes. Data were analysed as mean/litter and expressed as box and whisker plots. * $P < 0.05$, ** $P < 0.01$ IF versus control fetuses (two-way ANOVA followed by Tukey's post hoc test).
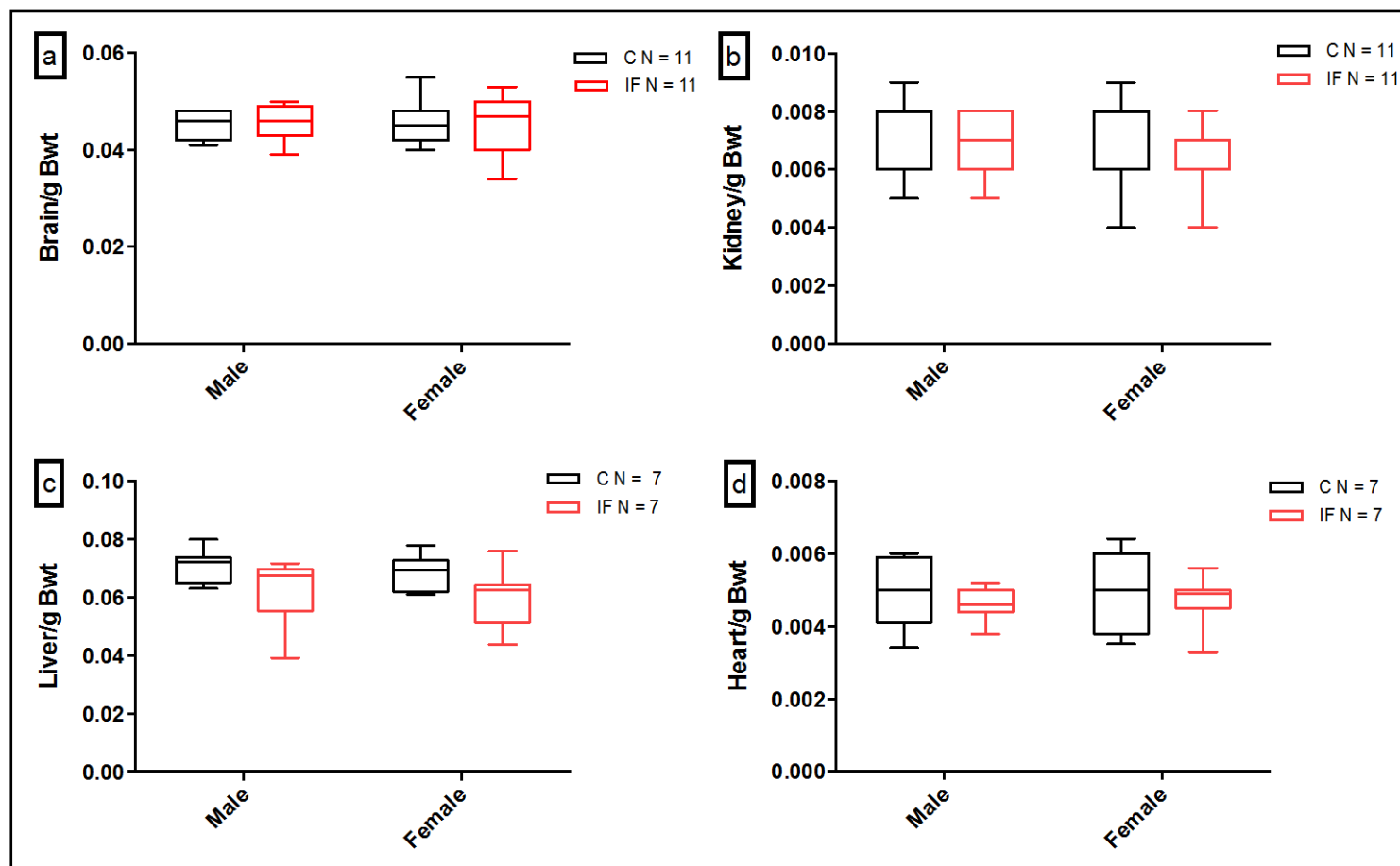
Figure 2.3.11 Fetal organ weights relative to fetal bodyweight (Bwt) at GD 21. **a.** brain, **b.** kidney, **c.** liver and **d.** heart. There were no significant differences in the relative fetal organ weights between control (black) and IF (red) groups for any fetal organ. Data were analysed as mean/litter and expressed as box and whisker plots. $P > 0.05$ for diet and sex comparisons (two-way ANOVA followed by Tukey's post hoc test).
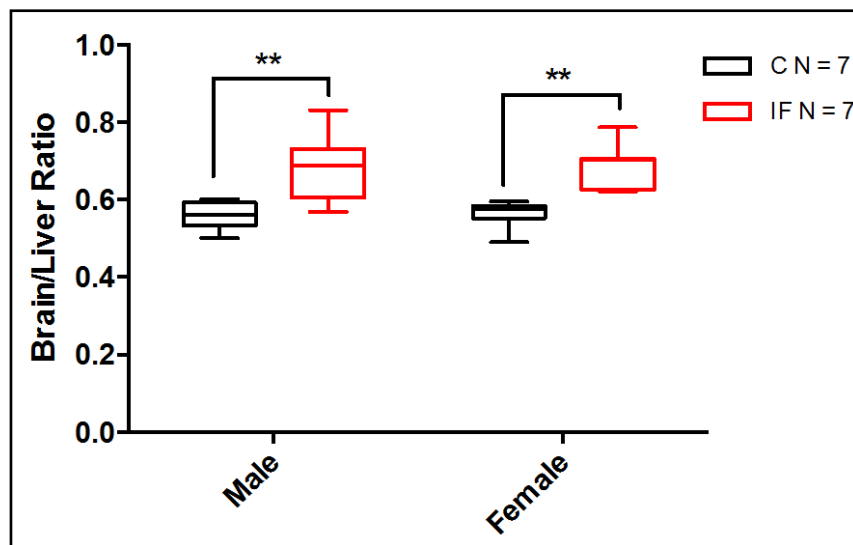
Figure 2.3.12 The effect of maternal intermittent fasting on the fetal brain/liver weight ratio at GD 21. The fetal brain/liver weight ratio increased in IF (red) compared to control (black) group. Data are analysed as mean/litter and expressed as box and whisker plots. ** P < 0.01 IF versus control group (two-way ANOVA followed by Tukey's post hoc test).

2.3.8 Maternal and fetal metabolites

The maternal intermittent fasting diet clearly impacted on maternal physiology, as manifest by the 33 ± 3% reduction in plasma glucose concentration (Figure 2.3.13 a). Associated with this, there was a 79 ± 6% reduction in maternal plasma glucagon concentration (Figure 2.3.13 b), yet surprisingly, maternal insulin concentration was unaffected (Figure 2.3.13 c). Additionally, maternal BCAA concentration was also significantly decreased in the IF group (P < 0.01, Figure 2.3.15).

The reduced maternal glucose concentration in fasted pregnant dams may influence maternal behaviour. It was noted that these dams were more anxious and hyperactive with a heightened state of agitation, leading them to jump and move constantly around their cages. It was also noted that it took a longer time for the IF dams to become anaesthetised using isoflurane and sodium thiobutabarbital, consistent with the notion that maternal metabolic capacity had been altered.
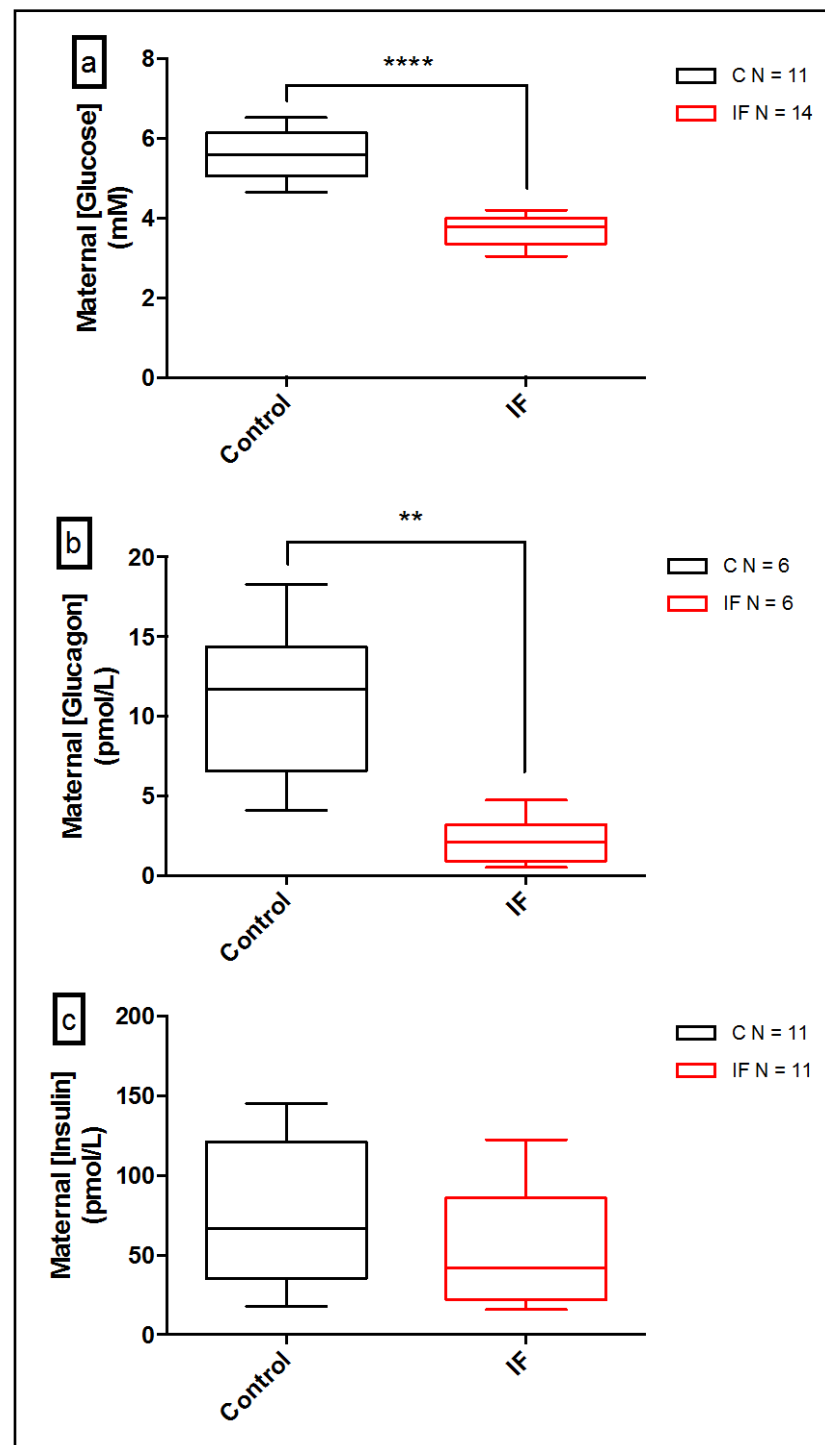
Figure 2.3.13 The effect of maternal intermittent fasting on maternal glucose, glucagon and insulin concentrations at GD 21. **a.** glucose, **b.** glucagon and **c.** insulin. In IF dams, glucose and glucagon concentrations were significantly lower compared to controls with no significant changes in insulin concentration. Data are expressed as box and whisker plots. ** P < 0.01, **** P < 0.0001 IF versus control dams (unpaired t-test).

The response of IF fetuses to maternal intermittent fasting was distinct to that of their mothers with regards to glucose concentration and the hormones measured above. No sex-related effects were found between male and female fetuses for these variables, so data were pooled from both sexes for both dietary groups. IF fetuses maintained plasma glucose and glucagon at similar concentrations to the control group whilst exhibiting a significant reduction in insulin concentration ($P < 0.05$; Figures 2.3.14 a, b and c). Hence, fetal plasma glucose was preserved in the face of a significantly lowered maternal glucose concentration. In contrast, the BCAA concentration of IF fetuses ($P < 0.0001$; Figure 2.3.15) mirrored the reduction observed in the maternal plasma of the IF group, with an attenuation of the materno-fetal transplacental BCAA concentration gradient (Figure 2.3.15).

2.3.9 Maternal and fetal amino acid concentrations

Maternal and fetal concentrations of individual amino acids were measured at GD 21. As shown in Table 2.3.2, amino acid concentrations were significantly higher in fetal plasma compared to maternal plasma. Several essential and non-essential amino acids were found to be altered by the IF regime in both dams and their fetuses (Table 2.3.2). As regards maternal plasma, alanine (-54 ± 4%), asparagine (-47 ± 3%), threonine (-45 ± 7%), histidine (-40 ± 6%), arginine (-39 ± 4%), serine (-32 ± 6%), valine (-24 ± 6%), methionine (-23 ± 4%) and tyrosine (-16 ± 3%) were significantly reduced in the plasma of IF dams compared to controls.

This impact on maternal environment was mirrored with a similar alteration in the fetal compartment for most of these amino acid concentrations with the exception of asparagine and tyrosine (Table 2.3.2). Histidine (~58 ± 4%), methionine (~17 ± 2%) and alanine (~25 ± 2%) were significantly reduced in both sexes of the IF dietary group compared to controls.

There was a sex-specific effect of maternal diet on certain fetal amino acids: threonine (-22 ± 6%), arginine (-28 ± 5%) and valine (-19 ± 2%) were significantly reduced in IF male fetuses compared to controls, while serine (-21 ± 3%) was significantly lowered in IF female fetuses compared to control females. Aspartate (-34 ± 8%) and isoleucine (-25 ± 3%) differed significantly in IF fetuses of both sexes compared to controls but not in their mothers.

Moreover, sex differences were found in certain amino acids. Lysine (-21 ± 3%), arginine (-18 ± 6%) and tyrosine (-16 ± 4%) were significantly lower in control female compared to control male fetuses, whereas phenylalanine (-17 ± 2%), tryptophan (-22 ± 6%) and tyrosine (12 ± 2%) were significantly reduced in IF females compared to their male counterparts. The HPLC analysis revealed that the reduction in maternal BCAA concentration in the IF group (measured by ELISA) was mainly attributable to a lowered valine concentration, whereas the diminished fetal BCAA concentration in the IF group, on other hand, was due to reductions in both valine and isoleucine concentration.

The fetal/maternal concentration ratio of amino acids in both control and IF dietary groups are shown in Figure 2.3.16. There were no differences in the ratio between the sexes, thus, the data were pooled from both sexes in the control and IF groups. The fetal/maternal amino acid concentration ratio varies in value from 1.5 to 7, with a more pronounced fetal/maternal ratio for certain amino acids such as tyrosine, phenylalanine, glutamate and aspartate. Surprisingly, fetal/maternal concentration ratios of most essential and non-essential amino acids were significantly increased in the IF dietary group (Figure 2.3.16). In contrast, ratios for branched-chain amino acids, methionine, histidine and some of the gluconeogenic amino acids (glutamate, aspartate and glycine) were comparable between the two dietary groups.
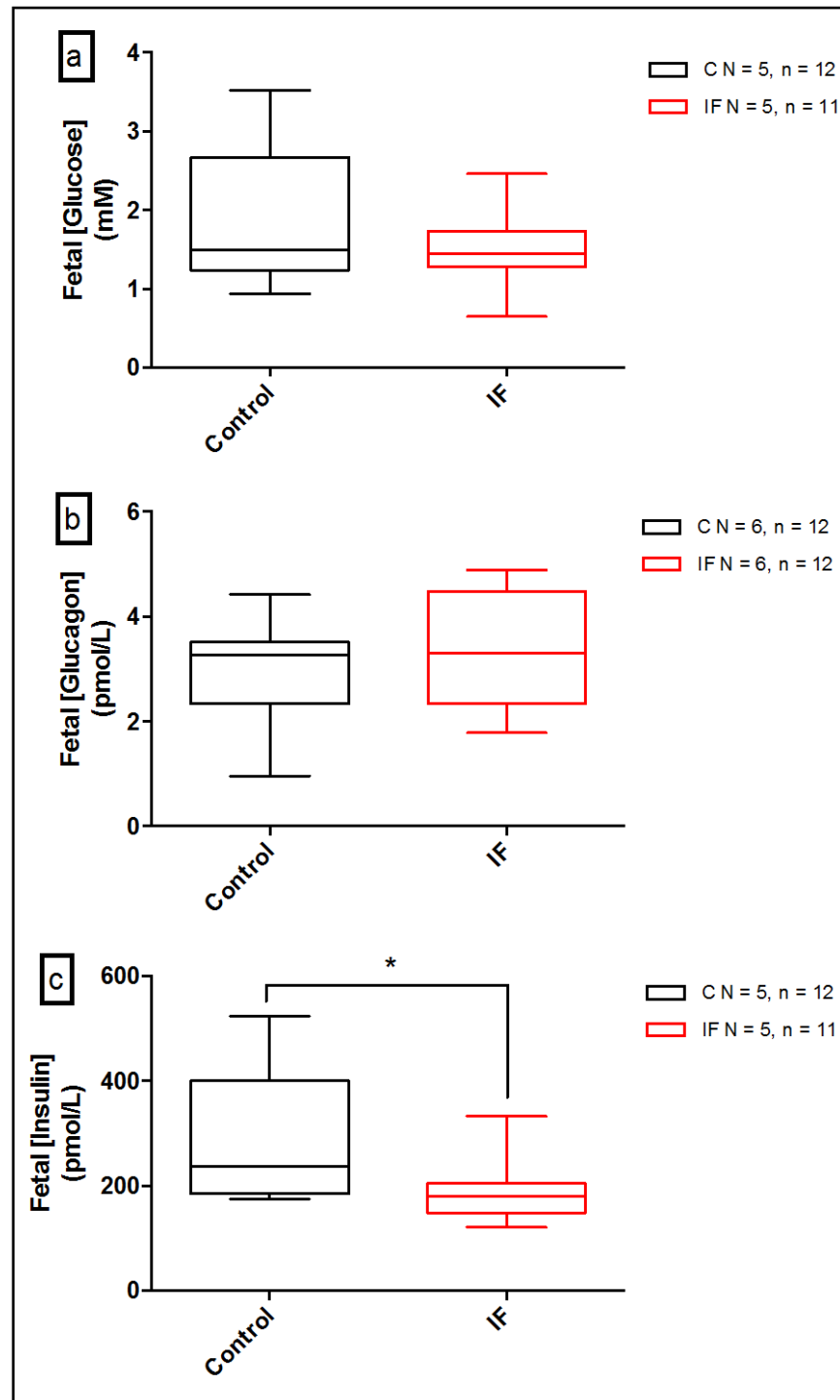
Figure 2.3.14 The effect of maternal intermittent fasting on fetal glucose, glucagon and insulin concentrations at GD 21. **a.** glucose, **b.** glucagon and **c.** insulin. Fetal plasma glucose, insulin and glucagon concentrations did not differ between sexes in each dietary group and therefore the data were pooled from both sexes in the control and IF groups. IF fetuses had normal glucose and glucagon concentrations, but in contrast, IF fetal insulin concentration was 30% lower than controls. Data are expressed as box and whisker plots. * $P < 0.05$ IF versus control fetuses (unpaired t-test).
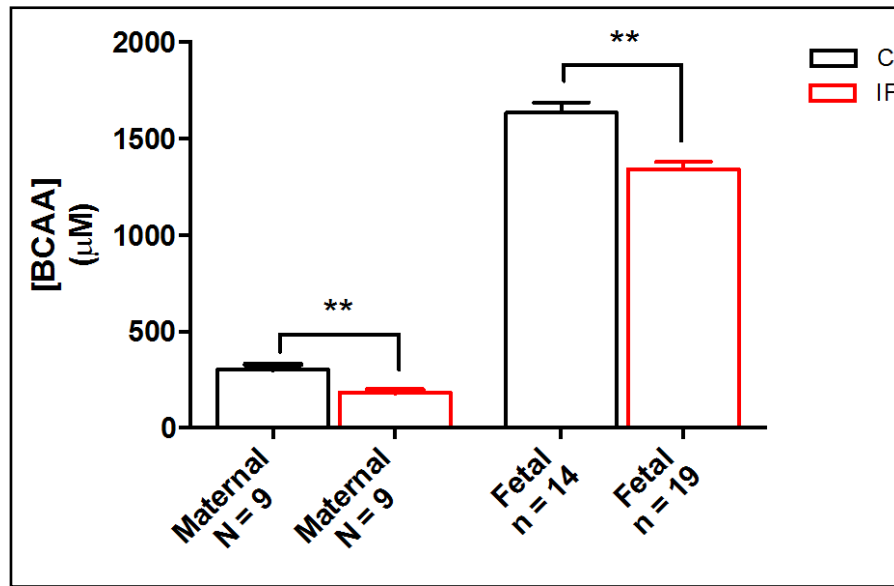
Figure 2.3.15 The effect of maternal intermittent fasting on maternal and fetal BCAA concentrations at GD 21. IF dams and their fetuses demonstrated 40 ± 5% and 18 ± 3% reduction, respectively, in plasma BCAA concentration. However, both fetal groups were able to maintain higher plasma BCAA concentrations relative to maternal plasma, consistent with the activity of carrier-mediated amino acid transport across the placenta. Data are expressed as mean + SEM. ** $P < 0.01$ IF versus control group (unpaired t-test).

Table 2.3.2 Maternal and fetal plasma amino acid concentrations at GD 21

	Maternal dams		Fetal Male		Fetal Female	
	C N = 6	IF N = 6	C N = 6	IF N = 7	C N = 6	IF N = 7
Essential Amino Acids						
His	49±3	30±2****	173±18	69±9****	137±13	61±8***
Ile	59±5	47±3	162±8	123±8**	136±5	100±3**
Leu	72±7	64±3	277±14	232±16	237±11	192±8
Lys	709±100	588±66	1695±79	1839±138	1345±51†	1471±73
Met	29±2	22±1*	99±4	80±3**	82±2	69±2*
Phe	39±1	36±1	212±9	241±9	181±12	200±5†
Thr	228±21	148±15**	327±18	255±19*	282±16	218±10
Trp	38±3	35±5	97±6	111±4	79±2	86±7†
Val	99±9	75±5*	328±21	264±14*	272±14	220±6
Non-Essential Amino Acids						
Ala	607±38	277±22****	1003±66	740±48**	889±50	682±18*
Arg	101±10	62±3**	215±10	154±10**	166±12†	131±8
Asn	80±4	42±2****	144±6	113±6	122±7	95±2
Asp	7±0.7	6±0.8	42±4	32±3	44±2	29±3*
Glu	55±5	53±4	262±24	245±25	262±32	244±31
Gln	529±36	437±22	793±19	791±30	716±27	736±24
Gly	122±6	126±8	226±9	241±10	200±18	227±8
Ser	223±17	151±13**	350±10	300±19	321±13	254±9*
Tyr	28±1	23±0.9*	157±4	166±4	132±6†	147±4†

Values are mean ± SEM, with all concentrations expressed as μM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 IF versus control group. † P < 0.05 female versus male fetuses. Statistical analysis for maternal concentration was carried out using unpaired t-test except for Glu, Gln, Gly, Thr, Tyr, Val, Phe and Ile in which Mann-Whitney test was used. For fetal concentrations, two-way ANOVA was used followed by Tukey's post hoc test except for Asn, Ser, Gln, Phe and Lys in which Kruskal-Wallis test with Dunn's post hoc was used.
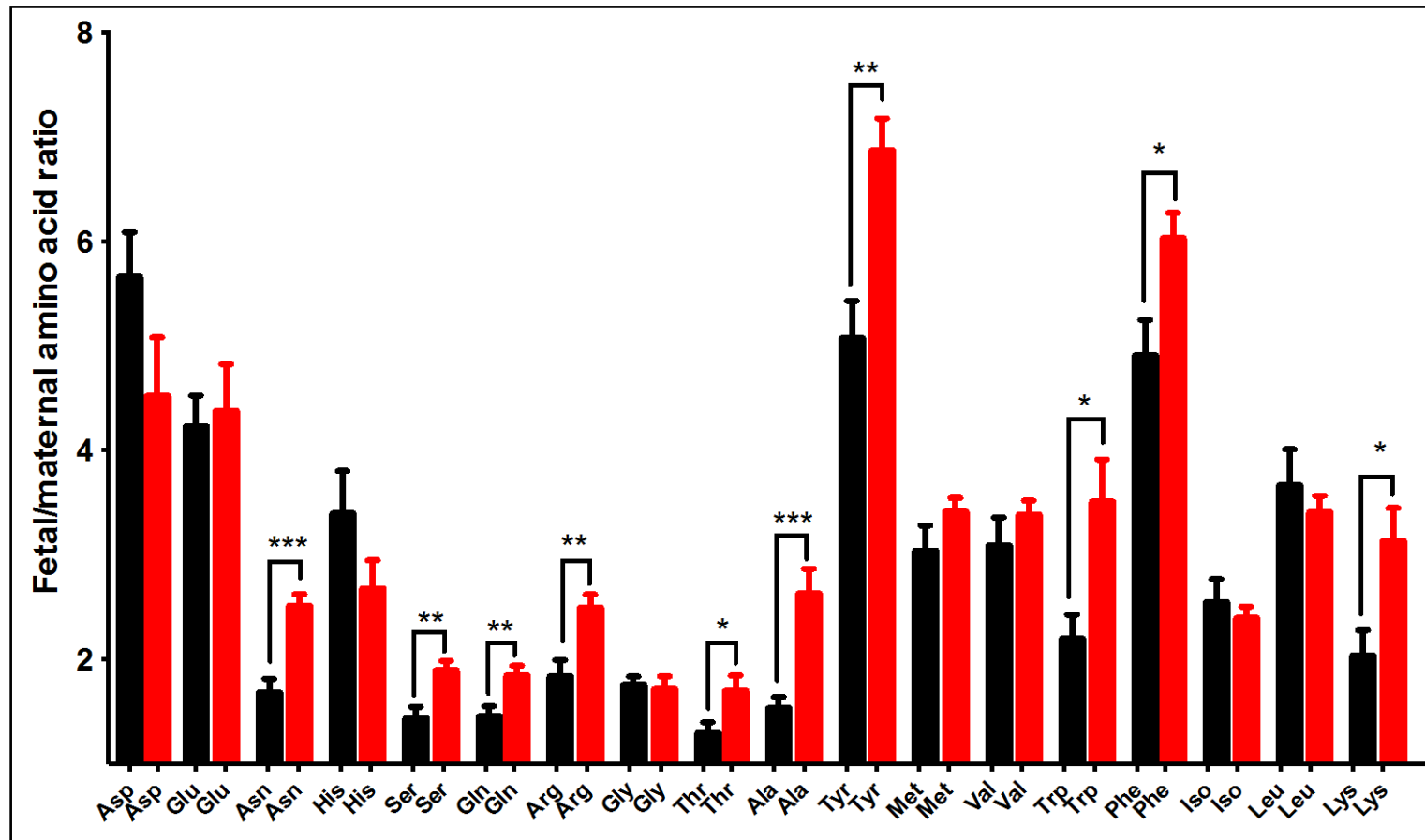
Figure 2.3.16 Fetal/maternal concentration ratio for plasma amino acids at GD 21. Data were pooled from both sexes in the control (black; N = 6) and IF (red; N = 6) groups since there were no significant differences between sexes in each dietary group. Data are expressed as bar mean + SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 IF versus control group (unpaired t-test was used except for Met, Mann-Whitney test was used instead).

2.3.10 Placental histology

Placental morphology was assessed to examine whether maternal intermittent fasting was underscored by changes in the junctional and/or labyrinth zones of the placenta (Figures 2.3.17 and 2.3.18). In keeping with an unaltered placental weight between control and IF groups (Figure 2.3.7), total placental area was similar between the control and the IF groups at GD 21. There were no differences seen in the percentage ratio of junctional zone area to total placental area or labyrinth zone area to total placental area between the dietary groups (Table 2.3.3, Figure 2.3.19). However, sex-dependent differences emerged, as females of both dietary groups had a significantly higher percentage ratio of the junctional zone to total area compared to their respective male counterparts and the converse was apparent as regards the percentage ratio of labyrinth zone to total area ($P_{Control} < 0.001$, $P_{IF} < 0.01$, Table 2.3.3, Figure 2.3.19).

2.3.11 Placental glycogen content

To address how fetal glucose concentration may be maintained in IF dams with lowered maternal plasma glucose concentrations, placental (junctional zone) and fetal hepatic glycogen contents were measured as a source of potential mobilisable glucose. These measurements in the placenta were restricted to the junctional zone, as it is the major part of the placenta that harbours clusters of glycogen cells (Coan et al., 2006). Placental glycogen content did not differ between fetuses of the control (2.3 ± 0.4 µg/mg placenta) and IF (2.0 ± 0.3 µg/mg placenta) groups (Figure 2.3.20 a). The mean glycogen content of the fetal liver from male and female control rats was 46 ± 4 µg/mg tissue and 42 ± 5 µg/mg tissue respectively, while that of the male and female IF rats was 24 ± 3 µg/mg tissue and 27 ± 3 µg/mg tissue respectively (Figure 2.3.20 b). This showed that maternal intermittent fasting exposure substantially reduced the glycogen content of the fetal liver by $48 \pm 6\%$ ($P < 0.01$) and $36 \pm 7\%$ ($P < 0.05$) in both male and female of IF fetuses compared to the controls.
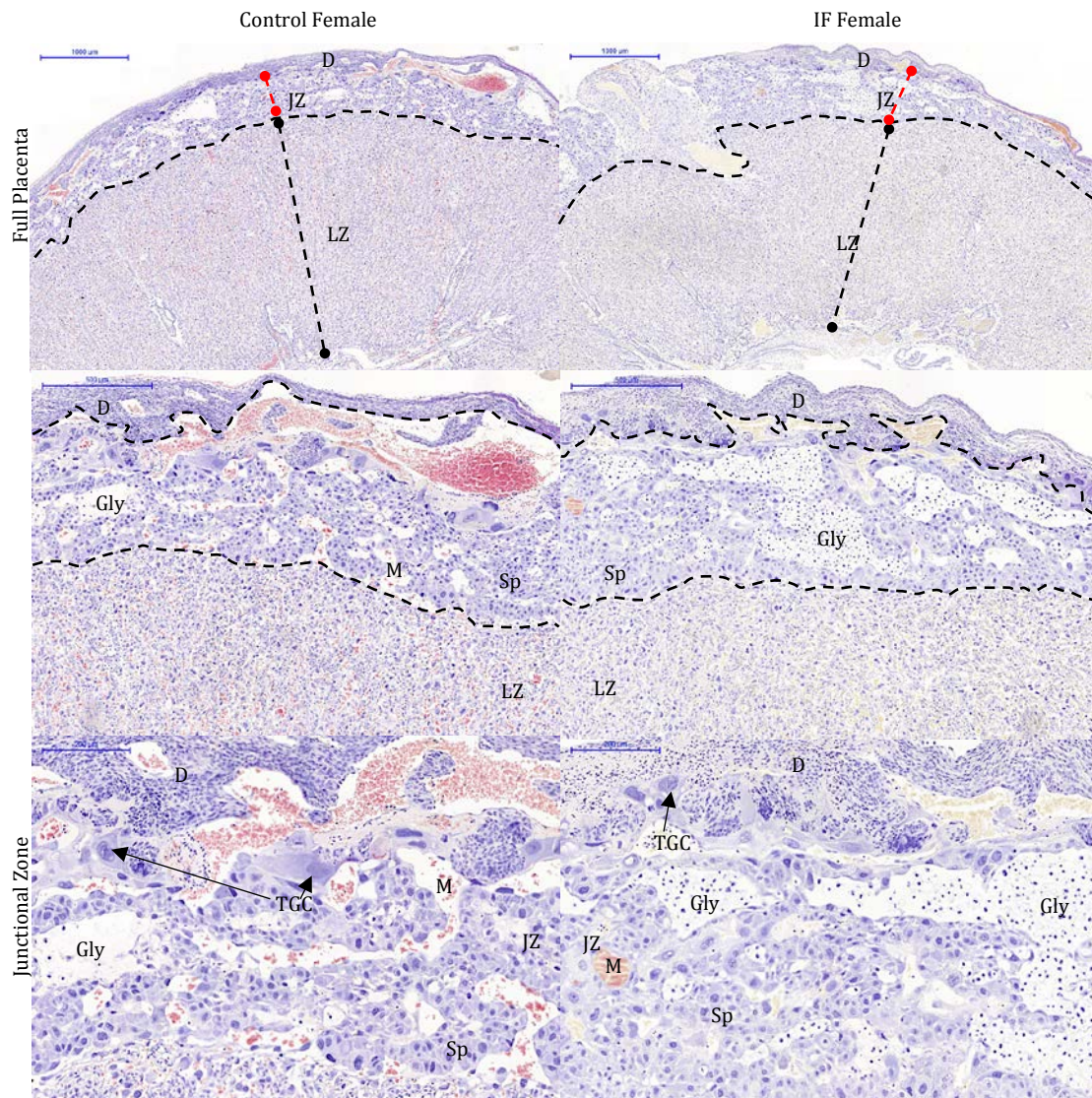
Figure 2.3.17 Representative images for histological comparison of rat placental morphology between the control and IF females at GD 21. Full placental sections show separation of junctional (JZ) which lies between decidua (D) and labyrinth zone (LZ) and contains maternal blood canals. Junctional zone sections show the three cell types of this layer: parietal trophoblast giant cells (TGC), glycogen cells (Gly) and spongiotrophoblast (Sp). Dashed line shows separation of JZ from LZ and D.
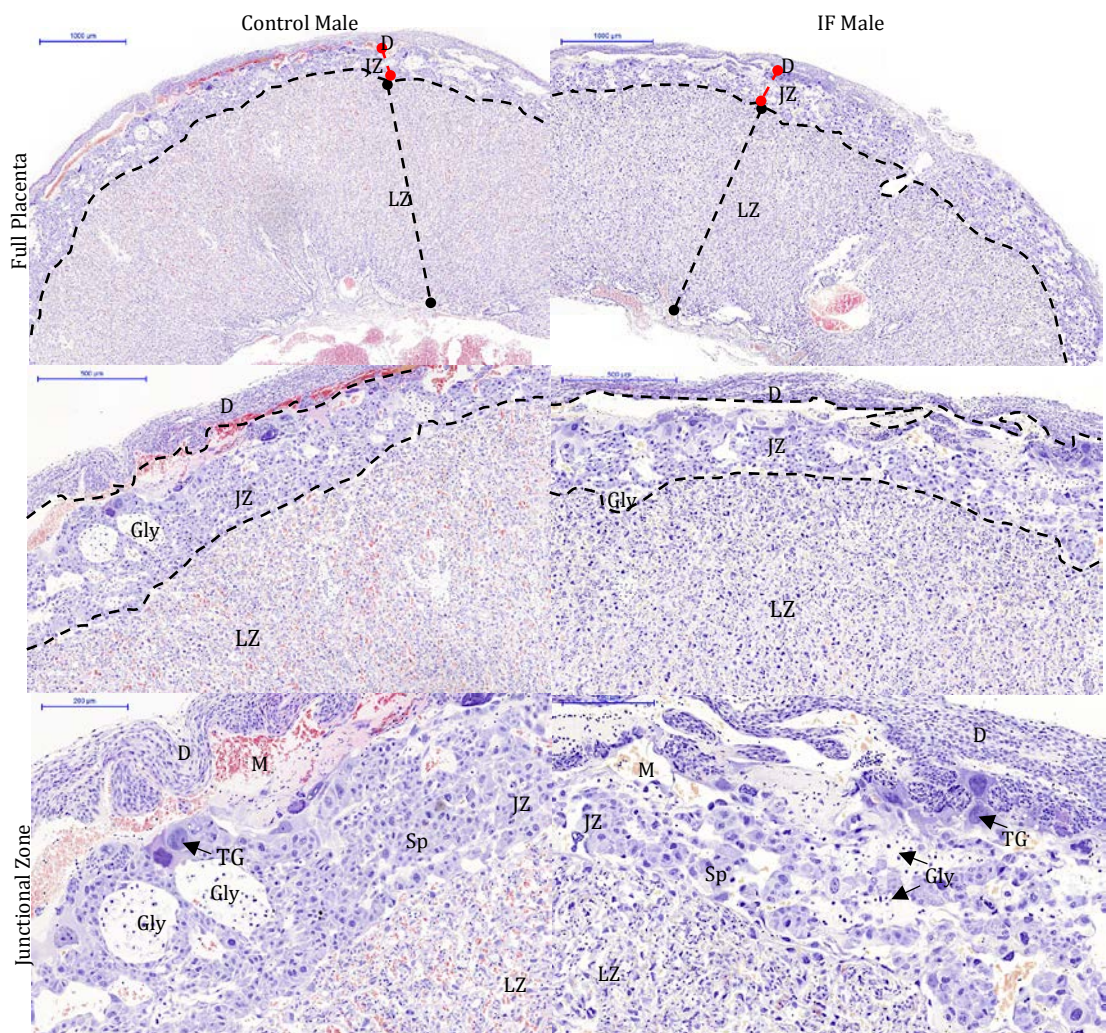
Figure 2.3.18 Representative images for histological comparison of rat placental morphology between the control and IF males at GD 21. Full placental sections show separation of junctional (JZ) which lies between decidua (D) and labyrinth zone (LZ) and contains maternal blood canals. Junctional zone sections show the three cell types of this layer: parietal trophoblast giant cells (TGC), glycogen cells (Gly) and spongiotrophoblast (Sp). Dashed line shows separation of JZ from LZ and D.
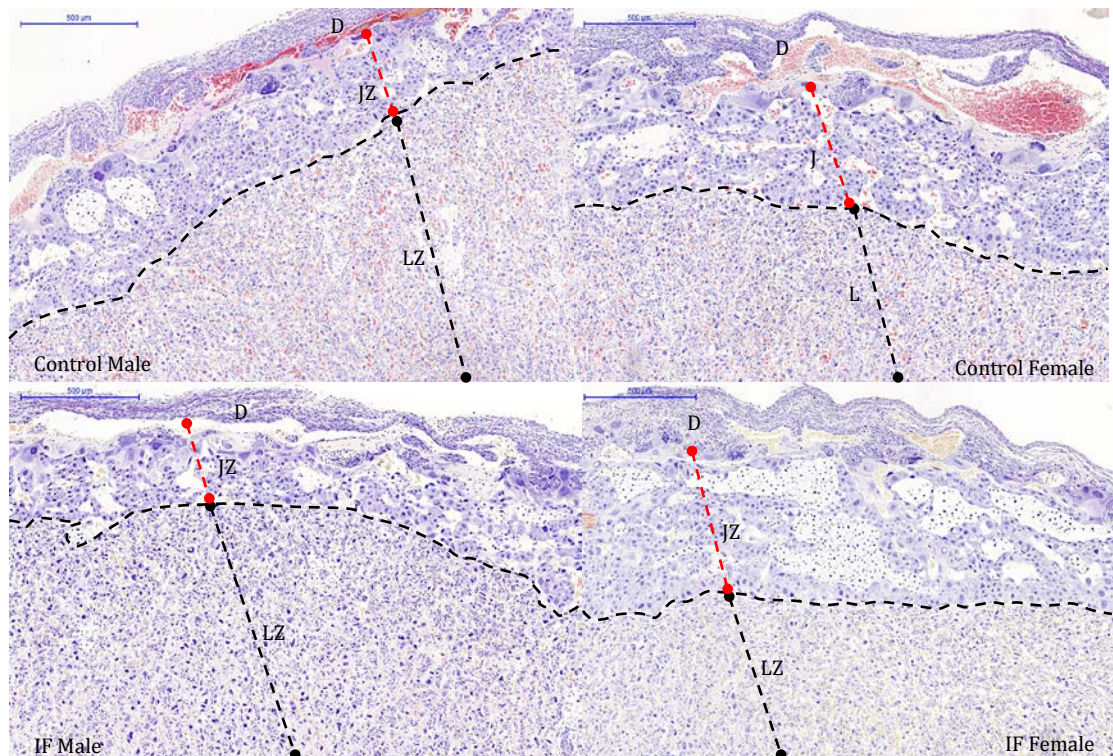
Figure 2.3.19 Representative images for histological comparison of rat placental morphology between both sexes of control and IF groups at GD 21. Junctional zone (JZ) appears thinner while labryinth zone (LZ) appears larger in male placentas of both dietary groups compared to their female counterparts. Dashed line shows separation of JZ and LZ. D, decidua.

Table 2.3.3 Placental area of control and IF groups at GD 21

	Control (n = 5 placentas/3 litters)		IF (n = 5 placentas/3 litters)	
	Male	Female	Male	Female
LZ:total area (%)	83.4 ± 1.4	75.2 ± 1.0***	80.2 ± 0.8	73.6 ± 0.6**
JZ:total area (%)	16.6 ± 1.4	24.8 ± 1.0***	19.8 ± 0.8	26.4 ± 0.6**
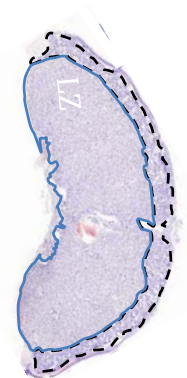
Values are mean ± SEM. ** $P_{IF} < 0.01$, *** $P_{Control} < 0.001$ females versus males (two-way ANOVA followed by Tukey's post hoc test).
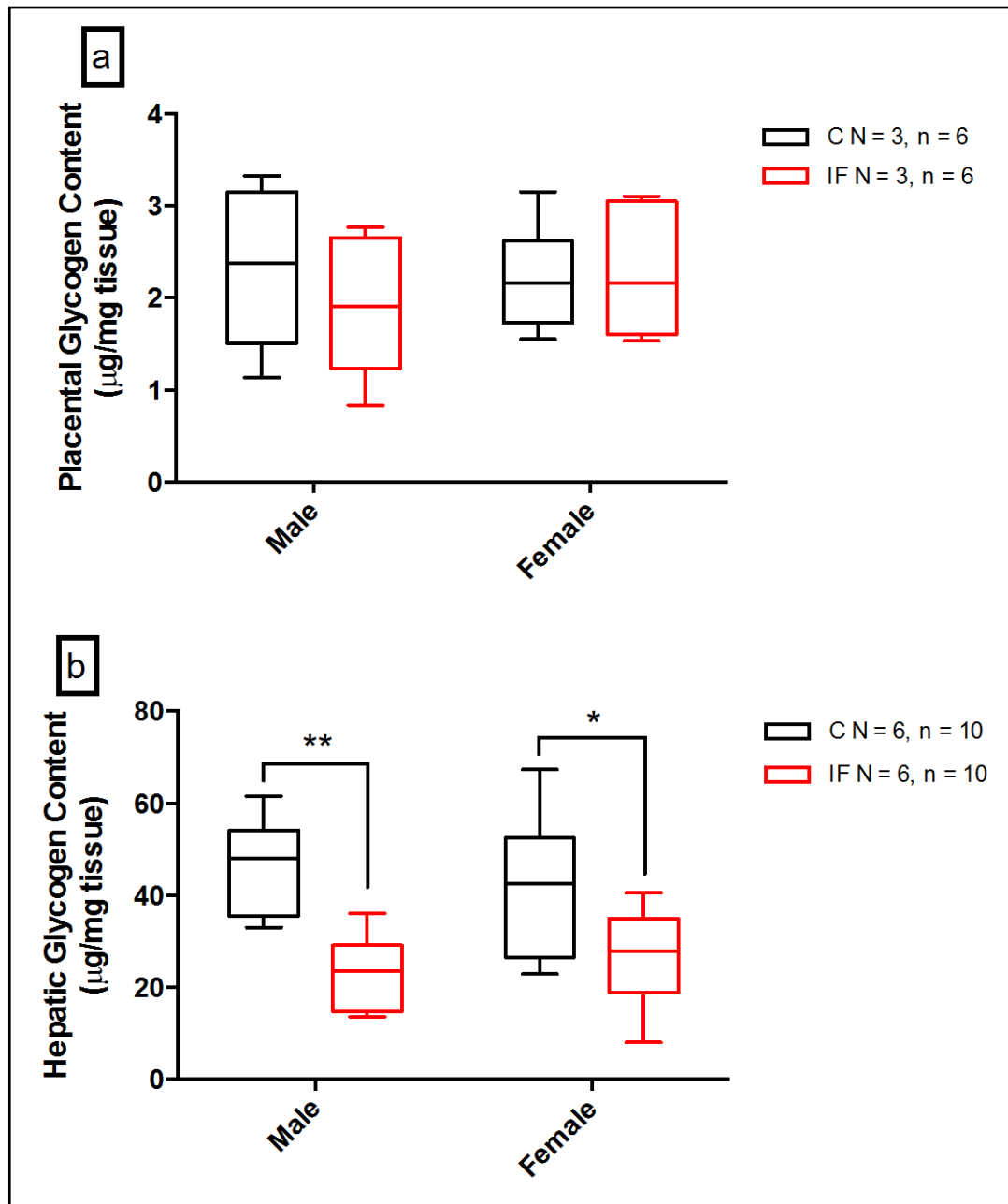
Figure 2.3.20 Glycogen content in **a.** placenta and **b.** fetal liver. Placental and fetal hepatic glycogen contents were normalised to mg of tissue wet weight. No differences in placental glycogen content were observed between control (black) and IF (red) dietary groups at GD 21. Fetal hepatic glycogen content was significantly lower in IF fetuses compared to controls. Data are expressed as box and whisker plots. * $P_{Female} < 0.05$, ** $P_{Male} < 0.01$ IF versus control fetuses. Statistical analysis for placental glycogen content was carried out using Kruskal-Wallis test followed by Dunn's multiple comparisons test whereas for fetal hepatic glycogen content two-way ANOVA followed by Tukey's post hoc test was used.

2.3.12 Metabolomics

To investigate whether maternal intermittent fasting altered placental metabolites, a wide range of metabolites in both the control and the IF groups were compared using one-way ANOVA. The results showed that intermittent fasting affected common metabolites in placentas of both sexes, as shown in Table 2.3.4. Twenty-seven metabolites, of different metabolite classes, were significantly altered in the placentas of both sexes of the IF group compared to controls, the majority (20 metabolites) demonstrating an increased placental concentration, with the most striking being ophthalmic acid, a marker of oxidative stress (Soga et al., 2006). However, in contrast, seven metabolites related to fatty acid and lipid metabolism, including diglycerides, glycerophospholipids, lysophosphatidylserine, steroids and phospholipids, were significantly lower in both sexes of the IF group compared to their counterparts in the control group. Interestingly, sex differences emerged in response to the IF dietary regime, with male placentas showing more profound and diverse metabolite changes than those of females (97 metabolites altered in males compared to 13 in females; data not shown). There was a significant increase in aromatic amino acids such as tryptophan, tyrosine and phenylalanine, and a wide range of trisaccharides in the IF male placentas compared to IF females (Table 2.3.5), with the glucosamine derivative isopentenyladenine-9-N-glucoside (Table 2.3.5) also raised in IF male placentas. Collectively, the placental metabolites altered in association with the IF dietary regime, as listed in Tables 2.3.4 and 2.3.5, suggest that divergent metabolic pathways are affected in the placenta, with evidence of amino acid, fatty acid, phospholipid, steroid and nucleotide metabolism being particularly perturbed.

2.3.13 Expression of *Slc38a* genes in the placenta

Based on the observed reduction in certain maternal and fetal amino acid concentrations (alanine, histidine and methionine, Table 2.3.2) associated with the IF diet, expression of the genes encoding placental SNAT proteins (SNAT1; *Slc38a1*, SNAT2; *Slc38a2* and SNAT4; *Slc38a4*) were measured. These genes were selected on the basis that the amino acids affected by intermittent fasting are transported by system A.

2.3.13.1 Expression of *Ywhaz* gene in the placenta

The housekeeping gene *Ywhaz* was expressed in the placentas of both dietary groups (Figure 2.3.21). The standard curve (standards 1 - 8) demonstrated a good linearity and PCR efficiency of 111.7% (Figure 2.3.21 a). All samples were amplified with no amplification of negative controls above the threshold (i.e. no Ct value) detected (Figure 2.3.21 b). The dissociation curve showed a single peak confirming amplification of a single PCR product (Figure 2.3.21 c). Figure 2.3.21 d, shows that *Ywhaz* expression was stable across the dietary groups and sexes

(P = 0.7). Therefore, *Ywhaz* was selected as the housekeeping gene for normalisation for the genes of interest.

2.3.13.2 Relative expression of *Slc38a* genes in the placenta

Each of the genes of interest, *Slc38a1*, *Slc38a2* and *Slc38a4* which encode the SNAT isoforms, were expressed in the placenta. The standard curve showed good linearity and PCR efficiencies falling between 106 and 111% (i.e. doubling of product for each Ct cycle, Figure 2.3.22). All samples were amplified between 18 and 25 cycles (Figure 2.3.23) and a dissociation curve was generated showing a single PCR product and primer specificity with no amplification of negative controls detected (Figure 2.3.24).

The expression of all the genes was normalised to the expression of the housekeeping gene, *Ywhaz*. The relative expression of the *Slc38a* genes was similar in the placentas of the control male fetuses (Figure 2.3.25). In contrast, expression of *Slc38a4* was significantly greater than that of *Slc38a1* in the placentas of the control female fetuses (P < 0.05, Figure 2.3.25). Furthermore, a sex-specific effect was observed in terms of *Slc38a4* expression, which was significantly higher in the placentas of the control female fetuses compared to their counterpart males (P < 0.05, Figure 2.3.25).

Maternal intermittent fasting induced changes in *Slc38a* gene expression in a sex-specific manner. The relative expression of *Slc38a1, Slc38a2 and Slc38a4* was significantly increased in IF male placentas by 65 ± 12% (P < 0.01), 41 ± 7% (P < 0.01) and 45 ± 12% (P < 0.05) respectively, whereas female placentas displayed no difference between groups (Figure 2.3.26).

2.3.14 Expression of SNAT isoforms

To examine whether the increased mRNA level of *Slc38a* genes observed in the placentas of the IF male fetuses (Figure 2.3.26) translated into increased protein levels, comparisons were made between placentas of both sexes from each dietary group. For SNAT1, an intense immunoreactive species at 52 kDa was observed in all samples, whereas for SNAT2, two distinct bands at approximately 60 and 150 kDa were visualized (Figures 2.3.27 a and c). Quantitation of immunoreactive species intensity revealed that expression of SNAT1 (52 kDa) and SNAT2 (both 60 and 150 kDa species) were similar between the control and IF groups for both sexes (P > 0.05, Figure 2.3.28). All immunoreactive signals were abolished in the absence of primary antibody (data not shown) or following pre-absorption of antibody with excess blocking peptide (Figure 7.1; Appendix).

Table 2.3.4 Metabolites with significant changes in IF placentas of both sexes compared to controls at GD 21

Metabolite	C M	IF M	C F	IF F	q-value
Ophthalmic acid	Not Detected	0.00982	Not Detected	0.00565	6.3E-15
Octahydroindole-2-Carboxylic Acid	Not Detected	0.01547	Not Detected	0.01016	9.0E-13
16alpha-Bromo-17beta-estradiol;N-(4-Guanidinobutyl)-4-hydroxycinnamide	0.00099	0.02146	Not Detected	0.01597	5.4E-11
1-(beta-D ribofuranosyl)nicotinamide	0.00197	0.02122	0.00251	0.01725	5.8E-07
1-(5-Phosphoribosyl)imidazole-4-acetate;5-Hydroxymethyluridine-2'-Deoxy-5'-Monophosphate;5-Methyluridine 5'-Monophosphate	Not Detected	0.01055	0.00264	0.00621	1.4E-05
2-Hydroxybutyric acid;3-Hydroxybutyric acid;4-Hydroxybutanoic acid	0.00096	0.00518	0.00099	0.00260	6.2E-05
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine;N-1-Methylheptylformamide	Not Detected	0.00198	Not Detected	0.00100	0.0002
(6R)-6-(L-erythro-1,2-Dihydroxypropyl)-7,8-dihydro-6H-pterin	0.00110	0.01115	0.00126	0.00559	0.0008
3-Sulfocatechol;4-Sulfocatechol	0.00919	0.00504	0.01485	0.00277	0.0010
3'-UMP;Pseudouridine 5'-phosphate;UMP;Uridine 2'-phosphate;uridine 3'-monophosphate	0.00211	0.00388	0.00139	0.00229	0.0014
gamma-Glutamyl-gamma-aminobutyrate;gamma-L-glutamyl-L-alpha-aminobutyrate;N2-Succinyl-L-ornithine;N6-Acetyl-LL-2,6-diaminoheptanedioate;N-acetyl-L,L-2,6-diaminopimelate	0.00123	0.00710	0.00116	0.00552	0.0019
6-O-Cyclohexylmethyl Guanine	0.02161	0.18231	0.02959	0.07945	0.0034
3,3-Dimethylglutaric acid;3-Methyladipic acid;6-Carboxyhexanoate	0.00453	0.01059	0.00471	0.00787	0.0034
LysoPS(20:4)	0.00139	Not Detected	0.00175	0.00277	0.0052
PA(12:0/12:0);PC(O-18:1/0:0);PC(O-18:1/0:0);PC(O-18:1/0:0)[S];PC(O-18:1/0:0);PC(P-18:0/0:0)	0.01787	0.00876	0.01426	0.01150	0.0067
MG (12:0);dihydroxy-pentadecanoic acid	0.00209	0.00679	0.00266	0.00415	0.0089
oxo-nonanoic acid;hydroxy-nonenoic acid	0.00099	0.01311	0.00241	0.00633	0.0089
3-Oxopregn-4-ene-20beta-carboxaldehyde dioxime;17-Propyl-5alpha-androst-2-en-17beta-ol	0.00202	0.00422	0.00050	0.00283	0.0113
Acetylcarnitine	0.00632	0.01186	0.00769	0.01178	0.0113
Tacrine(8)-4-aminoquinoline	0.00090	0.00359	0.00153	0.00113	0.0113
N6,N6-Dimethyladenosine	0.00559	0.01176	0.00753	0.01156	0.0118
QYNAD;Desmosine;Isodesmosine	0.00064	0.00256	0.00101	0.00145	0.0207
DG(21:0/22:6/0:0);DG(19:0/22:3/0:0);DG(20:3/21:0/0:0)	0.04872	Not Detected	0.06112	0.05306	0.0227
S-(11-hydroxy-9-deoxy-delta12-PGD2)-glutathione;S-(9-hydroxy-PGA1)-glutathione	0.00085	0.00372	0.00109	0.00185	0.0258
(22alpha)-hydroxy-cholestanol;3alpha,7alpha-Dihydroxy-5beta-cholestane;5beta-Cholestane-3alpha,12alpha-diol";"5beta-Cholestane-3alpha,26-diol;5-beta-cholestane-3-alpha,7-alpha-diol;6alpha-hydroxycholestanol;6-Deoxocastasterone	0.00246	0.00125	0.00245	0.00192	0.0426
3'-Deoxydihydrostreptomycin 3'-phosphate;3'-Deoxydihydrostreptomycin 6-phosphate	0.00553	0.00197	0.00578	0.00286	0.0437
PS(36:2);PG(38:7);PE(36:3);PE(38:6)	0.02944	0.01429	0.02446	0.02013	0.0437

Values shown are median. q value = P value control versus IF with black and red values indicating a significant increase and reduction respectively in those metabolites in IF placentas compared to control (one-way ANOVA followed by Tukey's post hoc test). C F, control female; C M, control male; DG, diglyceride; IF F, IF female; IF M, IF male; LysoPS, lysophosphatidylserine; PA, monoacylglycerophosphates; PC, glycerophosphatidylcholine; PE, glycerophosphatidylethanolamine; PG, phosphotidylglycerol; PS, glycerophosphotidylserine.

Table 2.3.5 Metabolite differences between sexes of IF placentas at GD 21

Metabolite	IF F	IF M	q-value
Phenylalanine	0.02676	0.06238	0.0437
Tryptophan	0.00737	0.01983	0.0148
Tyrosine	0.00679	0.00961	0.0393
Isopentenyladenine-9-N-glucoside	0.00163	0.00273	0.0438
Trisaccharide (1,6-beta-D-Glucan;1-alpha-D-{(1,4)-alpha-D-Glucosyl}(n-1)-alpha-D-glucopyranoside;1F-beta-D-Fructosylsucrose;1-kestotriose;3-Galactosyllactose;6-alpha-D-(1,4-alpha-D-Glucano)-glucan;6-alpha-Maltosylglucose;6F-alpha-D-Galactosylsucrose;6G-kestotriose;6-kestotriose;beta-D-Fructofuranosyl O-beta-D-glucopyranosyl-(1-6)-alpha-D-glucopyranoside;beta-D-Glucan;Cellotriose;Dextrin;D-Gal alpha 1->6D-Gal alpha 1->6D-Glucose;fagopyritol B2;Galactomannan;Isomaltotriose;Laminarin;Levan;Maltotriose;manninotriose;Melezitose;Panose;Polysaccharide;Raffinose;Umbelliferose)	0.00220	0.00363	0.0326

Values are shown as median. q value = P value, male versus female (one-way ANOVA followed by Tukey's post hoc test). IF F, IF female; IF M, IF male.
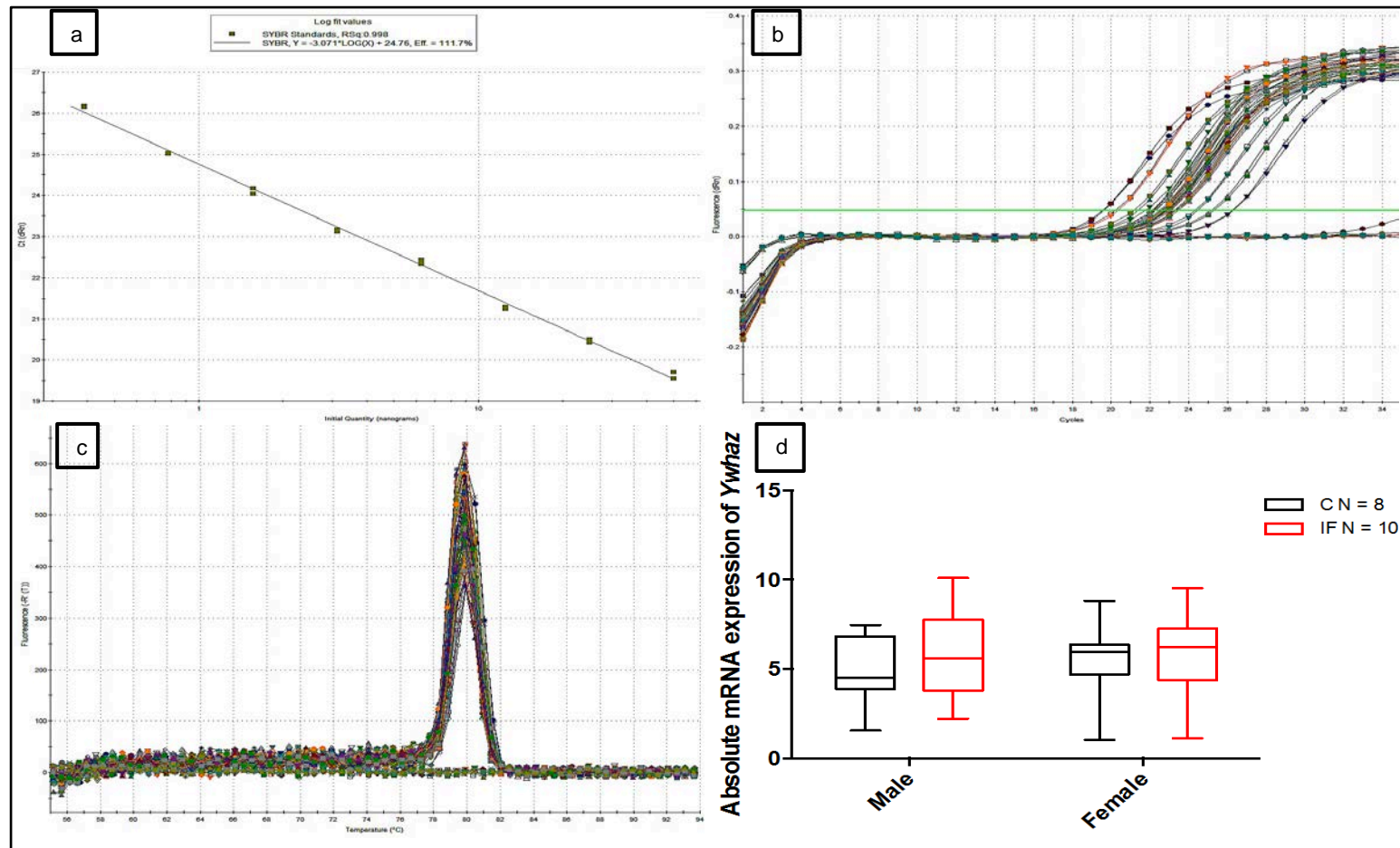
Figure 2.3.21 *Ywhaz* gene expression in the placenta at GD 21. *Ywhaz* mRNA was measured by qPCR in control (black) and IF (red) placentas as a control **a.** Standard curve, **b.** amplification plot, **c.** dissociation curve and **d.** *Ywhaz* expression in both sexes of control and IF dietary groups. No significant differences between dietary groups or sexes were observed. The data in **d.** are presented as box and whisker plot and were analysed using two-way ANOVA followed by Tukey's post hoc test.
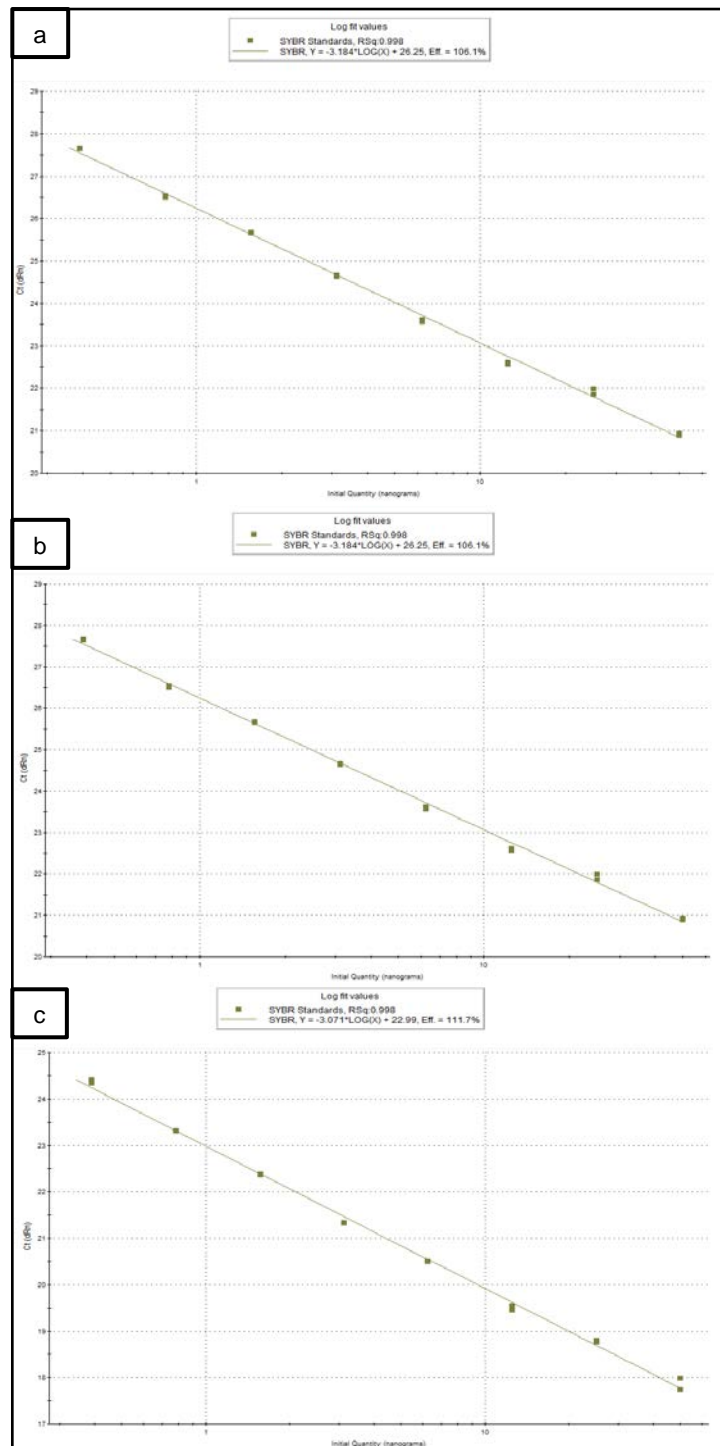
Figure 2.3.22 Standard curves of *Slc38a* genes in the placenta at GD 21. Standard curves between initial cDNA quantity in nanograms (x-axis) versus Ct value (y-axis). There was a good correlation between input cDNA and Ct value (r^2 = 0.998 for all three *Slc38a* genes) with a good efficiency of 106.1% for both **a.** *Slc38a1* and **b.** *Slc38a2* and 111.7% for **c.** *Slc38a4*. The efficiency of the PCR reactions was calculated by the qPCR software.
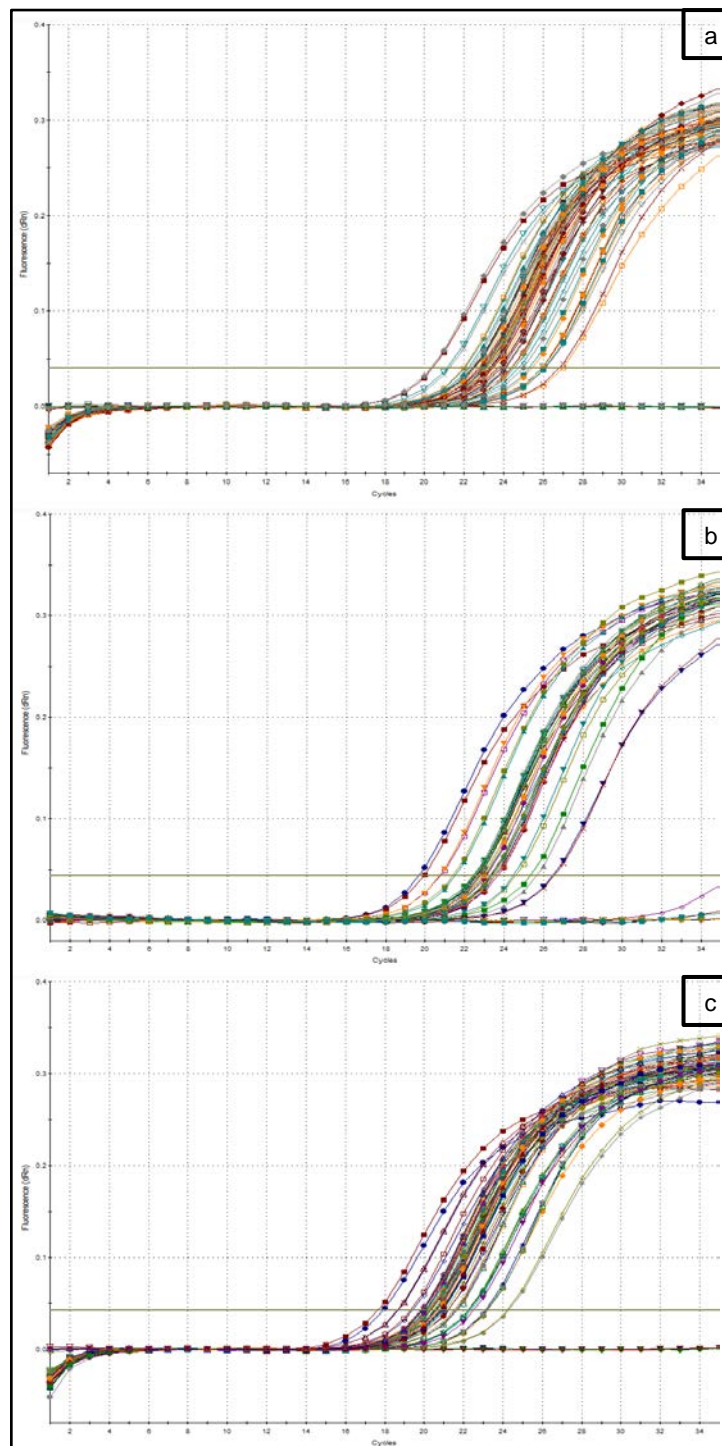
Figure 2.3.23 Amplification plots for qPCR of **a.** *Slc38a1*, **b.** *Slc38a2* and **c.** *Slc38a4* genes in the placenta at GD 21. PCR cycle number of 35 is shown on the x-axis and SYBR Green fluorescence intensity (normalised to ROX) on the y-axis.
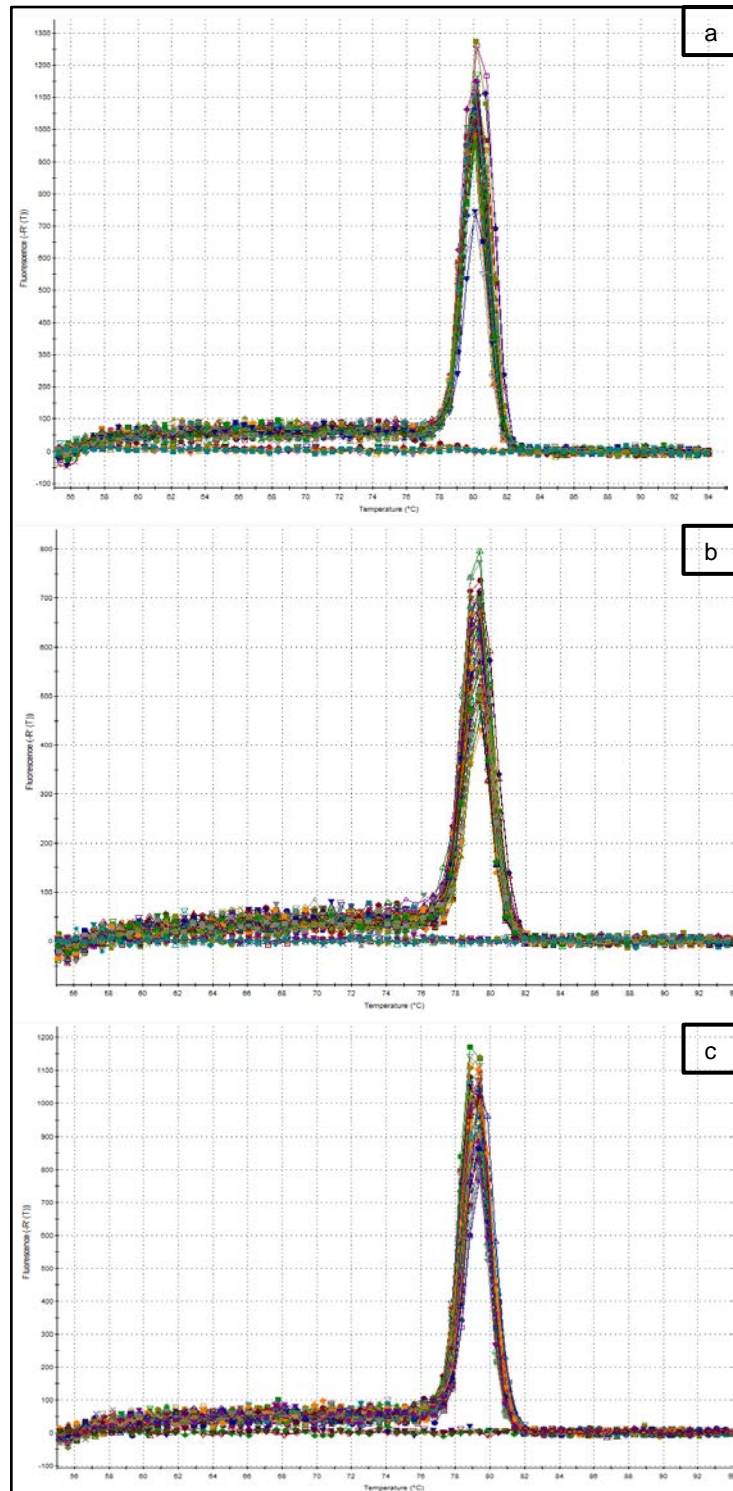
Figure 2.3.24 Dissociation curve (temperature versus fluorescence) for **a.** *Slc38a1*, **b.** *Slc38a2* and **c.** *Slc38a4* genes in the placenta at GD 21. The presence of a single peak demonstrates that each primer generated a single PCR product.
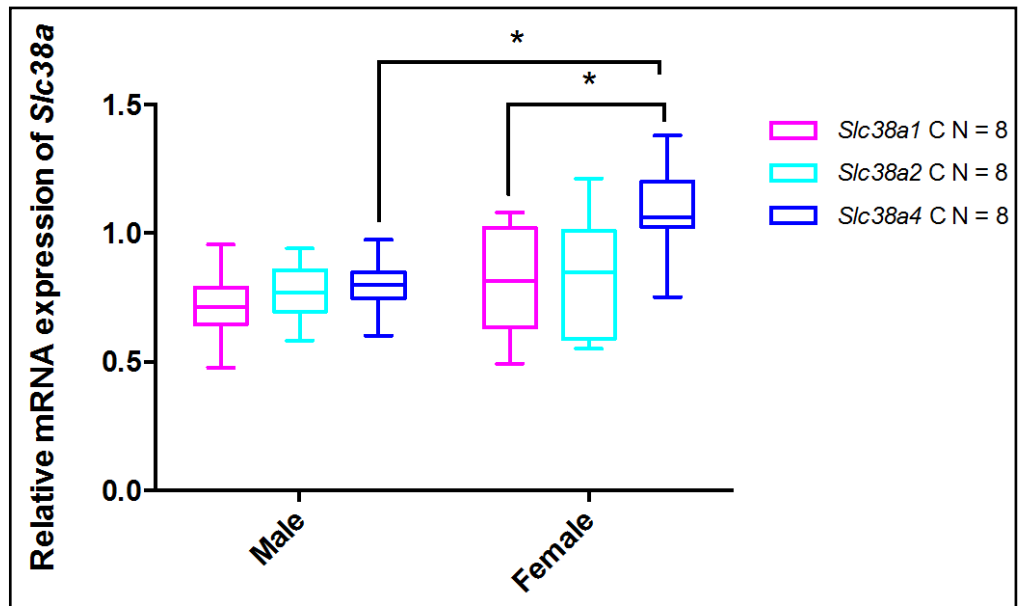
Figure 2.3.25 Comparison between relative mRNA expressions of **a.** *Slc38a1*, **b.** *Slc38a2* and **c.** *Slc38a4* in the placentas of the control dietary group at GD 21. No differences between the relative expression of *Slc38a1, Slc38a2 and Slc38a4* were observed in male placentas. The relative expression of *Slc38a4* was significantly higher in the placentas of female fetuses compared to males. Also, the relative expression of *Slc38a4* differed significantly to the relative expression of *Slc38a1* between the placentas of the control female fetuses. Data are presented as box and whisker plot. * P < 0.05 (two-way ANOVA followed by Tukey's post hoc test).
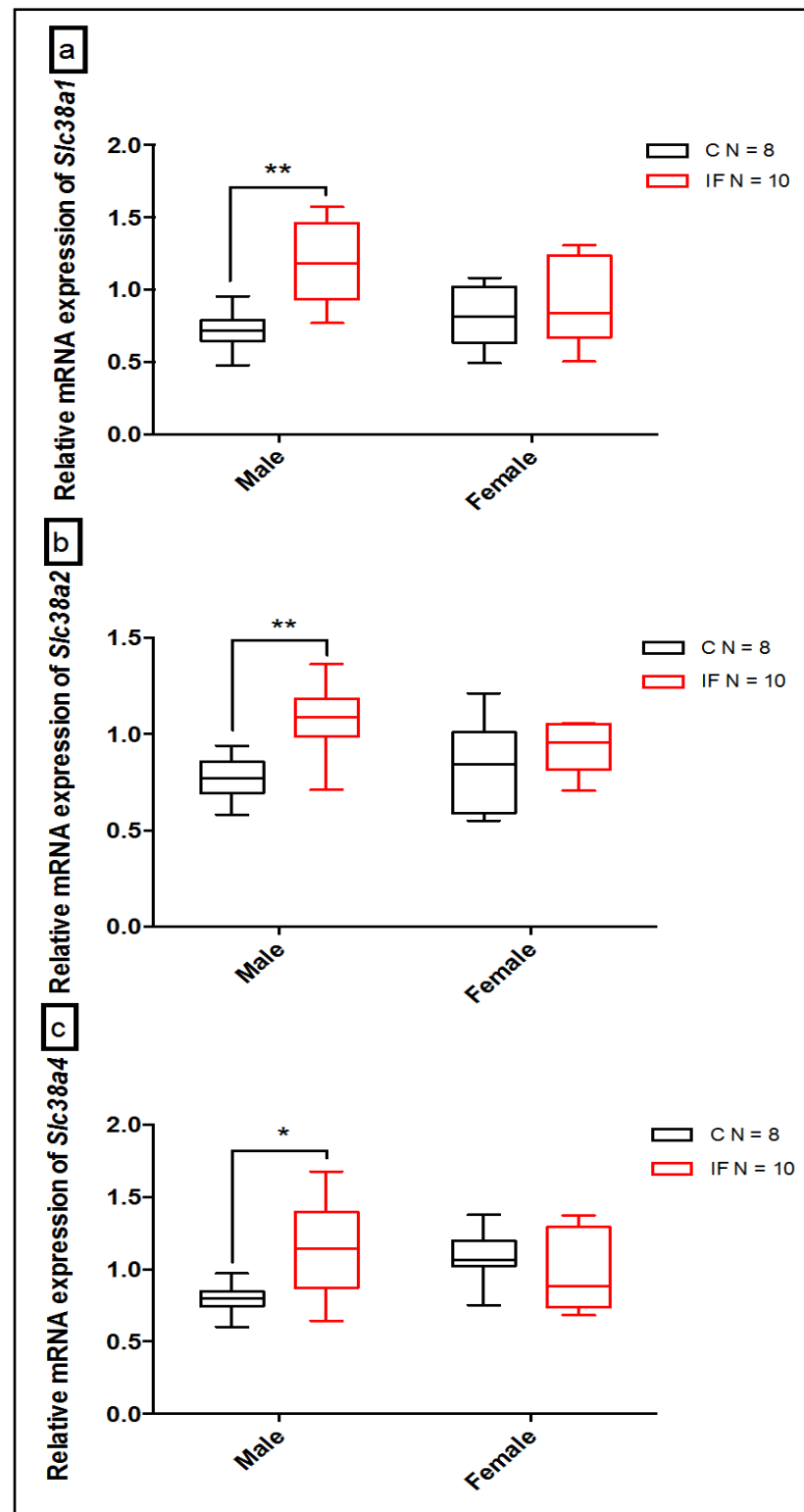
Figure 2.3.26 Relative mRNA expression of **a.** *Slc38a1*, **b.** *Slc38a2* and **c.** *Slc38a4* in the placenta at GD 21. The relative expressions of *Slc38a1, Slc38a2 and Slc38a4* were significantly increased in IF male placentas only. Data are presented as box and whisker plot. * $P < 0.05$, ** $P < 0.01$ IF male fetuses versus control males (two-way ANOVA followed by Tukey's post hoc test).
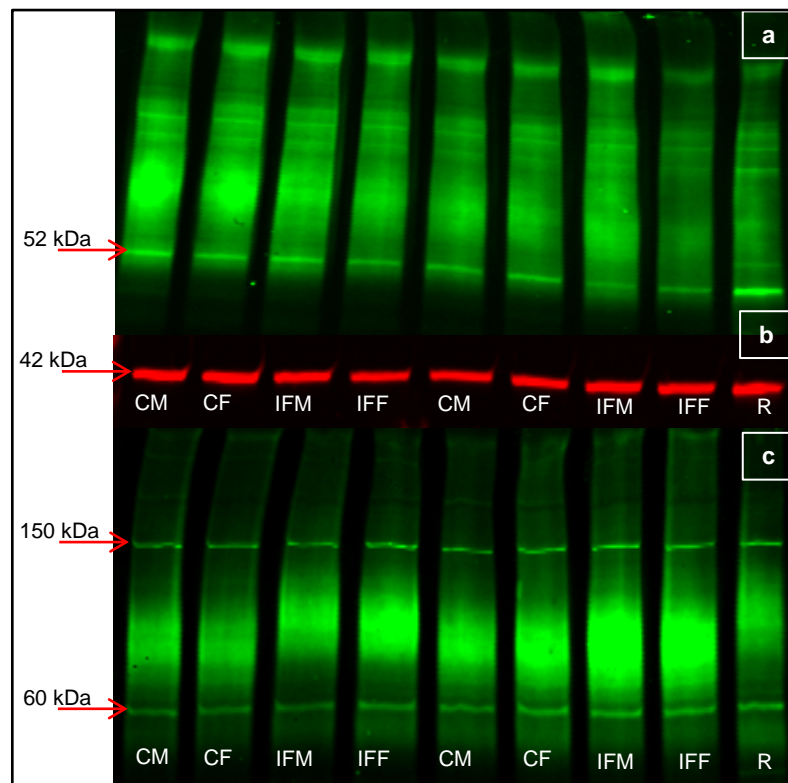
Figure 2.3.27 Western blot of SNAT1 and SNAT2 expression in rat placental vesicles isolated from the placentas of control and IF groups at GD 21 (20 µg protein/lane). **a.** SNAT1 showed a distinct band at the predicted size of 52 kDa. **b.** β-actin antibody generated an immunoreactive band at 42 kDa in all samples, of equal intensity across dietary groups and sexes (data not shown). **c.** SNAT2 showed two distinct bands at 60 and 150 kDa respectively. The blocking peptide and negative controls can be found in the Appendix (Figure 7.1). Abbreviations: CF, control female; CM, control male; IFF, IF female; IFM, IF male; R, reference sample of pooled placentas from control dams at GD 21. Representative of 4 blots.
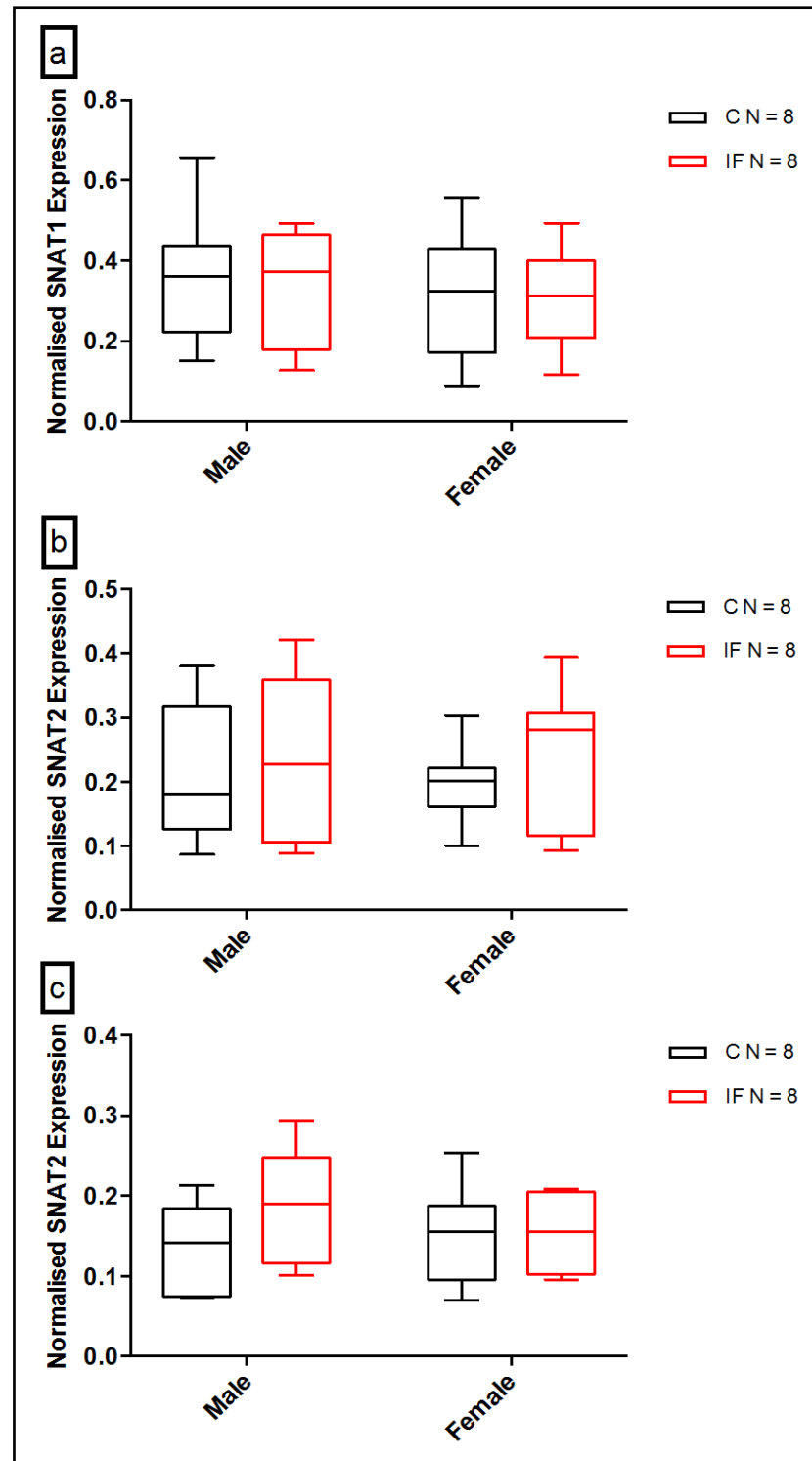
Figure 2.3.28 Normalised expression of **a.** SNAT1, **b.** SNAT2 (60 kDa) and **c.** SNAT2 (150 kDa) in rat placental vesicles at GD 21. The relative protein expression (normalised to β-actin signal intensity) of SNAT1 and SNAT2 was similar in rat placental vesicles between control and IF groups for both sexes. Data are presented as a box and whisker plots. P > 0.05 control versus IF (two-way ANOVA followed by Tukey's post hoc test).

2.3.15 Placental function and system A activity

To determine whether the FGR phenotype associated with the IF dietary regime was caused by an alteration in placental nutrient transport capacity, the activity of the system A amino acid transporter was examined. Diminished system A activity is associated with the severity of FGR (Glazier et al., 1997; Jansson et al., 2002b; Kusinski et al., 2012). In order to address this, placental system A amino acid transport capacity was measured *in vivo* as unidirectional maternofetal clearance of ^{14}C-MeAIB, a preferred substrate for system A amino acid transporter, and *in vitro* as the Na$^+$-dependent uptake of ^{14}C-MeAIB into rat placental vesicles at GD 21.

2.3.15.1 Unidirectional maternofetal clearance of ^{14}C-MeAIB across rat placenta

In vivo, the unidirectional clearance of ^{14}C-MeAIB ($^{MeAIB}K_{mf}$) was measured across the placenta at GD 21. The placental uptake of ^{14}C-MeAIB expressed as dpm placenta/g placenta, was not altered by the IF dietary regime (Figure 2.3.29 a). The placental transport of ^{14}C-MeAIB to the fetus as expressed as dpm fetus/g fetus, on the other hand, was significantly lower in females of the IF dietary group compared to controls ($P < 0.05$, Figure 2.3.30 b). However, this difference was not observed in male fetuses of the same litters, although there was a trend towards a lower concentration, but this failed to reach statistical significance ($P = 0.07$).

Sequential maternal plasma samples taken over 5 min following injection of ^{14}C-MeAIB showed there was no significant difference in plasma clearance between IF and control dams. Therefore, the collective data were pooled to generate a single maternal plasma ^{14}C-MeAIB disappearance curve, displaying the exponential decline of maternal isotope clearance over 5 min (Figure 2.3.30 a).

Based on these data, unidirectional maternofetal clearance of ^{14}C-MeAIB was calculated as the amount of tracer accumulated per fetus at 5 min over the isotope concentration in maternal plasma from time integral 0 to 5 min (calculated as the area under the curve) and divided by placenta weight. $^{MeAIB}K_{mf}$ was significantly reduced in IF males and females by $37 \pm 9\%$ and $41 \pm 8\%$ ($P < 0.05$ and $P < 0.01$) respectively compared to their control counterparts (Figure 2.3.30 b). This implies that altered system A activity could be associated with the FGR observed in IF dietary group.
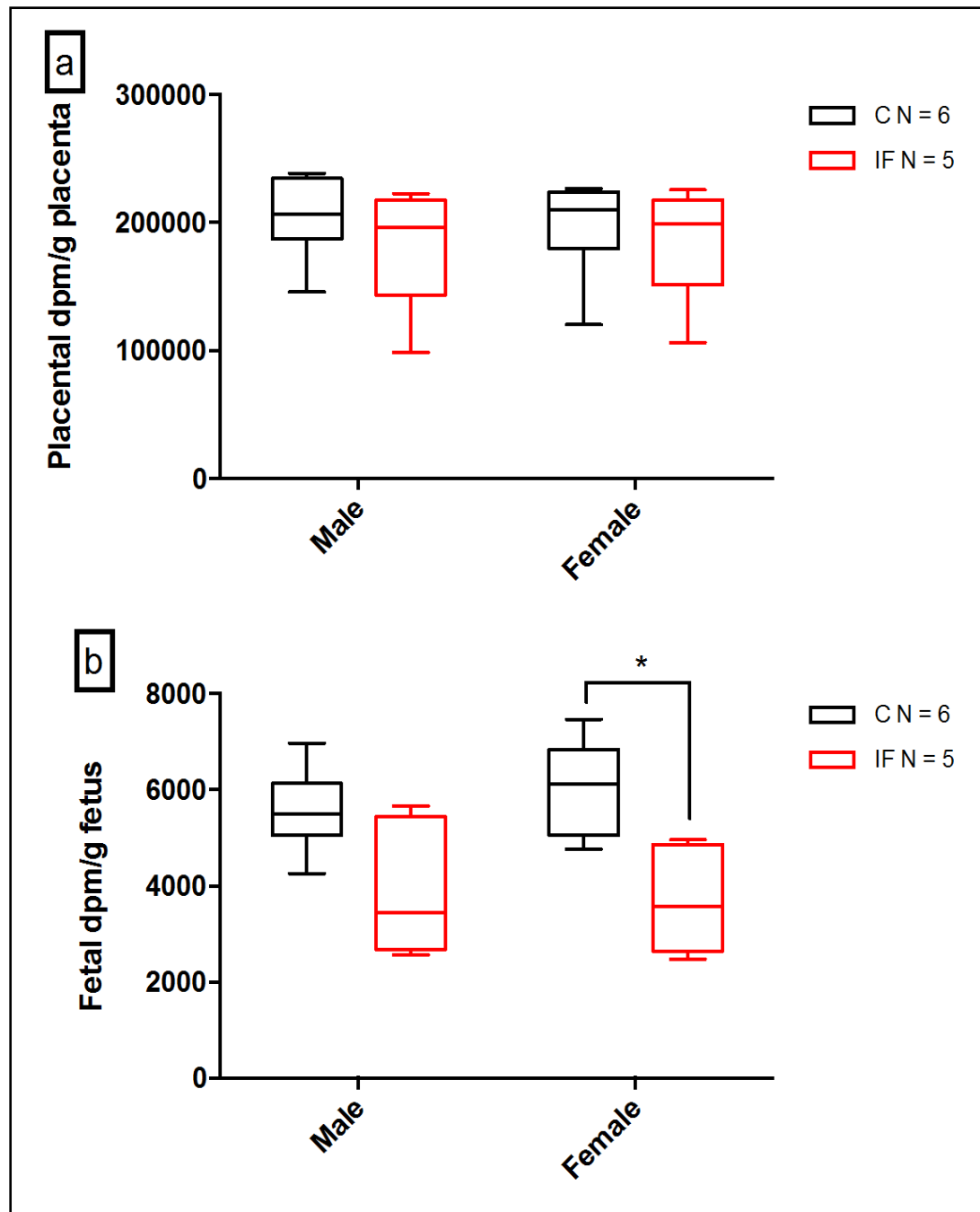
Figure 2.3.29 Placental transport of ^{14}C-MeAIB to the fetus at GD 21. **a.** The placental uptake (placental dpm/g placenta) of ^{14}C-MeAIB in the IF group (red) was similar to the control group (black) for both sexes. **b.** Placental transfer of ^{14}C-MeAIB to the fetus (fetal dpm/g fetus) was significantly diminished only in female fetuses of the IF group compared to controls. Data are presented for mean/litter as box and whisker plots. * $P < 0.05$ IF female fetuses compared to control females. For IF male versus control male comparison, $P = 0.07$. Statistical analysis for placental uptake was carried out using Kruskal-Wallis test followed by Dunn's multiple comparisons test whereas for placental transfer, two-way ANOVA followed by Tukey's post hoc test was used.
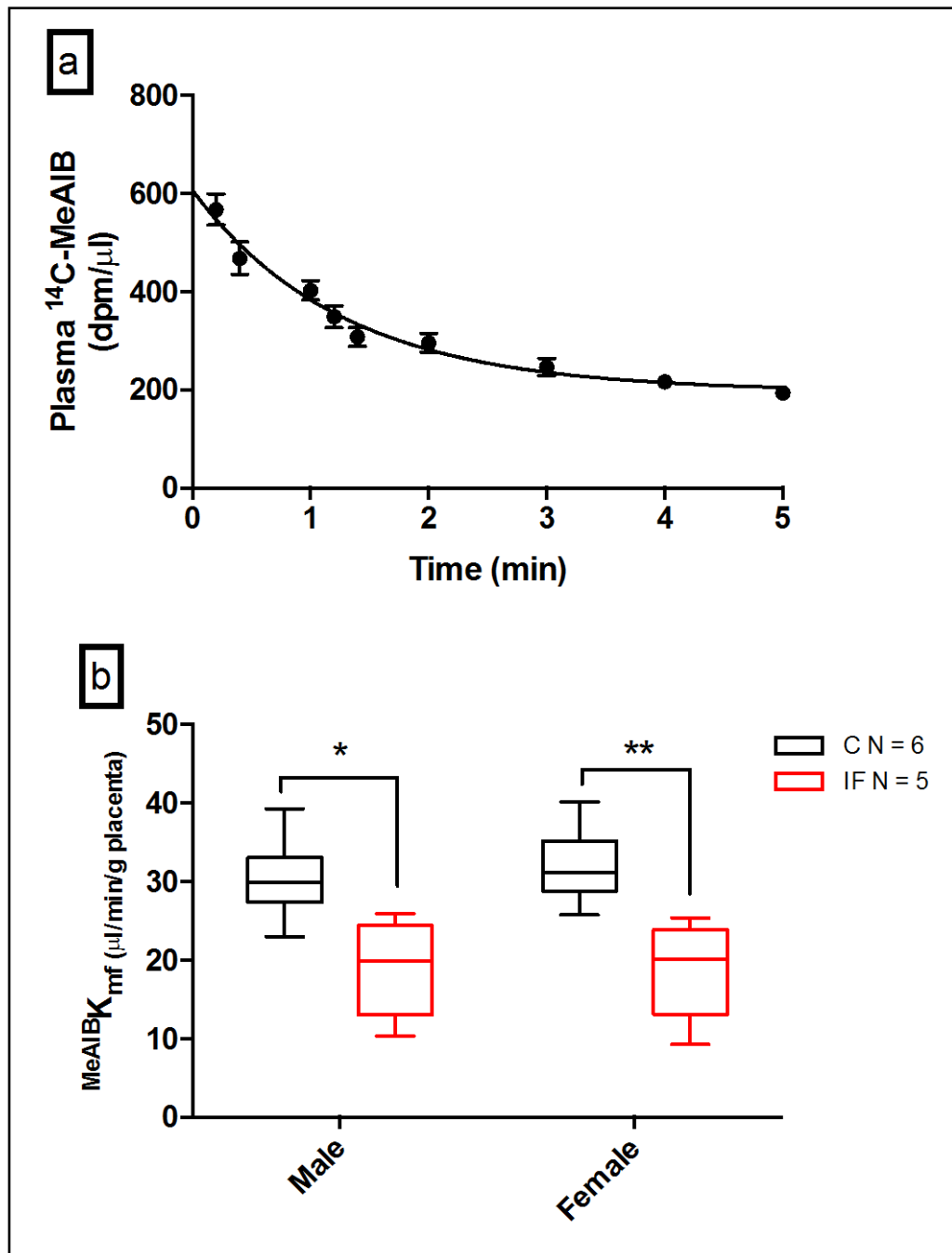
Figure 2.3.30 a. A curve depicting maternal plasma ^{14}C-MeAIB disappearance, constructed from isotope measured in maternal plasma from 6 and 5 dams from control and IF groups respectively, pooled together, and fitted to a one-phase exponential decay model. Data presented as mean ± SEM.

b. Unidirectional maternofetal clearance of ^{14}C-MeAIB ($^{MeAIB}K_{mf}$) across rat placenta at GD 21. $^{MeAIB}K_{mf}$ was significantly diminished in both sexes of the IF dietary group compared to controls. Data are shown for mean/litter as box and whisker plots. * $P_{Male} < 0.05$, ** $P_{Female} < 0.01$ IF versus control group (two-way ANOVA followed by Tukey's post hoc test).

2.3.15.2 System A activity in rat placental vesicles

The enrichment of alkaline phosphatase activity in rat placental vesicles (Table 2.3.6), as a marker of the maternal-facing plasma membrane of SynTB layer II, showed conformance with previously published data in rodents (Kusinski et al., 2010). This was achieved despite the isolation of the plasma membrane being performed from small placental numbers/litter (from 2 up to 12), with similar purity in both groups and sexes (Table 2.3.6). Furthermore, the amount of protein recovered in the rat placental membrane fraction was similar in both groups and sexes (Table 2.3.6).

Table 2.3.6 Vesicle alkaline phosphatase enrichment factors and protein recovery for control and IF rats at GD 21

	Control (N = 13)		IF (N = 15)	
	Male	Female	Male	Female
Alkaline phosphatase enrichment factor [§]	9.7 ± 0.6	9.3 ± 0.4	9.9 ± 0.6	8.4 ± 0.3
Protein recovery (mg/g placenta)	2.6 ± 0.1	2.9 ± 0.1	2.6 ± 0.2	2.6 ± 0.2

Kruskal-Wallis test followed by Dunn's multiple comparisons test. Values are given as mean ± SEM with N = litter number.

[§] Alkaline phosphatase enrichment factor is comparing the activity of alkaline phosphatase over 2 min normalised to the protein content of the vesicles to that of placental homogenate (ΔA410/2 min/µg protein).

Uptake of ^{14}C-MeAIB into rat placental vesicles was linear over 60 s for all conditions, groups and sexes (Figures 2.3.31 a - c, $r^2 \geq 0.98$, $P < 0.05$; linear regression for all analyses). Uptake of ^{14}C-MeAIB into rat placental vesicles for both control (N = 5) and IF (N = 7) dams increased with time over 15 - 60 s and was greater in the presence of Na^+ ($P < 0.0001$) than in its absence (Figures 2.3.31 a and b). Disruption of vesicle integrity with triton resulted in negligible ^{14}C-MeAIB uptake. The uptake in the presence of an inwardly directed Na^+ gradient was lower ($P < 0.0001$) in female fetuses than in male fetuses in the IF group (Figure 2.3.31 b).

The Na^+-dependent uptake of ^{14}C-MeAIB into rat placental vesicles (calculated as the difference between the presence and absence of Na^+) was linear over the time course of 60 s, therefore the uptakes at 60 s were chosen (Control N = 13, IF N = 14). The Na^+-dependent uptake of ^{14}C-MeAIB into rat placental vesicles demonstrated a lack of difference in the uptake by the placentas of IF fetuses compared to controls (Figure 2.3.32).
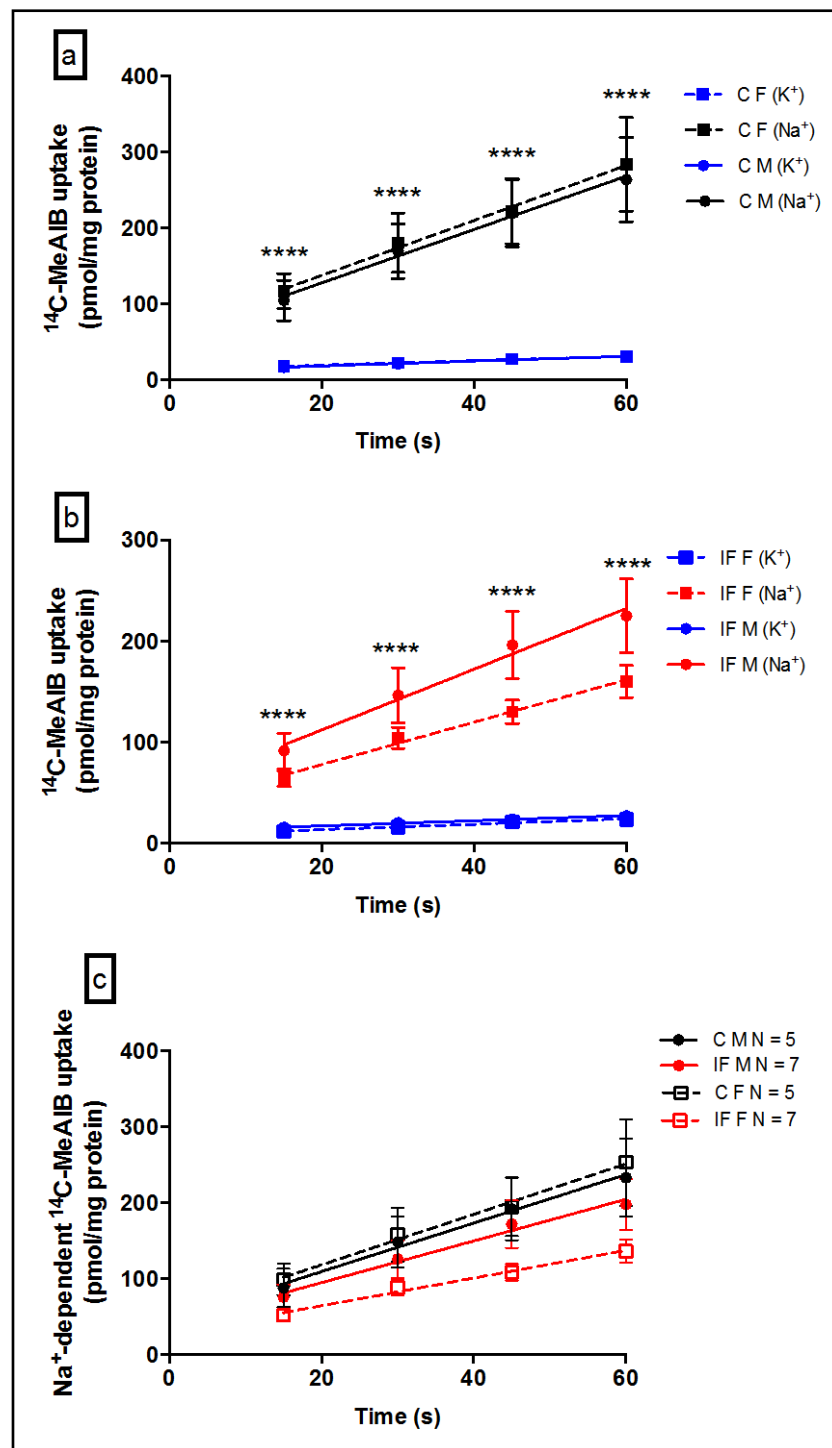
Figure 2.3.31 Time course of ^{14}C-MeAIB uptake into rat placental vesicles at GD 21 in the presence and absence of Na$^+$ (K$^+$ replacement): **a.** control (N = 5) and **b.** IF groups (N = 7) for both sexes. **c.** Time course of Na$^+$-dependent ^{14}C-MeAIB uptake at GD 21 in rat vesicles. Data are expressed as mean ± SEM. **** $P < 0.0001$, IF female (Na$^+$) versus IF male (Na$^+$), **** $P < 0.0001$, Na$^+$ versus K$^+$ uptake of both sexes in two dietary groups (repeated-measures two-way ANOVA followed by Tukey's post hoc test).
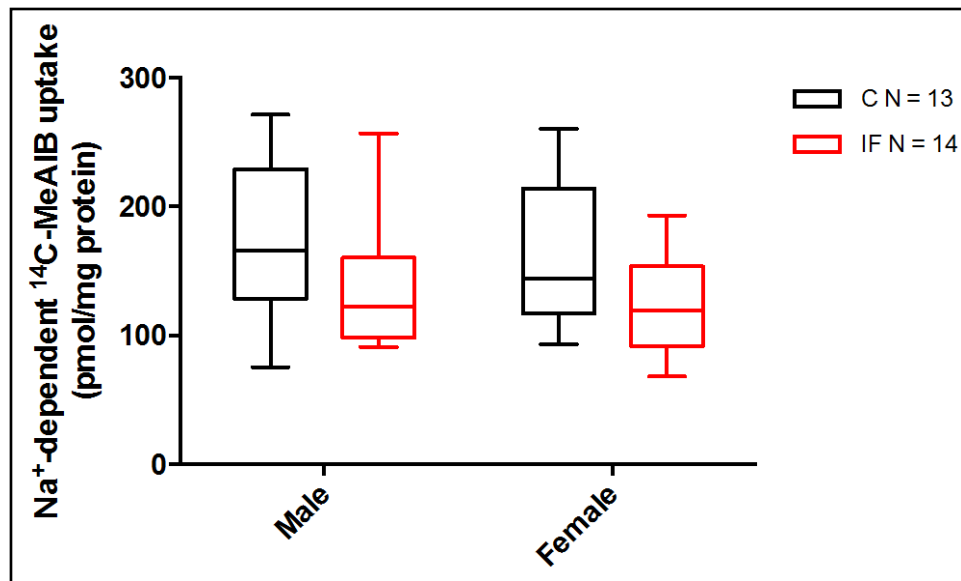
Figure 2.3.32 Na$^+$-dependent ^{14}C-MeAIB uptake at 60 s into rat placental vesicles at GD 21. No difference was observed in Na$^+$-dependent ^{14}C-MeAIB uptake into rat vesicles between the two dietary groups. Data are presented as box and whisker plots. P > 0.05 for diet and sex comparisons. Statistical analysis was carried out using two-way ANOVA followed by Tukey's post hoc test.

2.4 DISCUSSION

In the predominantly Muslim Arabic world, Ramadan fasting during the holy month of Ramadan is a religious practice that spans across the entire society. Pregnant Muslim women are exempted from daily fasting during that month; nevertheless, many pregnant women (70% - 90%) voluntarily participate in the fast during that period (Prentice et al., 1983; Malhotra et al., 1989; Arab and Nasrollahi, 2001; Joosoph et al., 2004; Mubeen et al., 2012). The detrimental effects of Ramadan fasting during pregnancy on maternal and fetal health have been documented by a limited number of research studies (Arab, 2004; Kiziltan et al. 2005; Alwasel et al., 2010a; Sakar et al., 2015).

For this reason, the primary aim of the present study was to recapitulate the dietary impact of Ramadan fasting during pregnancy in an animal model. Although the model employed in this study did not completely resemble Ramadan fasting in humans, in so much that the pregnant rats were deprived of food, but not water, for a period of 16 h each day throughout 21 days of gestation, it can be considered a step forward in understanding how intermittent fasting during pregnancy may affect maternal physiology, fetal development, metabolic profiles, placental morphology, metabolomics and function, particularly of system A amino acid transporter activity, and how fetal sex can interact with maternal diet *in utero*.

Overall, the study clearly showed that intermittent fasting had a compromising influence on the physiology of pregnant dams and the development of their fetuses, as well as the functionality of their placentas. Maternal food intake and bodyweight gain were significantly lower in the pregnant dams of the experimental group compared to controls during pregnancy. The maternal organs in the IF group were also altered: relative liver weight was decreased, while relative kidney weight was increased. Intermittent fasting also had a detrimental impact on maternal metabolic status, as plasma glucose, glucagon and amino acid concentrations were significantly reduced. The litter size was similar in both dietary groups, but IF fetal growth was impaired at GD 21, indicated by the wet weight and fetal anthropometric measurements. The relative fetal organ weights were unaltered by intermittent fasting. However, the brain/liver weight ratio was significantly higher in the IF group.

Maternal intermittent fasting also affected the fetal metabolite profiles in which plasma insulin and amino acid concentrations were reduced, while glucose and glucagon concentrations remained unaltered. The normal fetal glucose concentration in the presence of altered maternal plasma glucose was accompanied by a reduction in fetal liver glycogen contents. With regards to placenta, the wet weight and the ratio of the junctional zone and the labyrinth zone to the total placental area were similar in the two groups. The placental glycogen content in both dietary groups was also comparably similar. Interestingly, there were normal sex differences in the structure of the placenta, as females of both dietary groups had larger junctional and

smaller labyrinth zones than males. A striking impact of maternal intermittent fasting was seen in placental metabolomics; there were some obvious major alterations in amino acids, cysteine and methionine metabolism, glutathione metabolism, glycerophospholipids and nucleosides and nucleotides. There were also sex-specific changes in aromatic amino acids particularly phenylalanine, tyrosine and tryptophan, which were significantly higher in IF males than IF females. Looking at the placental system A amino acid transporter, *Slc38a* isoforms were significantly up-regulated only in IF male placentas, but not in females. This up-regulation was not mirrored by any changes in SNAT protein expression. The activity of the system A transporter, on the other hand, was significantly reduced *in vivo*: ^{14}C-MeAIB unidirectional maternofetal clearance was lower in IF placentas of both sexes, which contrasts with the trend seen in *in vitro* measurements of Na^{+}-dependent uptake of ^{14}C-MeAIB into rat placental vesicles.

2.4.1 The effect of intermittent fasting during pregnancy on maternal physiology

Dams subjected to intermittent fasting consumed 30% less food than controls. This means that the reintroduction of food in the morning did not provide the IF dams with enough opportunity to compensate for the food withdrawal during the night. This is attributable to the dams' inclination to sleep following their first feeding session in the morning and remain relatively inactive during the day. As a consequence, their overall food intake was not sufficient to sustain normal weight gain during pregnancy. The IF dams therefore exhibited reduced daily weight gain over the course of pregnancy starting from GD 8 onward.

The pregnancy period in rats is divided into two phases: anabolic and catabolic (Anderson et al., 1980). The first half of pregnancy is the anabolic phase when maternal weight gain reflects the body's nutrient store accumulation. The second half of pregnancy is the catabolic phase, where maternal weight gain is mostly due to rapid fetal growth. During the first half of gestation, IF dams showed nutrient accumulation indicated by increased bodyweight, albeit insufficient accumulation to maintain a weight comparable to control dams. During the second half of the pregnancy, IF dams' weight gain was also significantly lower than that of the control dams, therefore reflecting limited fetal growth. Since the water intake relative to 100 g bodyweight of the two dietary groups was similar, water intake did not have an impact on the lower weight observed in IF dams. These observations agree well with the findings of previous studies, where pregnant rats that were food-restricted for 19 h per day (from 2 pm until 9 am the second day) during 18 days of pregnancy ate 50% less and dropped weight compared to the control dams (Kawaguchi et al., 1994). The food-restricted model, imposed at any stage of pregnancy or throughout gestation, commonly leads to a reduction in maternal weight gain during pregnancy (Anderson et al., 1980; Lederman and Rosso, 1980; Chen and Chou, 2009; Coan et al., 2010; Belkacemi et al., 2011c). Despite protein-restricted dams showing increased food

consumption, their bodyweight gain was significantly lower than that of the control dams (Jansson et al., 2006; Coan et al., 2011; Rosario et al., 2011).

The current outcome has relevance to Ramadan fasting in humans as a study conducted by Arab (2004) found that of 91.9% of pregnant women participating in Ramadan fasting suffered daily calorific deficiencies of over 500 kcal with respect to the recommended daily calorific intake of ~2500 kcal. Another study by Cole (1993) found that fasting pregnant women gained weight over the first week of Ramadan, but then the participants gradually lost more than 1 kg of bodyweight over the remaining three weeks. Kiziltan et al. (2005) also reported that pregnant women exposed to Ramadan fasting showed reductions in calorific intake and bodyweight, especially if they fasted during the second and third trimesters. Hence, Ramadan fasting during pregnancy is associated with diminished maternal weight gain and reduced calorific intake. In addition, fasting pregnant women had significant increases in the serum levels of cortisol, which is a type of stress-related steroid hormone, due to changes in eating and sleeping patterns during Ramadan (Dikensoy et al., 2009). Therefore, as the IF dams were exposed to a repeated cycle of fasting overnight throughout pregnancy, this may have elicited stress responses associated with the pattern of regular food deprivation, which was clearly evidenced by the IF dams becoming more anxious and agitated. Thus, measuring maternal corticosterone levels in the future would be worthwhile.

In normal pregnant rats, both liver and kidney weights increase significantly with the advance of gestation (Davison and Lindheimer, 1980; Borlakoglu et al., 1993; Kuriyama et al., 2000; Cornock et al., 2010). The increase in liver weight was ascribed to a transitory swelling of parenchymal cells during pregnancy (Borlakoglu et al., 1993). However, intermittent fasting altered maternal organ weights such that relative liver weight was significantly reduced, whereas relative kidney weight was significantly increased; there was no effect on relative heart weight. In a study by Kuriyama et al. (2000), food-restricted pregnant rats showed less liver enlargement compared with the control group at GD 20 (term 22), which is in agreement with the findings of the present study. Hence, there is evidence that nutritional perturbations *in utero* can alter maternal liver growth and potentially function.

With regards to kidney weight, Garland et al. (1978) reported an increase in the dry kidney weight of pregnant rats accompanied by an increase in proximal tubule length. Davison and Lindheimer (1980) disputed this observation and suggested that renal hypertrophy in pregnant rats was due to an increase in water content rather than morphological changes. They showed that dry kidney weights were similar for both pregnant and non-pregnant rats and highlighted that changes occurring during pregnancy are functional in nature. As normal pregnancy progresses, plasma volume and glomerular filtration rate increase (Lindheimer and Katz, 1971; Rosso and Streeter, 1979; Davison and Lindheimer, 1980), the former as a result of changes in the feto-placental compartment (Van Mieghem et al., 2009). In protein-restricted pregnant

dams, kidney weights were similar to those of pregnant control dams; however, kidney function was altered (Cornock et al., 2010). The creatinine clearance, intra-renal AT_2 receptor expression, and vasopressin-related aquaporin 2 mRNA and protein expressions were significantly reduced in protein-restricted pregnant dams (Cornock et al., 2010). Therefore, the observed hypertrophy of the IF kidney may be due to increased water content and an alteration in renal function, an outcome that is worthy of further investigation.

Food and protein restriction during pregnancy have been reported to cause a reduction in maternal plasma volume that is correlated with FGR (Rosso and Streeter, 1979; Welham et al., 1998). In relation to Ramadan fasting during pregnancy, a Turkish study conducted during the summer of 2014, when food and water abstinence lasted for more than 17 h/d, showed an alteration in the renal function of fasting pregnant women indicated by a significant increase in haematocrit, blood urea nitrogen and potassium concentrations, and blood and urinary neutrophil gelatinase-associated lipocalin (NGAL), which is considered an early biomarker for renal injury (Bayoglu Tekin et al., 2016). The adverse effect extended to the fetus showing increased fetal renal artery Doppler indices. This underscores the elevated risk of renal dysfunction in pregnant women and their fetuses as a result of Ramadan fasting.

2.4.2 The effect of maternal intermittent fasting on fetal development during pregnancy

In this study, the reduced food intake of the IF group had no impact on either litter size or placental weight, yet fetal development was profoundly affected as evidenced by a significantly reduced fetal weight (15%), fetal length (crown: rump length) and head circumference. Males were heavier than females in the control groups, which is consistent with findings by Alwasel et al. (2011) in humans and Zhang et al. (2010) in rats, but the sex-differences no longer existed following intermittent fasting. This finding concurs with previous observations in pregnant rats that were food-restricted for periods of 12 h or longer before GD 10.5 (Ellington, 1980). It was found that fetal length was decreased in fasting animals compared to control animals, as well as the protein content of the embryo and somite number (Ellington, 1980), consistent with observations made in the present study. In addition, Kawaguchi et al. (1994) found that intermittent fasting had no effect on litter size, placental weight or duration of pregnancy. Likewise, a calorific-restricted diet provided a similar reduction in maternal weight gain with no effect on litter size; however, placental and fetal weights were significantly reduced (Ahokas et al., 1981; Woodall et al., 1996; Agale et al., 2010; Coan et al. 2010; Belkacemi et al., 2011c; Ganguly et al., 2012).

Several observations made here mimic fetal/neonatal outcomes in pregnant women who fasted during Ramadan. When the duration of Ramadan fasting was more than 17 h/d, significant decreases in fetal biparietal diameter, fetal head circumference and fetal femur length (Sakar et al., 2015) along with birth weight (Savitri et al., 2014) were observed. Apart from this, a

reduction in the amniotic fluid index of the pregnant women was also reported (Seckin et al., 2014; Sakar et al., 2015). Even when the duration of Ramadan fasting was only up to 10 h/d, a reduction in heart rate accelerations and fetal breathing movement in the fetuses of fasting pregnant women were recorded (Mirghani et al., 2004; 2005).

In an extensive study, Almond and Mazumder (2011) examined the short- and long-term effects of *in utero* exposure to Ramadan fasting with regard to birth outcomes. They found that mothers who exposed their unborn fetuses to Ramadan fasting during their pregnancies gave birth to children with birth weights reduced by 40 g (Almond and Mazumder, 2011). Additionally, census data from Iraq, Uganda and the United States reported that Ramadan fasting, especially during the first trimester during which organogenesis occurs, was associated with a higher number of sensory and mental disabilities in the children (Almond and Mazumder, 2011). These findings cast an interesting light on the smaller head circumference of IF fetuses observed in this study. Fetal organ weights were measured in the current study to investigate a link between observations in humans and rats. Fetal brain and kidney weights were significantly lighter in both sexes of IF fetuses, while fetal liver weight was reduced only in the female fetuses of the IF group. The organ weights relative to bodyweight revealed that the reduction was proportionate to growth restriction seen in IF fetuses. The fact that the organ weights to bodyweight ratios of the two groups are comparable does not necessarily reflect a normal structure of the organ in the IF group. For example, in rats protein-restricted during pregnancy, fetal liver structures were altered at GD 21.5 exhibiting increased apoptosis, glycogen granules and vacuolization of the hepatocytes (Ramadan et al., 2013).

One factor that is used as an index of intrauterine growth retardation is the fetal brain/liver weight ratio (Mitchell, 2001). It also indicates whether slow growth of fetal abdominal organs protects the development of the fetal brain. In the current study, IF fetuses of both sexes showed a significant increase in the fetal brain/liver weight ratio. Therefore, the IF brain is spared at the expense of the liver. One possible mechanism behind the phenomenon of the fetal brain being spared from the impact of food restriction is the compromise of glucose provision to the IF fetuses due to the low maternal glucose concentration, as observed in the study. Such a glucose deficit is a powerful stimulus that elicits responses to allow the fetus to adjust for this nutritional shortage by dispersing its available energy to more vital organs, particularly the brain, while neglecting other organs (Godfrey and Barker, 2001). Burrage et al. (2008) discovered that the carotid body plays a role in this mechanism, imitating the protection reponse of fetal vital organs to undernutrition in fetal sheep at the expense of liver growth. The carotid body is sensitive to low blood glucose (Zhang et al., 2007) and can employ neural mechanisms resembling hypoxia that trigger the redistribution of fetal cardiac output, shunting the blood flow towards essential vascular vessels, for example those supplying the brain, and away from peripheral vessels (Giussani et al., 1994).

Other studies have reported no correlation between malnutrition and fetal brain development (Wainwright et al., 1985; Agale et al., 2010). Fetuses exposed to 40% calorific-restriction during pregnancy had similar brain weights and antioxidant and fatty acid concentrations to fetuses of the control group (Agale et al., 2010). This raises the possibility that the fetal brain was spared under this condition. Furthermore, Wainwright et al. (1985) found similar fetal brain weights in dams on low protein (8%) and normal protein (20%) diets.

However, other researchers have commented that in the model where protein-restriction was imposed at conception and the first two weeks of gestation, astrogenesis, neuronal differentiation, production of extracellular matrix and programmed cell death in the cerebellum and in the cortex of offspring during the first 2 weeks of age were hindered (Gressens et al., 1997). Such a transient alteration of brain architecture no longer existed in adult brains, implying an incredible level of plasticity in the developing brain (Gressens et al., 1997). In Zhang et al. (2010), male offspring exposed to 50% food restriction during gestation and lactation showed impaired learning and memory function at postnatal day 70. This was in part due to the reduction in the density of nitric oxide synthase neurons in the hippocampus of those offspring's brains.

It is also interesting to note in the context of maternal hypoglycaemia and the reduced fetal head circumference observed here in the IF group, that brain cell number and myelination are positively related to the maternal glucose index (Saintonge and Côté, 1987). Taken together, this could imply that the intermittent pattern of fasting in the model employed here predisposes more profoundly to a damaging effect on the development of the fetal brain. In an epidemiological study of Ramadan fasting, the ratio of head circumference to bodyweight was significantly greater in boys than girls which revealed that in adverse conditions, boys favour brain growth at the cost of trunk growth (Alwasel et al., 2011).

Previous studies have revealed that male and female fetuses/neonates exhibit different patterns of adaptation to intrauterine insults. It is intriguing that the predisposition to exencephaly, snout face deformity and stillbirth observed in the present study occurred in female fetuses exposed to intermittent fasting. This may be a consequence of maternal low blood glucose which could invoke a state of 'accelerated starvation'. This phenomenon usually occurs during fasting, where the body attempts to compensate for the diminished fuel supply by mobilising fat stores, yielding ketones as a byproduct (Metzger et al., 1982). Ketones diffuse easily from the maternal circulation to the fetus (Hunter and Sadler, 1987). An *in vitro* study in mice embryos at GD 9 demonstrated that when these were exposed for 24 h to D-β-hydroxybutyrate, a ketone body, the embryos developed neurological malformations and growth restriction (Hunter and Sadler, 1987). Rat embryos cultured in the serum of food-restricted dams showed growth restriction, an effect diminished when glucose was added to the serum (Ellington, 1980). Runner and Miller (1956) demonstrated vertebral, costal deformities

and exencephaly in mice fetuses of dams exposed to food restriction for a period of 24 to 30 h during early gestation. Collectively, these studies highlight the importance of maintaining adequate glucose homeostasis and energy balance in pregnancy, and that fasting during pregnancy can have impacts on fetal development particularly with regards to brain development and long-term mental ability. This aspect was followed up in long-term longitudinal studies by investigating the impact of intermittent fasting on behaviour and cognition in the offspring (Dr Nick Ashton, personal communication).

Sex-specific changes have been reported in offspring exposed to Ramadan fasting in mid-gestation: boys were taller and girls had a shorter gestational period compared to fetuses that were not exposed to Ramadan fasting (Alwasel et al., 2011). Other perinatal outcomes exhibit sex-differences; for example, male fetuses have a higher risk of diabetes-related morbidity (Bracero et al., 1996) and preterm birth (Zisk et al., 2011) than females. Exposure to glucocorticoids in sheep during early pregnancy is associated with a higher incidence of growth restriction in female fetuses compared to male fetuses (Miller et al., 2012). Hence, fetal glucocorticoid exposure is one mechanism that could 'programme' or alter fetal growth trajectory in a sex-related manner. Indeed, overexposure of the fetus to glucocorticoids is proposed to be one of the major mechanisms by which 'developmental programming' occurs, associated with a slowed fetal growth, altered fetal organ development, epigenetic modification of gene expression and activation or changes in the activity of the hypothalamic-pituitary-adrenal axis in the offspring, programming for cardiometabolic disease later in life (Reynolds, 2013).

In the context of Ramadan fasting, it was shown that fasting pregnant women have higher cortisol concentrations due to their changes in eating and sleeping patterns during Ramadan, which affect the circadian rhythm of cortisol secretion (Dikensoy et al., 2008). It is possible that a similar case applied to this study. In the literature, pregnant rats subjected to 50% calorific-restriction demonstrated higher plasma corticosterone concentrations than control dams (Belkacemi et al., 2011c). This was accompanied by a significant reduction in 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2) activity in the placenta, whose specific purpose is to convert the bioactive corticosterone into the inactive form 11β-hydrocorticosterone (Belkacemi et al., 2011c). Overexposure to maternal corticosterone leads to FGR at GD 21 with a down-regulation of system A transporter proteins SNAT1 and SNAT2. The system A transporter is one of the major subjects of the current study that will be discussed in more detail below.

2.4.3 Maternal intermittent fasting during pregnancy altered metabolic characteristics of mothers and their fetuses

In this study intermittent fasting had, as expected, a negative impact on maternal glucose and glucagon concentrations which were significantly lower than in control dams. However, it must be noted that blood was collected from the IF dams in the morning before food was introduced after the overnight fast, whereas the control group had access to food at all times. Yet, IF maternal insulin concentration remained unchanged. Maternal glucose is not the only source of placental and fetal energy, but it is essential for fetal growth (Baumann et al., 2002). Several studies showed that the fetal growth restriction induced by restriction of maternal food for a period of 24 to 30 h was diminished when glucose was supplied (Runner and Miller, 1956; Ellington, 1980). Herrera et al. (1985) showed a positive correlation between maternal glycaemia and placental glucose transfer. Therefore, as a consequence of IF maternal hypoglycaemia, the maternal-to-fetal glucose concentration gradient is diminished with the possibility of a reduction in transplacental glucose transport. This was shown to be the case in a study by Lesage et al. (2002), in which pregnant dams exposed to 50% food restriction during the last trimester had reduced maternal plasma glucose content with corresponding reduced placental glucose transporter GLUT3 expression. GLUT3 is localised in the labyrinth zone (Shin et al., 1997) and its expression is increased during gestation to supply an adequate amount of glucose needed to support fetal growth (Zhou and Bondy, 1993).

During Ramadan, fasting pregnant women showed a significant reduction in fasting blood glucose and alanine concentrations consistent with finding in the present study (Prentice et al., 1983; Mirghani et al., 2004; 2005). Using an oral glucose tolerance test, 70.5% of fasting pregnant women during Ramadan and 50% after Ramadan showed higher blood glucose concentration compared to non-fasting pregnant women (Baynouna Al Ketbi et al., 2014). Thus, fasting pregnant women had an increased risk of gestational diabetes mellitus (Mirghani and Hamud, 2006). As pregnancy progresses, pregnant women become insulin resistant in order to provide the fetus with nutrients; this insulin resistance is aggravated by Ramadan fasting (Baynouna Al Ketbi et al., 2014). This could explain why there was no difference between IF and control maternal insulin concentrations.

During normal pregnancy fetal plasma glucose is lower and insulin is higher than in the maternal circulation (Girard et al., 1977; Lesage et al., 2002). This may be explained by the fact that fetal growth accelerates with the advance of pregnancy. As a consequence, the fetal demands for glucose increases, accompanied by an increase in the plasma insulin concentration, which promotes glucose utilisation by the insulin-sensitive fetal organs such as skeletal muscle, leading to relative fetal hypoglycaemia (Hay, 1991).

It was found in this study that fetal insulin concentration was significantly higher than maternal insulin concentration, as reported in other studies (Girard et al., 1977; Fernandez-Twinn et al., 2003), whereas plasma glucose concentration in the fetus was significantly lower than maternal glucose concentration similar to other published data (Girard et al., 1977; Lesage et al., 2002; Coan et al., 2010). Interestingly, fetuses exposed *in utero* to intermittent fasting suffered from hypoinsulinaemia, which possibly plays a role in the growth retardation observed in this study. An unexpected finding was that IF fetal plasma glucose and glucagon concentrations were not significantly different from those in controls. Lesage et al. (2002) have reported similar findings in 50% food-restricted fetuses.

In contrast, 20% calorific-restricted as well as protein-restricted fetuses displayed a significant reduction in blood glucose concentrations (Coan et al., 2010; 2011). This may provide evidence for the restoration of euglycaemia at term through counter-regulatory mechanisms in the IF fetus. The sustainment of fetal glucose levels may be induced by provisions from glycogen storage in the placenta (Barash and Shafrir, 1990) to maintain fetal glucose supply in the face of decreased maternal glucose availability and to fulfil the fetal metabolic demand (Girard et al., 1977); this seems improbable in this study as placental glycogen content was unaltered in the IF group.

Glycogen cells form a portion of the junctional zone of the placenta. The literature provides evidence of an inverse relationship between fetal weight and glycogen content of the placenta (Kurz et al., 1999). It is known that glycogen in the rodent placenta provides an additional energy source for the developing fetus near term or during parturition. Glucagon stimulates the breakdown of glycogen stores into glucose in order to meet the increase in fetal energy demands (Coan et al., 2006). In addition, a positive relationship between the junctional zone's size at term and the weight of the fetus was shown in previous studies, such as Coan et al. (2011) and Kurz et al. (1999). It is yet not fully established how the weight of the fetus and the junctional zone's size are interdependent, but it is believed that glycogen cells play a part by controlling the growth of the fetus. Kurz et al. (1999) conducted a study with interspecific mice, which highlighted that the weight of the fetus and the glycogen content of the placenta had a negative correlation. In the current study, placental glycogen content was similar in both IF and control groups, suggesting that the catabolism of stored placental glycogen to glucose is unlikely to be the source of fetal glucose homeostasis.

In rats, fetal liver glycogen content increases progressively over the last three days of gestation and is correlated with the increase in fetal weight (Goodner and Thompson, 1967; Greengard and Dewey, 1970). The liver glycogen stores are rapidly depleted within a few hours after birth (Greengard and Dewey, 1970). This glycogen storage is likely an energy source for the gap between the cessation of transplacental nutrition and the start of suckling. Several studies showed that gluconeogenesis is functional *in utero* in the fetal liver (Ballard and Oliver, 1964;

Goodner and Thompson, 1967). During short-term fasting, fetal liver glycogen is conserved by fetal gluconeogenesis, and maternal liver glycogen is the fetal glucose source (Goodner and Thompson, 1967). However, under prolonged starvation of pregnant animals (Goodner and Thompson, 1967) and anoxia (Dawes et al., 1959), the fetal glycogen storage is mobilised and liver glycogen content is depleted. It seems likely that in this study, fetal liver glycogen was the source of fetal plasma glucose. Maternal liver weight was significantly reduced in IF dams suggesting that maternal hepatic glycogen in this situation was an unlikely sustainable source of fetal glucose. There was a significant reduction in IF fetal liver glycogen contents compared with controls, which points to fetal hepatic glycogen as the source of glucose, in agreement with the above studies. Moreover, Goodner and Thompson (1967) argued that fasting activates gluconeogenesis *in utero* by utilising amino acids as the major gluconeogenetic precursors to synthesize glucose by the fetal liver at the expense of protein synthesis within the fetus. Insulin and glucagon play a role in this cycle; insulin is an anabolic hormone that plays a role in promoting fetal growth; glucagon, on the other hand, is important in the induction of hepatic gluconeogenesis (Girard et al., 1976). Girard et al. (1976) demonstrated that induction of glucagon or suppression of insulin in fetal and newborn rats caused a significant reduction in fetal plasma amino acid concentrations and activated the hepatic metabolism of amino acids. An identical case may be found in the current study as IF fetuses displayed hypoinsulinaemia and normo-glucagon with a significant reduction in a number of plasma essential and non-essential amino acid concentrations.

To my knowledge, this is the first analysis exploring the sex-specific differences in fetal amino acid concentrations in rodents and in response to maternal intermittent fasting *in utero*. Amino acids are essential for normal feto-placental growth during pregnancy. As IF maternal weight gain was significantly reduced and fetuses were growth restricted at GD 21, we speculated that there may be an alteration in amino acid concentrations in both the dams and their fetuses.

Maternal and fetal amino acid concentrations were similar to those that have been reported previously (Palou et al., 1977; Austic et al., 1999; Rees et al., 1999). Fetal amino acid concentrations were higher than their mothers, signifying the existence of active transport mechanisms from mother to fetus (Palou et al., 1977; Gude et al., 2004). In the fetuses of the control group, three amino acids were significantly higher in males compared with females: lysine, arginine and tyrosine. Intermittent fasting caused significant reduction in nine essential and non-essential amino acids in the pregnant dams (Table 2.3.2). Most of these amino acids were mirrored by similar reductions in the fetal compartment, with the exception of asparagine. The most profoundly affected amino acids in both mothers and fetuses was alanine, which is transported by system A. This observation supports the rationale for investigating system A in IF placentas, which will be discussed later in detail. Valine and methionine were significantly reduced in IF mothers and fetuses; isoleucine was also lower in IF fetuses, suggestive of an alteration in placental system L activity. Interestingly, phenylalanine, tryptophan and tyrosine,

also transported by system L, were significantly lower in IF females compared to their male counterparts. Methionine and phenylalanine have been reported to be lower at GD 21 in the amniotic fluid of rats fed 12% less glucose content during pregnancy (Gurekian and Koski, 2005).

A reduced concentration of branched-chain amino acids (BCAA) plasma concentrations in the dams and fetuses of the IF group were indicated initially by ELISA assay and confirmed by HPLC analysis. Gluconeogenic molecules such as aspartate, glutamine and glutamate were unaltered in both the IF mothers and the fetuses. Since enterocytes entirely oxidize these gluconeogenic molecules, their main plasma source is derived from utilisation of BCAA (Wu and Morris, 1998), which can lead to major reductions in BCAA in the fetus to overcome low maternal glucose. Intriguingly, a study by Zheng et al. (2009) was able to reverse FGR induced by 30% food restriction by introducing BCAA supplementation in the rat's diet.

Threonine was significantly reduced in IF dams and male fetuses. The reduction of threonine can be linked to its utilisation to synthesize glycine, an essential amino acid during pregnancy. Lenton et al. (1998) reported a negative relationship between head circumference at birth and 5-L-oxo-proline, a marker of glycine insufficiency. Glycine can influence growth functionally and structurally by being part of the synthesis of growth-related compounds such as bile salts, haem and collagen (Jackson, 1991).

Similar to the findings of this study, rats exposed to protein restriction showed a significant increase in glycine, glutamic acid and glutamine whereas BCAA and threonine were significantly decreased in maternal serum at GD 21 (Rees et al., 1999; Jansson et al., 2006). In contrast, Malandro et al. (1996) demonstrated that low-protein dams entered a catabolic phase and were able to maintain normal plasma amino acid concentrations, similar to, or higher than, control dams. Moreover, elevatations in glycine have been reported in 30% calorific-restricted baboons (McDonald et al., 2013) and in small-for-gestational fetuses in human pregnancy (Economides et al., 1989). Furthermore, offspring which underwent 24 h starvation at postnatal day 20 showed a similar reduction in plasma amino acids (Arola et al., 1984). In contrast, lysine was maintained in both the maternal and fetal compartments, consistent with an earlier report in the protein-restricted model (Rees et al., 1999).

Calculating the fetal to maternal ratio of amino acid concentrations indicated a more pronounced difference in certain amino acids such as tyrosine, phenylalanine, glutamate and aspartate as reported previously by Palou et al. (1977). Remarkably, fetal to maternal concentration ratios of most essential and non-essential amino acids were significantly increased in the IF dietary group thus reflecting the depletion in maternal amino acid content in the face of intermittent fasting presumably to maintain an adequate supply to sustain fetal growth. This observation differs from a human study, in which maternal essential amino acid

concentrations were significantly increased in intrauterine growth restriction compared with appropriate-for-gestational-age pregnancies (Cetin et al., 1996). However, their fetuses showed opposite outcomes displaying a reduction in amino acid concentrations, thus reducing the fetal -to-maternal ratio (Cetin et al., 1996).

2.4.4 Maternal intermittent fasting during pregnancy altered placental metabolomics

Metabolomics, an analysis that captures smaller molecular compounds, is currently emerging as a technology to elucidate changes in specific biomarkers in metabolic pathways during normal pregnancy as well as in preeclampsia (Dunn et al., 2009; 2012a) and undernourished animals (Shen et al., 2008; van Vliet et al., 2013). In this study, both the IF mothers and their fetuses displayed alterations in their plasma metabolic profiles, providing a rationale for identifying and characterising changes in placental metabolites in response to maternal intermittent fasting. The results showed two distinct outcomes. First, intermittent fasting caused changes in 28 placental metabolites (mostly increases) in both sexes compared to the control group. Second, males and females exhibited different adaptations in response to intermittent fasting, as males showed more profound changes in placental metabolites than their female counterparts. Two of these metabolites were markedly increased in the placentas of both IF sexes: ophthalmic acid and 1-(beta-D-ribofuranosyl)-nicotinamide.

Ophthalmic acid is a marker of oxidative stress (Soga et al., 2006). Oxidative stress exists because of the overproduction of reactive oxygen species (ROS) that exceed the antioxidative defence in the body, and therefore play a role in the manifestation of various human diseases (Montuschi et al., 2004). ROS include superoxide anion, hydrogen peroxide and hydroxyl radical and are generated by several pathways such as mitochondrial respiration and by products of fatty acid oxidation (Shao et al., 2012). Hypoxia can lead to oxidative stress by releasing superoxide anion from mitochondria that easily oxidises phospholipids and amino acids, therefore damaging the structure and function of organelles (Semenza, 2007). In normal pregnancy, there is an increase in serum lipid hydroperoxides, a marker for oxidative stress, which rises as the pregnancy progresses to reach the highest concentration at the end of pregnancy and then drops to the baseline value during the postnatal period (Toescu et al., 2002).

In Ramadan fasting, oxidative stress was assessed in healthy men and non-pregnant women by measuring urinary 15-F_{2t}-Isoprostaine, a marker of oxidative stress (Faris et al., 2012). The study concluded that an increase in oxidative stress towards the last third of Ramadan fasting was associated with an increase in bodyweight and that this effect extended a month after Ramadan (Faris et al., 2012). Pregnancy as a factor by itself induces oxidative stress that can be amplified by Ramadan fasting and cause impairment of the feto-placental compartment, as is the case in this study. In rats that were 50% food-restricted throughout pregnancy,

antioxidant markers in the undernourished-placentas were decreased, which provides evidence that maternal undernutrition induced oxidative stress causing feto-placental damage (San Martin et al., 2015). Moreover, 35% food restriction imposed on rats during the last week of pregnancy caused disproportionate fetal growth restriction and reduction in placental efficiency at GD 20 that was reversed by melatonin treatment, a potent antioxidant, leading to a significant increase in placental expressions of catalase and manganese superoxide dismutase (Richter et al., 2009). Therefore, an upregulation in placental antioxidant can oppose the effect of maternal undernutrition.

The second metabolite that was significantly increased in the placenta of the IF group is 1-(beta-D-ribofuranosyl)-nicotinamide, a nicotinamide adenine dinucleotide (NAD^+) precursor. NAD^+ is a vital metabolic cofactor in redox processes and a second-messenger metabolite that plays a central role in energy metabolism of all living cells (Jiménez-Garcia et al., 2016). Under stresses such as inflammation and DNA damage, NAD^+ biosynthesis pathways are stimulated (Bogan and Brenner, 2008). This was shown in preeclampsia in which placental NAD^+ was significantly elevated (Jarabak et al., 1987). In normal pregnancy, both placental 11β-HSD-2 and 11β-HSD-1 markedly increased during the last phase of pregnancy, as a consequence of a significant rise in glucocorticoids and progesterone, respectively (Michael et al., 2003). Both 11β-HSDs are NAD^+-dependent isoforms. Since Ramadan fasting is associated with an increase in cortisol concentrations in fasting pregnant women (Dikensoy et al., 2009), it is tempting to speculate that such an increase in glucocorticoids is coupled with an alteration in placental 11β-HSDs and therefore an induction of NAD^+ precursor.

With regards to sex-differences in placental metabolomics, males showed distinctive responses to the maternal insult, compared to females, with a wide range of changes in metabolites. One of the most striking differences was the increase in aromatic amino acids (tryptophan, phenylalanine and tyrosine) in male IF placentas; in contrast they were not altered in female fetuses of the same group. These amino acids have the common property of being transported by system L. Maternal plasma concentrations of tryptophan and phenylalanine were unaltered in the IF group; however tyrosine was significantly reduced. In the IF fetal compartment, on other hand, these amino acids showed a trend towards an increase in both sexes; however this failed to reach statistical significance. Intriguingly, sex differences emerged among the IF group with males showing significantly higher aromatic amino acid concentrations compared to their female littermates. This is in line with the placental metabolomics findings. These aromatic amino acids are involved in glucogenic and ketogenic pathways, whereas tryptophan comprises one of the major substrates for NAD^+ synthesis (Bogan and Brenner, 2008), which was increased in the placentas of the IF group.

One important question that arises is whether system L activity is up-regulated in the male placentas of IF group. System L acts as a 1:1 stoichiometric exchanger of amino acids (Verrey,

2003; Widdows et al., 2015) in concert with system A, which accumulates the intracellular amino acids to be exchanged by system L with extracellular amino acids (Cleal and Lewis, 2008). In this study, system A activity in placental plasma membrane vesicles was unaltered, which gives rise to the speculation that system L activity on this membrane was possibly unaffected or perhaps upregulated in the males of IF group, a point that needs further investigation.

From the perspective of FGR, several studies have shown the association between FGR and a reduction in system L activity, as system L activity is an essential transporter for fetal development. Jansson et al. (1998) reported that, system L activity was impaired in both isolated MVM and BM vesicles of human placenta from FGR pregnancies, indicating the direct association between reduced placental system L activity and diminished fetal growth. In another study, Rosario et al. (2011) showed that in dams exposed to a protein restricted diet, system L activity as well as LAT1 and LAT2 expression in rat placenta in the maternal-facing plasma membrane of syncytiotrophoblast layer II were reduced at GD 19 and 21 (Rosario et al., 2011).

Collectively, intermittent fasting resulted in different phenotypes compared to other dietary regimens, with males adapted differently than females. Females in the IF group showed little change in placental metabolites, had lower amino acid concentrations than their male littermates and more congenital malformations. However, the question of whether females fail to adapt, or adapt within a different temporal profile, to nutritional insult *in utero* requires future investigation.

Most of the changes in the metabolite profiles observed in the placentas of the IF group are similar to those that have been reported in preeclampsia, particularly with regard to glycerophospholipids and tryptophan (Dunn et al., 2009; 2012a). Dunn et al. (2009) examined the changes in metabolite profile of normal and preeclamptic placental tissue cultured in different oxygen levels. The normal placental tissue cultured in 1% oxygen (hypoxia) and the preeclamptic tissue cultured at 6% oxygen (nomoxia) displayed similar metabolite profiles, therefore pointing towards a vital part played by hypoxia in the development of preeclampsia. These changes were also reported in placentas of small-for-gestational age (SGA) offspring cultured in 1%, 6% and 20% oxygen for 96 h, as compared to placentas of the control group exposed to similar conditions (Horgan et al., 2010), which underlines the part played by oxidative stress and hypoxia in SGA and preeclampsia.

2.4.5 The effect of maternal intermittent fasting during pregnancy on placental development and function

Maternal intermittent fasting did not affect placental weight, which was comparable to the control group. The placental phenotype of this study differed from most previous calorific- and protein-restricted models in which placental weights were reduced (Ahokas et al., 1981; Malandro et al., 1996; Belkacemi et al., 2011c; Jansson et al., 2006; Coan et al., 2010). The normal placental weight of the IF animals was associated with unaltered junctional and the labyrinth area, as a percentage of the total placental area. Thus placental morphology was unaltered by the dietary regimen; however, performing *in situ* hybridisation using markers to detect different trophoblast cell types could be a useful tool to assess the effect of intermittent fasting on placental structural alterations. It is worth noting that despite the lack of impact of maternal diet, the study revealed sex-specific differences, whereby female placentas comprised larger junctional, but smaller labyrinth regions, relative to total placental area than males in both dietary groups. The fact that the labyrinth zone's purpose is nutrient exchange may go some way to explain why males are heavier than females. Such sex differences were also seen in Sprague-Dawley rats at GD 18 with almost similar percentages as observed in this study (Reynolds et al., 2015). In spiny mice (*Acomys cahirinus*), however, contrasting observations were reported in that female placenta contained a smaller junctional zone and bigger labyrinth zone than male placenta (O'Connell et al., 2013). A study on 321 pregnant Saudi women by Alwasel et al., (2014) demonstrated that male offspring's placentas had greater thickness than that of the female offspring, hence invading more deeply into the spiral arteries. The placentas of female offspring, on the other hand, displayed regional and geographical placental surface differentiation (Alwasel et al., 2014). Unfortunately a histological examination of placental structure was not conducted in Alwasel's study.

Fetal growth and development and fetal to placental weight ratio, a proxy of placental transport efficiency, were diminished by maternal intermittent fasting. For this reason, the study moved to investigating aspects of placental transport to elucidate potential underlying mechanisms. The placental system A amino acid transporter was selected as a putative mechanism that might be modulated by the IF regime, based on previous observations linking an altered dietary intake to changed system A activity in the placenta (Malandro et al., 1996; Glazier et al., 1997; Jansson et al., 2006; Coan et al., 2010; 2011) and its association with fetal growth restriction (Mahendran et al., 1993; Cramer et al., 2002; Jansson et al., 2006). This aspect was investigated employing *in vivo* and *in vitro* studies using ^{14}C-MeAIB, a specific substrate of system A. *In vivo* maternofetal clearance of ^{14}C-MeAIB across the placentas of IF fetuses was significantly reduced in both sexes compared to that of control fetuses, with a more pronounced reduction in IF females (41%) than males (37%). In contrast, system A-mediated uptake of ^{14}C-MeAIB into isolated plasma membrane vesicles *in vitro* was unaltered in the placentas of IF fetuses.

Assessment of placental system A activity by unidirectional maternofetal clearance of ^{14}C-MeAIB *in vivo* and Na$^+$-dependent uptake of ^{14}C-MeAIB *in vitro* did not produce comparable results. This is in contrast to other studies using these methods which have demonstrated good correspondence between these two approaches measuring system A activity (Kusinski et al., 2011). It should be noted that in these methods the data were normalised to different denominators: placental weight for maternofetal clearance of ^{14}C-MeAIB and vesicle protein content for vesicle uptakes. Unidirectional maternofetal clearance of ^{14}C-MeAIB measures the transplacental passage of ^{14}C-MeAIB, with placental uptake driven by the inwardly directed Na$^+$-gradient. Of interest, placental uptake of ^{14}C-MeAIB (i.e. dpm/placenta) was comparable between IF and control groups, with a significant reduction in fetal ^{14}C-MeAIB accumulation expressed as dpm fetus/g fetus only in IF females. The transcellular mechanisms implicated in the exit of ^{14}C-MeAIB from the placenta to the fetus *in vivo* remain unclear, but such mechanisms could potentially be influenced by intermittent fasting leading to the reduction seen in maternofetal clearance of ^{14}C-MeAIB. However, some maternofetal transfer of ^{14}C-MeAIB could also occur by the paracellular pathway, although this is anticipated to be of relatively low magnitude compared with the transcellular pathway, as ^{14}C-MeAIB is actively transported by system A. However, it cannot be discounted that the paracellular transfer of ^{14}C-MeAIB is altered by intermittent fasting. Measurement of *in vivo* paracellular permeability using markers such as radiolabelled mannitol or inulin may assist in addressing this issue (Sibley et al., 2004).

Similar down-regulation of *in vivo* system A transporter activity has been reported in the literature. In 50% calorific restriction from day 5 until 20 days of gestation, placental transfer and fetal AIB accumulation were significantly reduced in the calorific-restricted group. However, imposing calorific restriction at the last week of gestation did not alter placental transfer in those undernourished-rats (Ahokas et al., 1981). In a study by Coan et al. (2011) exposing pregnant dams to different severities of protein restriction, the authors found that with moderate protein deprivation, placental MeAIB clearance was significantly reduced at GD 19 in mice with no changes observed among severe protein restricted dams. Jansson et al. (2006) stated that a reduction in system A transporter activity precedes the onset of FGR in a low protein (LP) model (4%). In their study, at GD 19, LP rats showed a reduction in placental transport capacity of ^{14}C-MeAIB along with fetal accumulation, but placental accumulation of tracer was unchanged (Jansson et al., 2006); this is similar to the current findings in the IF model. However, at GD 21 when LP fetuses and placentas were growth restricted, placental transport capacity of ^{14}C-MeAIB and fetal and placental accumulation of tracer were reduced concomitantly (Jansson et al., 2006). In contrast, 20% calorific restriction from GD 3 until GD 19 in mice showed significant increases in unidirectional maternofetal clearance of ^{14}C-MeAIB and fetal accumulation. In another study of 50% calorific restriction in mice, placental accumulation of ^{14}C-MeAIB was increased at GD 18.5 with no differences in the transplacental transfer to the fetus (Ganguly et al., 2012). These different outcomes indicate that different

types of placental adaptation occur, dependent on the degree and type of nutrient restriction as well as the stage of pregnancy. They also indicate that there may not be a direct correspondence between placental tracer accumulation and transplacental tracer flux within the timeframe of the measurements performed; this notion would be compatible with the data obtained in the present study.

The ability to isolate the maternal-facing plasma membrane of SynTB layer II from both IF and control groups was consistent, without any difference between protein recovery and purity as measured by the enrichment of alkaline phosphatase activity localised to this plasma membrane (Glazier et al., 1990). The amounts of protein recovered and the purity were in agreement with previously published data in rodents (Kusinski et al., 2010). Furthermore, system A activity in this plasma membrane, measured as Na^+-dependent uptake of ^{14}C-MeAIB into plasma membrane vesicles, was similar to previously published data. For example, the system A activity measured in this study was comparable with that reported by Glazier et al. (1996) who showed that placentas from FGR fetuses induced by uterine artery ligation had similar system A activity to the sham-ligated group.

It is difficult to reconcile the lack of down-regulation in system A activity with the growth restriction observed in IF fetuses which, by definition, must reflect a reduced net placental solute flux. This raises the possibility that fetuses in the IF group are able to stimulate system A activity within the maternal-facing plasma membrane of SynTB layer II, although this seems improbable as this was not reflected by the maternofetal transfer of ^{14}C-MeAIB. It should however be acknowledged that the data obtained only represent examination of one stage of gestation, and it is not known if there was any alteration in placental transport prior to this stage. Also, it needs to be kept in mind that in the *in vitro* study with placental vesicles, despite the usefulness of this model to identify of transporters functioning on this membrane, there is a disadvantage: the loss of environmental regulatory components that may contribute to the down-regulation in placental system A activity observed in the *in vivo* study. Future investigation could be directed to assessing placental transporter activity on different days of gestation. Additionally, other amino acid transport systems such as system L could be investigated.

To further evaluate this conundrum regarding system A activity, SNAT isoform expression at the mRNA and protein levels were examined. Rodent placenta expresses all three genes encoding for the SNAT1, 2 and 4 isoforms of system A, the expression of which increases towards the final stage of rat gestation (Novak et al., 1996; 2006) corresponding with the increase in system A transporter activity to support fetal growth (Novak et al., 1996; 2006; Coan et al., 2010). The male placentas of the IF group showed significant increases in *Slc38a1*, *Slc38a2* and *Slc38a4* mRNA, contrasting with no changes observed in IF females. However, the up-regulation in mRNA levels did not translate to increased SNAT expression in

protein levels in IF male placentas. The absence of a relationship between protein and mRNA has been documented before; for example there was discordance between SNAT4 gene and protein expression in the human placenta (Desforges et al., 2006). It seems most likely that post-translational modifications of SNAT proteins or the presence of endogenous regulators play a significant part in modulating system A transporter mechanisms. A mis-match between gene expression and transporter activity has also been documented in animal models. Placental transport of ^{14}C-MeAIB has been reported to be down-regulated in protein-restricted rats at GD 19; this occurred 5 days after a preceding reduction in placental *Slc38a2* mRNA expression and occurred at a time when gene expression was already normalised (Jansson et al., 2006). In another study, protein-restricted mice showed a reduction in the *Slc38a2* isoform at GD 16 and by GD 19 both *Slc38a1* and *Slc38a4* isoform expression were down-regulated; however, these changes in gene expression did not result in any alteration in ^{14}C-MeAIB maternofetal clearance (Coan et al., 2011).

Although SNAT1 expression is more abundant in placenta, SNAT2 is more likely to be the isoform altered by nutrient perturbation, mainly amino acid deprivation, due to a tripartite amino acid response element in the first intron of the *Slc38a2* gene (Palii et al., 2006). In calorific-restricted mice (20% - 50%) or high-fat-fed mice, system A transporter activity was increased in parallel with the upregulation of *Slc38a2* expression (Jones et al., 2009b; Coan et al., 2010; Ganguly et al., 2012). In contrast to the observations of the IF study, *in utero* nutrient-manipulated models showed either down-regulated or unchanged *Slc38a4* expression in placenta (Coan et al., 2010; 2011; King et al., 2013; Reynolds et al., 2015). *Slc38a4* is predominant in the junctional zone of rodent placenta (Angiolini et al., 2009), and although *Slc38a4* expression was increased in the placenta of male IF fetuses, this did not parallel any differences seen in junctional zone growth as judged by the comparability of relative junctional zone area between dietary groups. By decreasing amino acid uptake into this placental region, available amino acids for transport to the fetus are spared (Coan et al., 2010; 2011). Sex-specific effects of maternal nutrient intake on *Slc38a* isoforms were reported previously. Pregnant rats exposed to high fat (HF), high-salt (HS), or high fat and salt (HFSD) diet displayed significant increases in *Slc38a2* in male placentas only; *Slc38a4* was also significantly increased in HFSD male placentas with no changes observed in female placentas (Reynolds et al., 2015).

In this study, SNAT1 and SNAT2 protein expression in the placentas of the two dietary groups were comparable and this is consistent with the similar system A-mediated uptake of ^{14}C-MeAIB into isolated plasma membrane vesicles *in vitro*. Hence, it is inferred that the reduction in maternofetal clearance of ^{14}C-MeAIB is occurring downstream of this plasma membrane, or that the presence of an endogenous modulator is required. It is also worthy of mention that classic system A amino acid substrates were not altered in IF placentas, as indicated by

placental metabolomics, and that placental ^{14}C-MeAIB tracer accumulation was similar between control and IF groups.

Western blot analysis of SNAT1 and SNAT2 protein in rat placental vesicles and lysates revealed molecular weight species that consistently differed from other fetal rat tissues such as brain and kidney or mouse wild-type placental lysate which exhibited a single immunoreactive signal (Chapter 7, Appendix). Nevertheless, the specificity of each SNAT antibody was confirmed both by the abolition of signal in the presence of excess blocking peptide and the clear absence of immunoreactive signal in the placentas of [SNAT1 + SNAT2] knockout mice. Several optimisations clearly showed an intense immunoreactive signal at 52 kDa for SNAT1. This band size accords with previous literature which reported that the predicted size of SNAT1 protein in placenta was between 50 and 58 kDa appearing either as a single band in rat placental lysate and MVM of baboon placenta (Haafiz et al., 2010; Belkacemi et al., 2011c; Gaccioli et al., 2013; Kavitha et al., 2014) or double bands in rat placental vesicles (Rosario et al., 2011). In the latter study, rats exposed to a low protein diet exhibited reduced SNAT1 protein expression at GD 21 coupled with a reduction in system A transport activity (Rosario et al., 2011). In Gaccioli et al. (2013), a high fat diet employed pre- and throughout pregnancy in rats elicited no changes in system A transport activity even though SNAT1 protein expression was reduced.

SNAT2, on other hand, appeared to be expressed as different molecular weight species in different tissues (Chapter 7, Appendix). The SNAT2 antibody revealed two distinct bands at approximately 60 and 150 kDa in rat placental vesicles. The lower molecular weight size in line with the predicted size between 50 and 60 kDa, as earlier reported, showing either a single band in the placental lysate and placental vesicles of rat as well as baboon (Haafiz et al., 2010; Belkacemi et al., 2011c; Gaccioli et al., 2013; Kavitha et al., 2014) or double bands in the rat placental lysate (Jansson et al., 2006).

It is feasible that the SNAT2 transporter may be glycosylated in rat placental vesicles in this study, with a corresponding increase in molecular weight. However, treating the rat placental plasma membrane with endoglycosidase F did not change the pattern or reduce the band to the predicted size (data not shown). Nishimura et al. (2014) reported a similar pattern for SNAT2 of diffuse multiple bands with higher molecular weight in the plasma membrane of TR-TBT 18d-1 cells. One observation is that blots obtained from 'fresh' vesicles isolated immediately from harvested placentas appeared cleaner with less background and a more intense band at 52 kDa than when the placenta was left in a tissue-preserving solution (Belzer) overnight, while the fetal tail PCR was performed to sex-type the fetuses (Chapter 7, Appendix). To rule out the possibilities that the preservative solution and the procedure to extract the placental membrane were the problems, the samples were probed with β-actin antibody and other transport system antibodies, for example folate receptor α and system L

light chains (Hussain and Owaydhah; personal communications). Those antibodies showed only a single immunoreactive signal for the target protein of predicted size, thus assuring that the SNAT banding pattern observed here is specific for this transporter in placenta since it was not seen in other tissues (Chapter 7, Appendix), or for other protein epitopes suggesting that it did not reflect protein degradation. In the calorific- and protein-restricted model, down-regulation of system A activity was mostly associated with a reduction in SNAT2 protein (Jansson et al., 2006; Rosario et al., 2011; Kavitha et al., 2014; Pantham et al., 2015). In rats exposed to a high fat diet, system A transporter activity and SNAT2 protein expression were unaltered even with a reduction of SNAT1 protein expression. Hence, this highlights the importance of evaluating each isoform of system A transporter as well as system A activity.

2.5 SUMMARY

Intermittent fasting *in utero* has a negative impact on maternal physiology reflected by reductions in weight gain and food intake during pregnancy and alterations in relative liver and kidney weights. In addition, IF mothers demonstrated hypoglycaemia, hypoglucagonaemia and hypoaminoacidaemia profiles. Fetuses exposed *in utero* to intermittent fasting exhibit growth restriction, not only in fetal size but also with regards to brain growth and the growth of visceral organs. Brains of fetuses in the IF group seem to be spared, reflected by an increased brain/liver weight ratio. Fetuses in the IF group mirrored their mothers and suffered from reductions in certain essential and non-essential amino acids. Interestingly, sex-specific differences emerged in both groups, with females having a lower concentration of certain amino acids compared to males. IF fetuses were able to maintain normal glucose and glucagon concentrations while insulin concentrations were reduced. The normal glucose was coupled with a reduction in fetal liver glycogen content. Sub-optimal maternal nutrition induced by intermittent fasting was associated with a down-regulation in transplacental ^{14}C-MeAIB flux measured *in vivo*. This alteration was not observed in system A activity in the maternal-facing plasma membrane of SynTB layer II, suggesting that the basal plasma membrane may be the limiting step, although regulation of placental transport *in vivo* by circulating factors may also exert an influence. Placental weight near-term was unaltered by intermittent fasting and this was associated with unchanged junctional and labyrinth area ratios. However, sex-specific differences appeared in both dietary groups, whereby females had higher junctional to placental area ratios and lower labyrinth-to-placental area ratios compared with males.

These data have implications for pregnant women who observe the practice of Ramadan fasting, corroborating the previously reported adverse effects on maternal physiology and fetal growth and development, with the potential for longer term effects on the child later in life. The following chapters will address the effects of prenatal intermittent fasting on the growth trajectory of the offspring and long-term impact on cardio-renal function.

CHAPTER 3

CARDIO-RENAL FUNCTION IN OFFSPRING EXPOSED TO INTERMITTENT FASTING *IN UTERO*

3.1 INTRODUCTION

In the previous chapter, data were presented which show that fetuses that were exposed to intermittent fasting throughout gestation were growth restricted and exhibited an altered metabolic status at GD 21. This is a phenotype that has been reported previously in the literature following a number of different types of maternal dietary intervention (Lucas et al., 1997; Woods et al., 2001a; Lesage et al., 2002; Jansson et al., 2006). A large body of epidemiological data demonstrated that adverse maternal milieu in pregnancy are associated with short-term complications including altered fetal growth as well as long-term adverse consequences for the health of the offspring. Barker et al. (1989a) were first to outline an inverse relationship between birth weight and risk of death from cardiovascular disease in adulthood. This outcome attracted world-wide attention, leading to an explosion of studies investigating the correlation between early neonatal growth patterns and the risk of developing cardiovascular disease in adulthood. Interestingly, these studies provided evidence that even within a normal range of birth weights, those born smaller had a greater risk of developing cardiovascular disease later in life (Barker et al., 1993; Leon et al., 1996; Stein et al., 1996; Forsén et al., 1997; Moore et al., 1999).

Animal models of fetal growth restriction (FGR) induced by maternal global nutrient restriction or a low protein diet (LP) during pregnancy support a role for the programming of a number of adulthood diseases such as hypertension (Langley-Evans et al., 1994; Woodall et al., 1996; Ozaki et al., 2001), impaired glucose tolerance (Ozanne and Hales, 1999; Burdge et al., 2008; Sutton et al., 2010), decreased insulin sensitivity (Erhuma et al., 2007) and altered renal development and function (Lucas et al., 1997; Langley-Evans et al., 1999; Nwagwu et al., 2000; Sahajpal and Ashton, 2003). Furthermore, both human and animal studies indicate that kidney remodelling, caused by an impairment of nephrogenesis, may play a significant role in the programming of hypertension and renal dysfunction in adult offspring (Mackenzie and Brenner, 1995; Almeida and Mandarim-de-Lacerda, 2005; Woods et al., 2004).

Glomerular filtration rate (GFR), which is the rate of flow of filtered fluid through the kidney, is a major indicator of renal function (O'Callaghan, 2009). In humans, GFR reaches a peak at around the age of approximately 25 years and then starts to decline progressively as a part of the normal ageing process as nephrons cease to function (Poggio et al., 2009). FGR and premature birth are both associated with a low nephron endowment (Rodríguez et al., 2004), which in turn may result in a lower total GFR (Schreuder et al., 2009). Amikacin and creatinine clearances are used as markers of renal haemodynamic function and as a proxy for the GFR. In a study by Schreuder et al. (2009), 1 day-old neonates born premature or small-for-gestational age (SGA) were shown to have lower GFRs (Amikacin clearance) compared to normal birth weight neonates. Low birth weight children, studied at 6 - 12 years of age, were

also found to have lower GFR (creatinine clearance) and higher serum creatinine concentration compared with age-matched normal birth weight children (Rodríguez-Soriano et al., 2005).

These findings in human studies have also been replicated in animal models. Woods et al. (2001a; 2004) showed that intrauterine protein restriction (moderate to severe) caused a nephron deficit and a reduction in total GFR. However, single nephron GFR was increased, driven by an increase in mean arterial pressure (MAP) of 10 mmHg. Furthermore, rat offspring exposed to 50% calorific restriction either throughout or for half of gestation showed a reduction in both glomerular number, indicative of diminished nephrogenesis, and GFR (Lucas et al., 1997) which was associated with the development of hypertension (Almeida and Mandarim-de-Lacerda, 2005).

In our laboratory, the LP model has been used to investigate the impact of maternal diet on kidney development and later renal function in the offspring. In 4 week-old LP offspring, GFR was normal although the nephron number was reduced compared with controls (Alwasel and Ashton, 2009; Sahajpal and Ashton, 2003). However, the administration of a non-pressor dose of angiotensin II caused a greater reduction in GFR in the LP rats than in the control group, due to an increase in glomerular AT_1 receptor expression (Sahajpal and Ashton, 2003; 2005). Furthermore, alterations in renal electrolyte handling were reported in the LP rats (Ashton et al., 2007; Alwasel and Ashton, 2009). Together these data suggest that the kidney is vulnerable during development and may be affected by the intrauterine environment.

Human offspring exposed to Ramadan fasting *in utero* are known to be at greater risk of low birth weight (Almond and Mazumder, 2011) and symptoms of cardiovascular disease and type 2 diabetes in adulthood (van Ewijk, 2011; Savitri et al., 2014). However, the impact of Ramadan fasting or intermittent fasting on renal development has not been reported. Therefore, this study followed a cohort of rats which had been exposed to prenatal intermittent fasting throughout gestation from birth to adulthood to observe whether programming of the fetus translated into altered renal function and hypertension in the adult. Since metabolic disease has been observed in humans exposed to Ramadan fasting *in utero* (van Ewijk, 2011; Savitri et al., 2014), glucose and insulin tolerance were also assessed in these animals

There is considerable evidence that maternal nutrition can have sex-specific effects on offspring renal and cardiovascular function. For example, animal studies have demonstrated that hypertension develops earlier in males than in females exposed to food or protein deprivation *in utero* (Kwong et al., 2000; Ozaki et al., 2001). Similarly, moderate maternal protein restriction caused a reduction in nephron number in male but not in female offspring (Woods et al., 2005). For this reason, both male and female offspring were studied to determine whether exposure to intermittent fasting results in sex-specific programming of adult diseases in this model.

In summary, the objectives of this chapter were to determine whether:

- Rat offspring which had been exposed *in utero* to maternal intermittent fasting were growth restricted at birth and whether they showed differences in growth trajectory.
- Maternal intermittent fasting during pregnancy affects glucose and insulin tolerance and systolic blood pressure in adulthood (12 weeks of age).
- Maternal intermittent fasting is associated with impaired renal function in adulthood (14 weeks of age).
- Outcomes in the offspring exhibit sex-dependent specificity.

3.2 MATERIALS AND METHODS

3.2.1 Progeny from control and intermittent fasting dietary regimen pregnancies

Virgin Wistar rats were treated as described in section 2.2.2. Upon confirmation of mating, dams were allocated randomly into two groups: either control, fed *ad libitum* (N = 10) or IF, food restricted overnight throughout most of pregnancy (N = 13). IF dams had food restricted from conception until the day before parturition (term, day 23) to avoid the potential for cannibalism of pups by hungry dams. For both dietary groups, the dams' food and water intake and bodyweight were recorded daily at approximately the same time (5:00 pm), before food withdrawal from the IF group. From birth onwards, all mothers had food *ad libitum* (standard rat chow diet). At birth, litter weight and size were recorded and accordingly litter weight per number of pups was calculated. Litters were then reduced to a maximum of eight pups per dam (four per sex where possible, determined by measuring the anogenital distance which is shorter in females than males) within 10 h of delivery to ensure consistent litter size and optimal growth. Dams with litter sizes less than 8 pups were excluded (N = 2). The culled pups' organs (brain, heart, liver and kidney) were retained, weighed and stored either in RNA*later*® (Sigma Aldrich, Dorset, UK) or 10% NBF for further analysis.

3.2.2 Postnatal growth

Offspring were caged with their mothers and litters were weighed daily from birth until two weeks of age then every alternate day until weaning at 4 weeks of age. Rats were then housed with a maximum of 4 females or males in one cage. Postnatal pup weights were measured weekly from week 5 until week 12 and then again at week 14 when the experiment was terminated. As the experimental manipulation was of the dams, rather than the offspring, the offspring were assigned to different experimental groups as shown schematically in Figure 3.2.1.
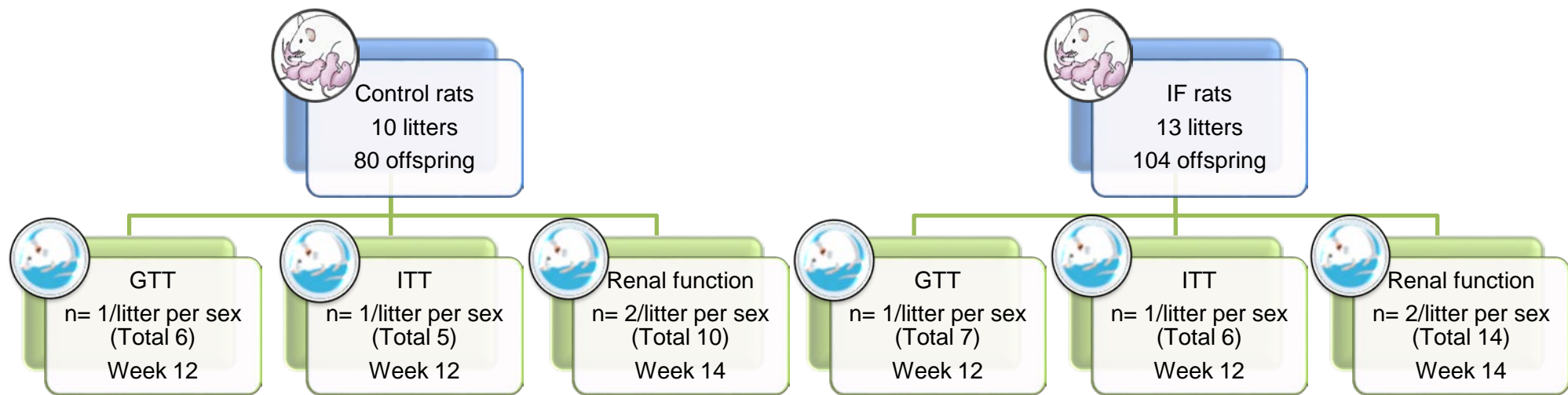
Figure 3.2.1 Study design for investigation of metabolic and renal function in control and IF offspring. The number of litters and offspring in each group are indicated. All offspring were used for systolic blood pressure (SBP) measurements at weeks 5, 7 and 10. Abbreviations: GTT, glucose tolerance test; ITT, insulin tolerance test.

3.2.3 Non-invasive systolic blood pressure measurements using tail-cuff plethysmography

Systolic blood pressure (SBP) measurements were performed by myself and Dr Heather Eyre. SBP was measured in a total of 80 control and 104 IF conscious offspring using a non-invasive blood pressure system (model LE5001, PanLab, Spain) at 5, 7 and 10 weeks of age. First, rats were transferred to a darkened room at 22 - 24 °C and allowed to acclimatize for 30 min before measurements were recorded. Rats were then either placed on the arm in a cradle position or in a restraint tube placed on a Thermopad heated mat (only at the beginning before placing the tail-cuff device; Harvard Apparatus, Kent, UK). A tail-cuff occlusion device and pulse transducer apparatus were placed on the rat's tail for 15 min prior to recording SBP measurements (Woodall et al., 1996). Data were consistent between the two approaches to handling and restraint. Twenty SBP and heart rate measurements were determined for each rat and the average taken.

3.2.4 Glucose tolerance tests

Glucose tolerance tests (GTTs) were performed at 12 weeks of age (Andrikopoulos et al., 2008; Figure 3.2.1). One offspring per sex was taken from each litter (C, N = 6; IF, N = 7). Rats were housed singly and fasted overnight for 16 h between 5:00 pm and 9:00 am with *ad libitum* access to water. First, one tail vein prick was made using a 25 gauge needle and the initial blood glucose concentration was measured using an Accu-Chek Mobile blood glucose monitoring system (Roche Diagnostics, West Sussex, UK). This was followed by intraperitioneal injection of a freshly prepared sterilized glucose solution (10% glucose in 0.9% saline (0.154 M NaCl)) at a dose of 1 g/kg bodyweight. Blood glucose concentration was measured at 15, 30, 60 and 120 min post-injection. At the end of the experiment, rats were euthanased by cervical dislocation under anaesthesia (inhalation of isoflurane, 4% in oxygen at 2 L/min). The area under the glucose concentration curve (AUC) was calculated for each individual rat using GraphPad Prism. The mean AUC values were calculated for each group and used in statistical analyses.

3.2.5 Insulin tolerance tests

Insulin tolerance tests (ITTs) were performed at 12 weeks of age in a separate group of rats (Liu et al., 2009). One offspring per sex was taken from each litter (C, N = 5; IF, N = 6), housed singly and fasted for 16 h overnight with free access to water. Initial blood glucose concentrations were measured via tail vein blood using an Accu-Chek Mobile blood glucose monitoring system (Roche Diagnostics, West Sussex, UK). Immediately afterwards, rats were injected intraperitioneally with 0.75 unit human insulin/kg bodyweight (I9278, Sigma Aldrich). Blood glucose concentration at 15, 30, 60, 90 and 120 min post-injection was recorded. At the

end of the experiment, rats were euthanased by cervical dislocation under anaesthesia (inhalation of isoflurane, 4% in oxygen at 2 L/min). AUC values were calculated for each individual rat using GraphPad Prism. The mean AUC values for each group were calculated and used in statistical analyses.

3.2.6 Renal function in anaesthetised rats: renal clearance

Two 14 week-old offspring of each sex from control (N = 5) and IF (N = 7) litters were used to determine the effects of *in utero* exposure to maternal intermittent fasting on renal function.

3.2.6.1 Surgery and experimental procedure

On the day of surgery, rats were weighed, anaesthetised with isoflurane by inhalation (4% in oxygen at 2 L/min), and then with sodium thiobutabarbital (Inactin® hydrate T133-1G, Sigma Aldrich) administered at 100 mg/kg bodyweight intraperitoneally. The anaesthetised rat was then placed on a heated surgical table to maintain body temperature at 37 °C. Surgery was performed within 30 - 45 min following induction of anaesthesia as described previously (Ahmed et al., 2003). Initially, a small incision was made in the neck; the right external jugular vein was exposed and freed from the surrounding tissue. A catheter (Portex polythene tubing ID 0.58 mm, OD 0.96 mm, Portex Ltd) was inserted through a small incision in the jugular vein and secured for saline and clearance marker infusion. Supplementary anaesthetic (Inactin 10 mg/kg bodyweight) was administered via the jugular line if required. A catheter containing heparinised saline (1000 IU/mL) was also inserted into a small incision in the left carotid artery, enabling direct measurements of arterial blood pressure and the withdrawal of arterial blood samples for clearance measurements. This was followed by a tracheotomy to assist breathing. Through a small abdominal incision, a cannula was inserted through a puncture hole at the apex of the bladder and secured for the collection of urine samples.

^{3}H-inulin and para-aminohippuric acid (PAH) were used as clearance markers to measure glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), respectively. Therefore, after surgery a priming bolus dose of ^{3}H-inulin (0.148 MBq (4 µCi), PerkinElmer) and (12 mg PAH, Sigma Aldrich) in 0.2 mL 0.9% saline (0.154 M NaCl) was injected via the jugular vein catheter. Animals were then infused continuously, at a rate of 50 µL/min, with ^{3}H-inulin (0.0999MBq/h (2.7 µCi/h)) and PAH (3 mg/h) dissolved in 0.9% saline for the whole 6 h experimental duration. Mean arterial blood pressure was recorded continuously using a PowerLab/4SP transducer and a PowerLab data acquisition system (Power Lab, ADInstruments Ltd, Chalgrove, Oxfordshire, UK, Chart5 software). Animals were allowed a 3 h equilibration period, after which urine samples were collected every 15 min into pre-weighed 1.5 mL eppendorf tubes for the remaining 3 h. A single blood sample (~ 400 µL) was collected at the mid-point of each hour. Immediately after blood sampling, a bolus of saline equal to the

volume of the blood sample was administered via the venous catheter to restore the circulating blood volume. The blood sample was centrifuged at 14000 xg for 2 min (Sigma 1-14K, Germany) at room temperature. 50 µL plasma was deproteinised by adding 50 µL 1.8% zinc sulphate heptahydrate ($ZnSO_4.7H_2O$; Sigma Aldrich) and 400 µL 0.1M NaOH; after 45 min the sample was centrifuged at 14000 xg for 2 min, and the supernatant collected and stored at -20 °C until PAH analysis. The remaining plasma and urine samples were stored at 4 °C for ^{3}H-inulin counting and electrolyte analyses. At the end of the experiment, terminal blood samples were collected into heparinised capillary tubes (Fisherbrand™ Microhematocrit capillary tubes, Loughborough, UK) and centrifuged at 15290 xg for 1 min (haematocrit Centrifugette 4203, ALC International Srl., Italy) for haematocrit determination. Figure 3.2.2 depicts the experimental procedure for renal clearance measurements. A limited number of animals have been excluded from this experiment due to blood loss from the external jugular vein (n = 2) and anaesthetic overdose (n = 1).

3.2.6.2 Tissue harvesting

After the end of the experiment, the animals were killed by cervical dislocation. The heart, lung, liver and kidneys were harvested and weighed. Kidneys were cut into half along the sagittal plane, placed in 4% PFA overnight at 4 °C, then washed in PBS, transferred to 70% IMS and stored at 4 °C for future use for histological study.

3.2.6.3 Urine and plasma analysis

Analyses were performed on urine and plasma samples from the control and IF male and female offspring to measure ^{3}H-inulin radioactivity counts, osmolality, and the concentrations of sodium, potassium and chloride.
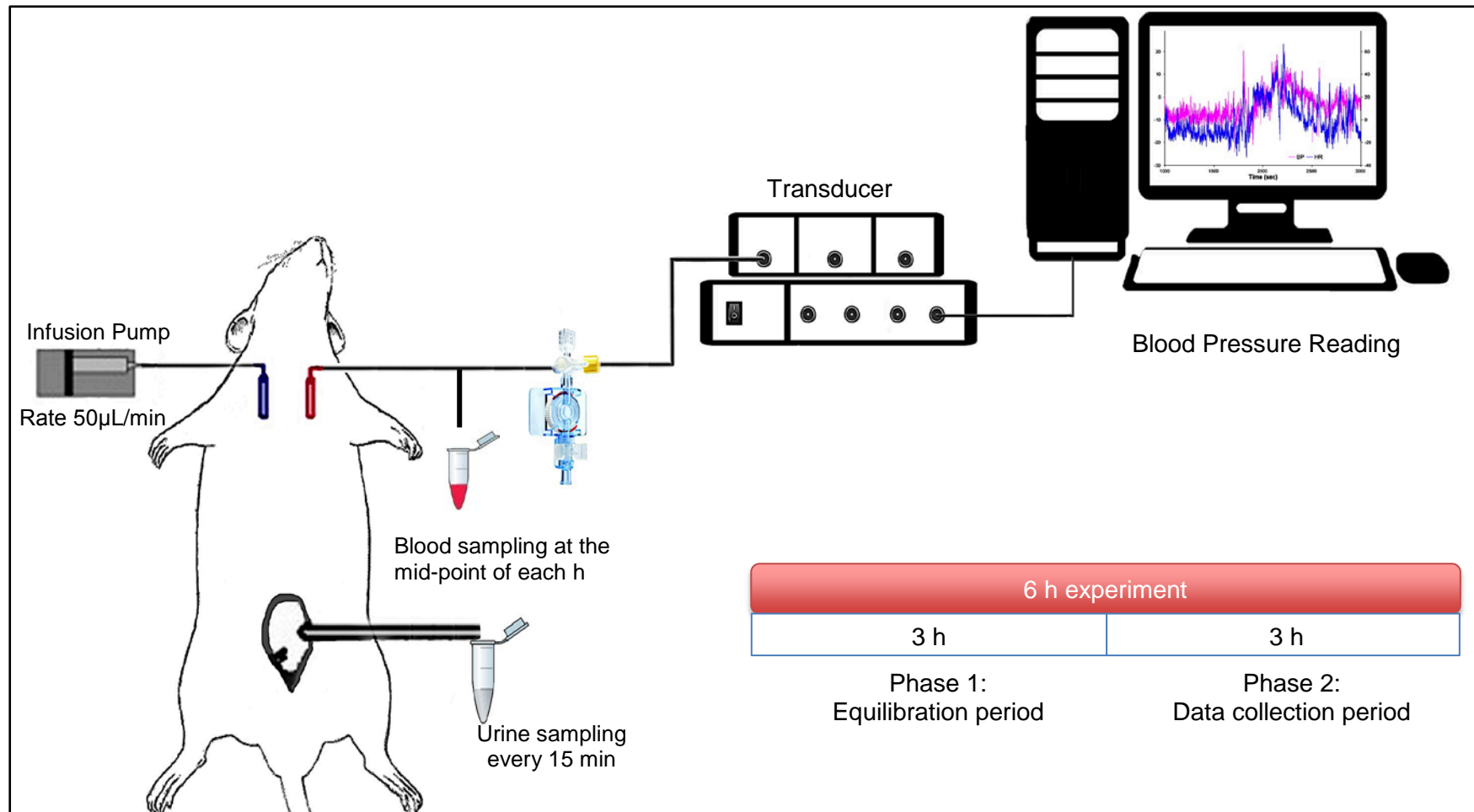
Figure 3.2.2 A diagram illustrating the renal clearance experiment. ^{3}H-inulin and PAH are infused into the jugular vein (blue-coloured vessel) and blood samples collected from the carotid artery (red-coloured vessel) which was connected to a transducer for arterial blood pressure recording. The bladder was cannulated and urine collected at regular intervals.

3.2.6.3.1 ^{3}H-inulin radioactivity analysis

For ^{3}H-inulin radioactivity analysis, 10 µL plasma or urine were added to 12 mL liquid scintillation fluid (Optiphase Hisafe II, Fisher Scientific Ltd). The samples were then counted in a liquid scintillation counter (Packard 2000CA) for 5 min using standard windows for ^{3}H with water blanks (background count). All of the sample counts were adjusted for background counts. The ^{3}H-inulin counts in plasma and urine samples were used to determine GFR (see Section 3.2.6.4.2).

3.2.6.3.2 Chloride concentration

The chloride concentration in urine and plasma samples was determined by electrometric titration (Chloride analyzer 925, Corning Scientific & Medical Ltd, Manchester, UK). The chloride analyzer was calibrated with 20 µL 100 mmol/L Chloride Meter Standard Solution (Sherwood Scientific Ltd, Cambridge, UK). 20 µL urine or plasma was added to 5 mL Combined Acid Buffer (Sherwood Scientific Ltd, Cambridge, UK) and the outcome reading recorded. In some samples, it was necessary to dilute the urine with dH$_2$O (1:2); the reading was then corrected for the dilution factor.

3.2.6.3.3 Sodium and potassium concentrations

Sodium and potassium concentrations in urine and plasma samples were determined using a Flame Photometer Model 420 (Sherwood Scientific Ltd, Cambridge, UK). First, the flame photometer was calibrated with a blank solution made up of Flame Photometer Standard 1000 ppm Li diluted 1:1000 in Flame Photometer Diluent Concentrate as a reference. This was followed by calibration using either a plasma (140 mmol/L Na and 5 mmol/L K, diluted1:200 in blank solution) or a urine (160 mmol/L Na and 80 mmol/L K, diluted 1:5 in dH$_2$O and then 1:200 in blank solution) standard solution (MutliCal™, Chiron Diagnostics, Spain). Plasma samples (diluted 1:200 in blank solution) and urine samples (diluted 1:5 in dH$_2$O and then 1:200 in blank solution) were then measured.

3.2.6.3.4 Osmolality

Osmolality measurements were determined using a Wescor 5500 Vapor Pressure Osmometer (Wescor, Inc, Logan, USA). The osmometer was calibrated with 10 µL each of 100, 290 and 1000 mOsmol/kg H$_2$O osmolality standard solutions (Opti-mole, Wescor Inc, Logan, USA) placed on filter paper discs. 10 µL urine or plasma were then placed on filter paper discs and osmolality (mOsmol/kg) measured.

3.2.6.3.5 Plasma protein concentration

To measure plasma protein concentration (mg/mL), the spectrophotometric absorbance of 2 µL plasma was measured at 280 nm using a NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific Inc.).

3.2.6.3.6 PAH analysis

To measure effective renal plasma flow (ERPF), urine and plasma PAH concentrations were measured by a standard colorimetric assay. A standard curve (0, 0.1, 0.25, 0.5, 1, 2.5 and 5 mg/100 mL) was prepared from a stock solution of 10 mg/100 mL PAH (Sigma Aldrich). 50 µL standards, deproteinised plasma (undiluted) or urine samples (diluted 1:100) were added to wells of a 96 well plate with 20 µL 1M hydrochloric acid and 20 µL 0.1% sodium nitrite (Sigma Aldrich) and incubated for 5 min at room temperature. 20 µL 0.5% ammonium sulphamate (Sigma Aldrich) was added to each well and incubated for 5 min at room temperature. 50 µL 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma Aldrich) was then added and incubated for 15 min at room temperature. Absorbance was measured at 546 nm (ThermoSpectronic Aquamate visible light spectrometer, Cole-Parmer instrument company Ltd, London, UK) and then analysed using Gen5 software (BioTek Instruments, Inc). PAH concentration (mg/mL) was determined by interpolation following linear regression analysis of the standard curve, multiplied by the dilution factor, as appropriate. The intra-assay coefficient of variance was 1.98% (n = 10 replicates).

3.2.6.4 Calculations

To calculate urine flow rate (UV), GFR, effective renal blood flow (ERBF), afferent and efferent arteriole resistances, electrolyte excretion rate ($U_{electrolyte}V$) and fractional excretion (FE), the equations detailed below were used.

3.2.6.4.1 Urine flow rate

The urine collection tubes were weighed before and after sample collection and it was assumed that 1 g = 1 mL, so that the UV per 100 g bodyweight (Bwt) could be determined according to the following equation:

$$UV \ (\mu L/min/100 \ g \ Bwt) = \frac{(\text{final tube weight- initial tube weight})/15 \ min}{(Bwt/100)}$$

3.2.6.4.2 Glomerular filtration rate

GFR was calculated per 100 g Bwt from UV and ^{3}H-inulin clearance according to the following equation:

$$\text{GFR (mL/min/100 g Bwt)} = \frac{\text{Urine } ^3\text{H-inulin (cpm/10 μL) x UV (mL/min/100 g Bwt)}}{\text{Plasma } ^3\text{H-inulin (cpm/10 μL)}}$$

3.2.6.4.3 Effective renal blood flow

ERBF was calculated per 100 g Bwt using the following equation:

$$\text{ERBF (mL/min/100 g Bwt)} = \frac{\text{Effective renal plasma flow (mL/min/100 g Bwt)}}{\text{(1- haematocrit)}}$$

Where effective renal plasma flow (ERPF) is the volume of plasma cleared of PAH per unit time, calculated using the following equation:

$$\text{ERPF (mL/min/100 g Bwt)} = \frac{\text{Urine PAH (mg/mL) x UV (mL/min/100 g Bwt)}}{\text{Plasma PAH (mg/mL)}}$$

3.2.6.4.4 Electrolyte excretion rate

Excretion rates of urinary electrolytes (Na$^+$, K$^+$ and Cl$^-$) were calculated using the following equation:

$$U_{\text{electrolyte}}V \text{ (μmol/min/100 g Bwt)} =$$
$$\text{Urine electrolyte concentration (mmol/L) x UV (mL/min/100 g Bwt)}$$

$$\text{Osmolar excretion rate } (U_{\text{Osm}}V; \text{ μOsmol/min/100 g Bwt)} =$$
$$\text{Urine osmolality (mOsmol/kg) x UV (mL/min/100 g Bwt)}$$

3.2.6.4.5. Arteriole resistance

Renal afferent and efferent arteriole resistances were calculated using the following equations taken from Kobrin et al. (1985):

Afferent arteriole resistance (mmHg/mL/min) =

$$\frac{\text{MAP (mmHg)} - P_G \text{ (mmHg)}}{\text{RBF (mL/min/g kidney)}}$$

Efferent arteriole resistance (mmHg/mL/min) =

$$\frac{P_G \text{ (mmHg)} - P_C \text{ (mmHg)}}{\text{GFR (mL/min/g kidney)} - \text{RBF (mL/min/g kidney)}}$$

where MAP is the mean arterial pressure, P_G is the glomerular capillary osmotic pressure, P_C is the peritubular capillary hydrostatic pressure and RBF is the renal blood flow.

P_C and P_G were calculated as follows:

P_C (mmHg) = P_T (mmHg) - 4 (mmHg) and P_G (mmHg) = π_e (mmHg) + P_T (mmHg)

where P_T is the proximal tubular hydrostatic pressure and calculated from the following equation:

P_T (mmHg) = 7.5 + (0.131 x GFR)

and π_e is the efferent colloid osmotic pressure and was calculated from the plasma protein concentration [P] using the equation bellow:

π_e (mmHg) = 2.1 x [P] + (0.16 x ([P]^2)) + (0.009 x ([P]^3))

3.2.6.4.6 Fractional excretion

FE of each electrolyte was calculated according to the following equation and expressed as a percentage:

FE electrolyte (%) =

$$\frac{U_{electrolyte}V \text{ (µmol/min/100 g Bwt)}}{\text{Plasma electrolyte concentration (mmol/L) x GFR (mL/min/100 g Bwt)}} \times 100$$

3.2.7 Statistical analysis

The data were analysed using the IBM SPSS statistical package (version 22, New York, US) and GraphPad Prism® software (La Jolla, USA). After applying Shapiro-Wilks normality

distribution tests, the data are presented as mean ± SEM and parametric analyses were used. Where data did not conform to a normal distribution, data are expressed as box and whisker plots unless stated otherwise and non-parametric analyses were used. The boxes mark the interval between the 25th and 75th percentile, the lines inside the box represent the median, and the whiskers denote the interval between the 5th and 95th percentiles. For all analyses, N = number of litters and n = number of individual offspring. $P < 0.05$ was considered statistically significant.

3.2.7.1 Maternal daily measurement and offspring postnatal body growth

Maternal daily food and water intake and weight gain and offspring postnatal body growth were assessed by repeated-measures two-way ANOVA with Tukey's post hoc test.

3.2.7.2 Neonatal organ weights and brain/liver weight ratio

Absolute and relative neonatal organ weights to bodyweights as well as brain/liver weight ratio were analysed by two-way ANOVA with Tukey's post hoc test.

3.2.7.3 Offspring organ weights, blood pressure and heart rate

Offspring (14 weeks of age) organ weights were expressed relative to bodyweights. Offspring organ weights and blood pressure (at 5, 7 and 10 weeks of age) were analysed by two-way ANOVA with Tukey's post hoc test. Heart rate was analysed by Kruskal-Wallis test followed by Dunn's multiple comparison test.

3.2.7.4 Glucose and insulin tolerance tests

For the glucose tolerance test, two-way ANOVA with Tukey's post hoc test was used to assess area under the curve (AUC) whereas for insulin tolerance test, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to assess AUC.

3.2.7.5 Renal function

Differences in renal function between the dietary groups were assessed by a two-way ANOVA with Tukey's post hoc test. The effect of time on renal function within dietary groups was analysed using a repeated measures two-way ANOVA with Tukey's post hoc test. The Kruskal-Wallis test followed by Dunn's multiple comparison test was used to analyse plasma and urine osmolality, afferent arteriole resistance and sodium excretion and fractional excretion of chloride.

3.3 RESULTS

3.3.1 Maternal food intake

Food intake was measured daily in both control and IF dams. Whereas the control group had free access to food, the IF dams were food restricted for 16 hours per day throughout most of gestation (see Section 3.2.1). At GD 22, a day before parturition, the food restriction was halted to avoid the potential for cannibalism of the neonates by the food-restricted dams.

As mentioned earlier (Section 2.3.1), both dietary groups gradually increased their food consumption over the course of pregnancy (P < 0.0001, Figure 3.3.1). At the day before parturition, there was a significant drop in food intake in both dietary groups of approximately 15 ± 3% compared to GD 21. Overall, IF pregnant dams ate 30 ± 1% less food compared to the control group (P < 0.0001, Figure 3.3.1).

3.3.2 Maternal water intake

Both IF and control dams had free access to water. In both dietary groups, the water intake increased substantially as pregnancy advanced (P < 0.0001, Figure 3.3.2) until GD 20, after which water consumption decreased in the last two days before birth, by approximately 21 ± 5%. While IF dams had similar water intake to control dams over the first two days, there was a trend to a lower water consumption with 4 - 12.5 mL less consumed between GD 4 - 20, which achieved statistically significance on various days (P < 0.05 and P < 0.01, Figure 3.3.2).

3.3.3 Maternal weight gain

Pre-pregnant dams in both dietary groups were of similar weights (250 ± 2 g). Both control and IF dams gained weight daily over the course of pregnancy (P < 0.0001, Figure 3.3.3). However, the lower food intake of the latter group had an impact on maternal weight gain such that the IF dams gained less weight than the controls; this difference was significant from GD 18 onwards (P < 0.0001, Figure 3.3.3).
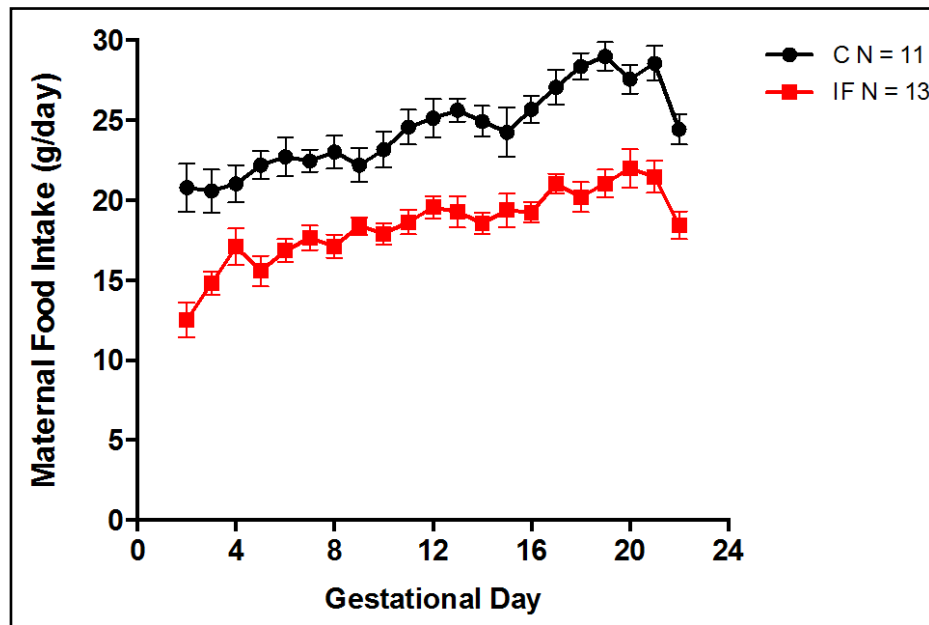
Figure 3.3.1 Daily maternal food intake of pregnant dams throughout gestation. Control dams (●) had free access to food throughout pregnancy. IF dams (■) had food restricted for 16 h per day from 5:00 pm to 9:00 am daily throughout pregnancy until GD 22 (a day before parturition). No data are shown for day 1, as this did not reflect a complete 24 h period. Daily maternal food intake was significantly lower in IF dams compared to controls throughout pregnancy. In the last day of pregnancy, there was a significant drop in the food intake in both dietary groups. Data are presented as mean ± SEM. $P_{Day} < 0.0001$, $P_{Diet} < 0.0001$ (repeated-measures two-way ANOVA followed by Tukey's post hoc test).
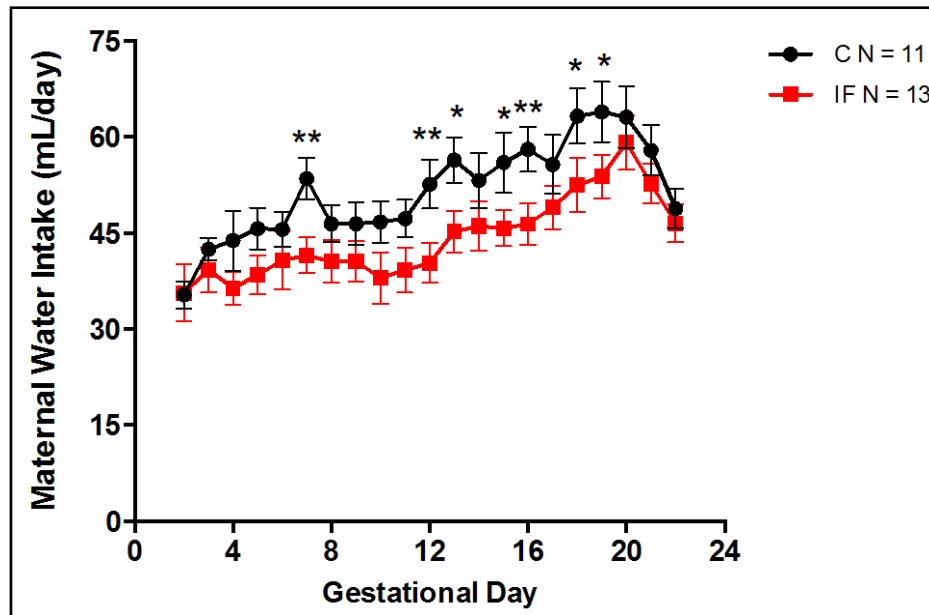
Figure 3.3.2 Daily maternal water intake by pregnant dams throughout gestation. Control (●) and IF (■) rats had unrestricted access to water throughout pregnancy. No data are shown for day 1, as this did not reflect a full 24 h period. Daily water intake increased as pregnancy progressed in both dietary groups. However, the water consumption by both dietary groups was decreased over the last two days of gestation. IF dams drank significantly less water than control dams from GD 12 until 19. Data are presented as mean ± SEM. $P_{Day} < 0.0001$, ** $P_{Diet} < 0.01$ at GD 7, 12 and 16, * $P_{Diet} < 0.05$ from GD 13, 15, 18 and 19. Statistical analysis was carried out using repeated-measures two-way ANOVA followed by Tukey's post hoc test.
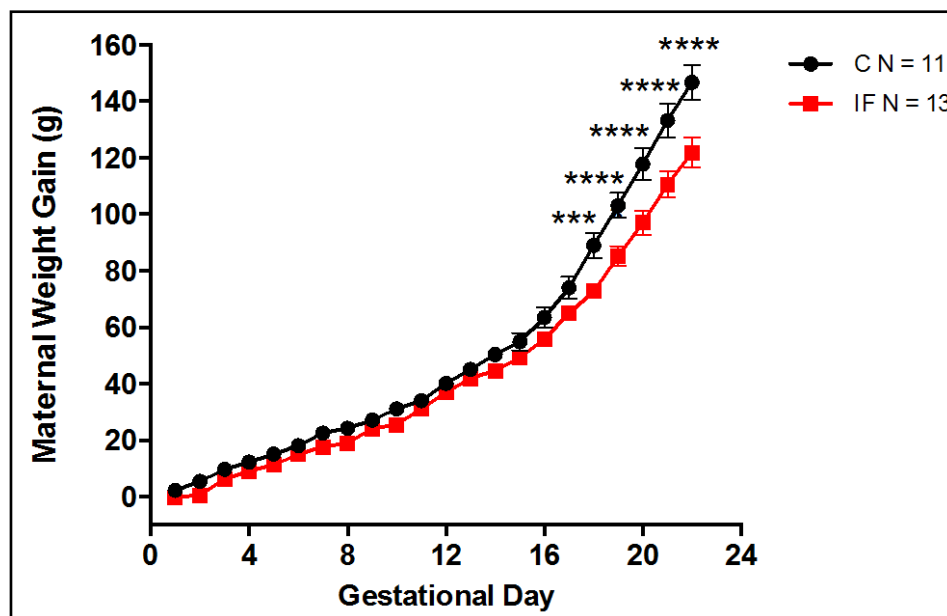
Figure 3.3.3 Daily weight gain in pregnant dams during gestation. Weight gain was calculated as increase in weight per day minus baseline (pre-pregnancy) weight. IF dams (■) gained significantly less weight than control dams (●) from GD 18 onwards. Date are presented as mean ± SEM. $P_{Day}< 0.0001$, *** $P_{Diet}< 0.001$ at GD 18, **** $P_{Diet}< 0.0001$ from GD 19 onwards. Statistical analysis was carried out using repeated-measures two-way ANOVA followed by Tukey's post hoc test.

3.3.4 Litter size and mean birth weight

All of the pregnant dams in the IF group had preterm delivery by half a day. The litter size did not vary significantly between the dietary groups, but showed a tendency to be lower in IF dams (P = 0.06, Table 3.3.1) compared to controls; however this difference failed to reach statistical significance.

Table 3.3.1 Litter size for control and IF dams

Experimental group	Control (N = 11)	IF (N = 13)
Litter size	15 ± 1	13 ± 1

Values are mean ± SEM with P > 0.05 (Mann-Whitney test)

Moreover, there was greater birth mortality in the IF group: two entire litters that had been exposed to the intermittent fasting regime *in utero* died shortly after birth. Interestingly, the mean neonatal weight at postnatal day 1 (PD 1) was similar between control and IF dams (P = 0.4, Figure 3.3.4). As mentioned in section 2.3.5, fetuses from the IF group were significantly growth restricted at GD 21. This suggests that IF fetuses underwent rapid growth during the last 2 days of gestation, with an increase of 97 ± 5% in bodyweight to achieve a similar weight

to that of the control group by PD 1. The bodyweight of the fetuses of the control group, on the other hand, increased by 79 ± 6% over the same period. Immediately after birth, the litters were standardised to 8 pups per dam (4 of each sex per litter where possible).
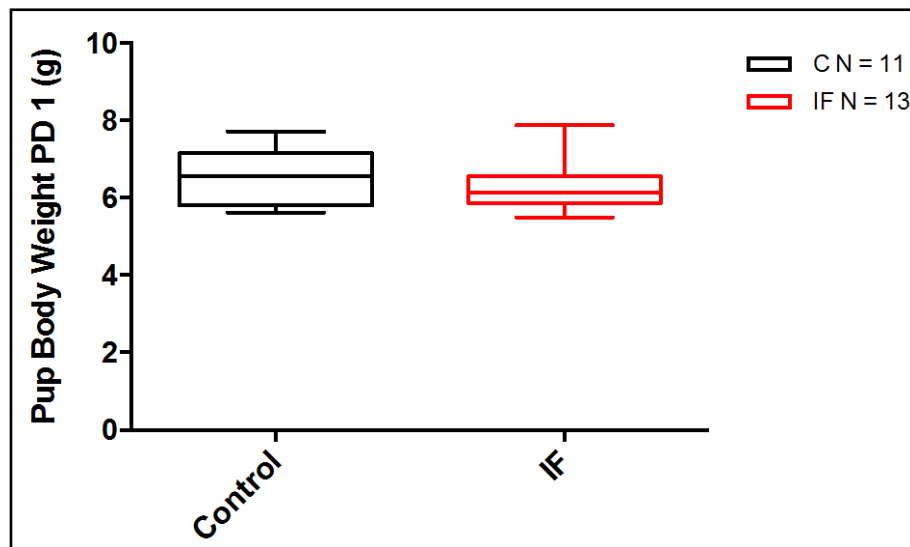
Figure 3.3.4 The effect of maternal intermittent fasting on mean neonatal weight. The mean pup weight at PD 1 was similar between controls (black) and IF (red). Data are expressed as box and whisker plots, where the lines inside the box represent the median. The boxes mark the interval between the 25th and 75th percentile, and the whiskers denote the interval between the 5th and 95th percentile. P > 0.05 IF versus C group. Statistical analysis was carried out using a Mann-Whitney test.

3.3.5 Neonatal organ weights

Neonatal organs (brain, heart, liver and kidney) were harvested from the culled pups at PD 1. The weights of brain (P < 0.0001, Figure 3.3.5 a) and kidney (P < 0.0001, Figure 3.3.5 b) in both sexes from the IF group, and heart of the IF female neonates (P < 0.05, Figure 3.3.5 c) were significantly reduced compared to controls. In contrast, the liver weights of both sexes from the IF group and heart weights of IF male neonates were similar to controls (Figures 3.3.5 c and d). When the tissue weights were normalised to neonatal bodyweight at PD 1, the kidney weights of both sexes of the IF group (Figure 3.3.6 b) and the brain weights of IF female neonates (Figure 3.3.6 a) remained significantly lower than the control group, whereas the other organs had comparable weights between groups (Figures 3.3.6 a, c and d).

Between GD 21 and PD 1, the brain weight had increased in males and females by 67 ± 4% and 73 ± 2% in the control group and by 60 ± 4% and 68 ± 3% in the IF group, respectively. Kidney weight, on the other hand, had increased in males and females by 162 ± 8% and 172 ± 9% in the control group and by 165 ± 9% and 148 ± 8% in the IF group respectively. The weight of the heart in the males and females from the control group increased by 87 ± 8% and

65 ± 21% and in liver by 9 ± 3% and 15 ± 11% respectively. In IF neonates, however, the growth of these organs was greater, as the heart's weight increased by 98 ± 5% and 92 ± 3% and the liver by 17 ± 4% and 21 ± 6% in males and females respectively.

Calculation of the neonatal brain/liver weight ratio, as an index of FGR and an indicator of brain growth relative to the peripheral organs (Mitchell, 2001), revealed an adverse effect caused by maternal intermittent fasting: there was a significant reduction in the weight ratios of IF males and females, by 15 ± 2% and 13 ± 2% respectively ($P < 0.05$), compared to controls (Figure 3.3.7). The reduced ratio is attributable to a deficit in the brain mass rather than the liver.

3.3.6 Growth trajectories of control and IF offspring

The pups remained with their mothers until 4 weeks of age. Up to 16 days of age, no differences in postnatal bodyweight were observed between the dietary groups. However, the IF offspring started to diverge thereafter, demonstrating a significant reduction in bodyweight from PD 18 until the end of 4 weeks ($P < 0.0001$, Figure 3.3.8).

Post-weaning, the offspring were split according to sex and bodyweight was monitored weekly until 12 weeks of age. As expected, male offspring were heavier than females in both dietary groups ($P < 0.0001$, Figure 3.3.9). The bodyweights of the male offspring in the IF group were significantly lower than control males until 10 weeks of age ($P < 0.01$, Figure 3.3.9). Thereafter, male offspring in the IF group were of comparable weight to control, indicating a 'catch-up' phenomenon. In contrast, the bodyweights of the female offspring of both dietary groups were similar during the entire post-weaning period up to 12 weeks of age.

It is worth noting that 2 females of the IF group from 2 different litters had seizure attacks, one at 7 weeks of age and the other at 9 weeks of age. They were subsequently excluded from the study.
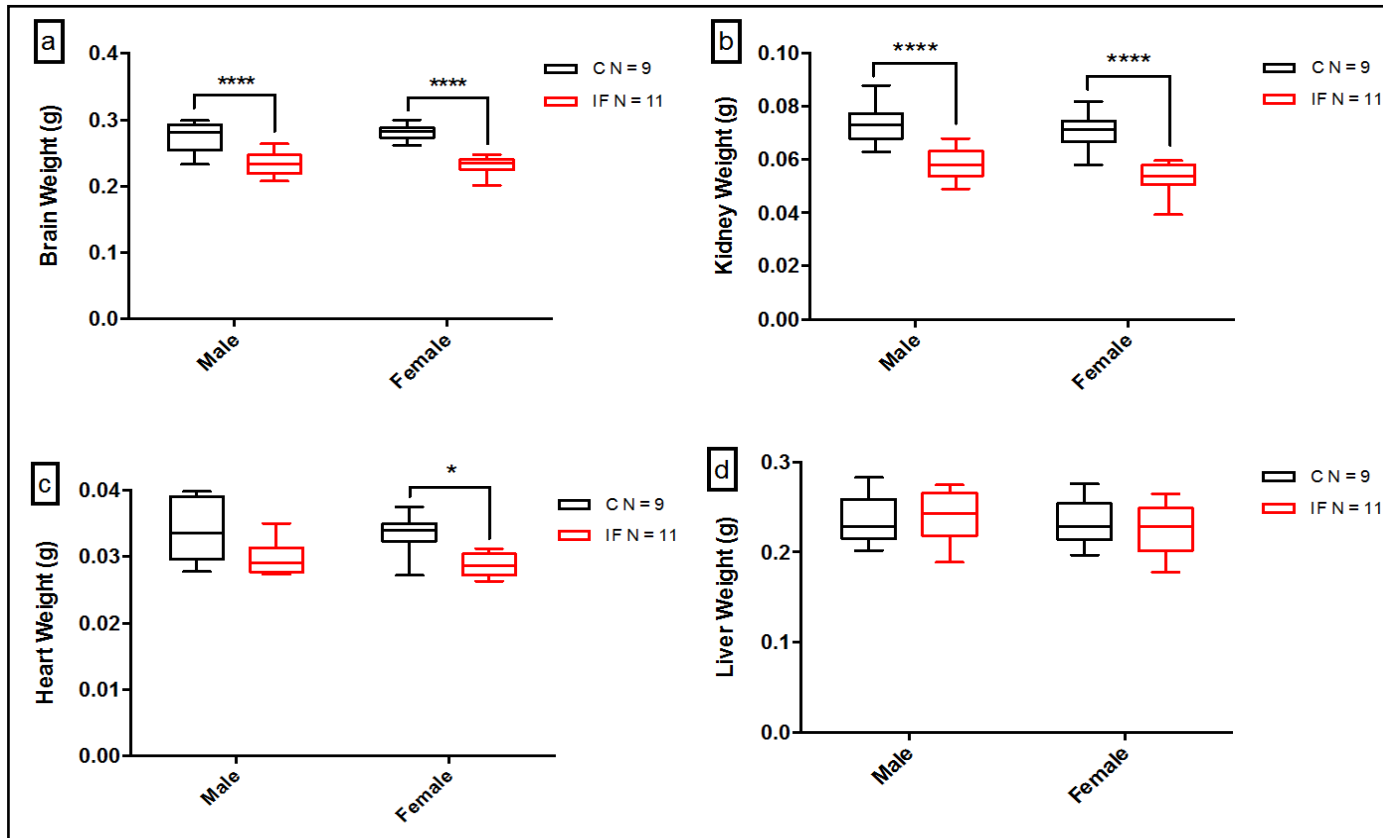
Figure 3.3.5 The effect of maternal intermittent fasting on neonatal organ weights at PD 1. **a.** brain, **b.** kidney, **c.** heart and **d.** liver. Control (black) and IF (red) groups. There was a significant decrease in brain and kidney weights of both sexes in the IF group and a significant decrease in heart weight for IF female neonates only. Liver weight was comparable between both dietary groups for both neonatal sexes. Data were analysed as mean/litter and expressed as box and whisker plots. * P < 0.05, **** P < 0.0001 IF versus control neonates (two-way ANOVA followed by Tukey's post hoc test).
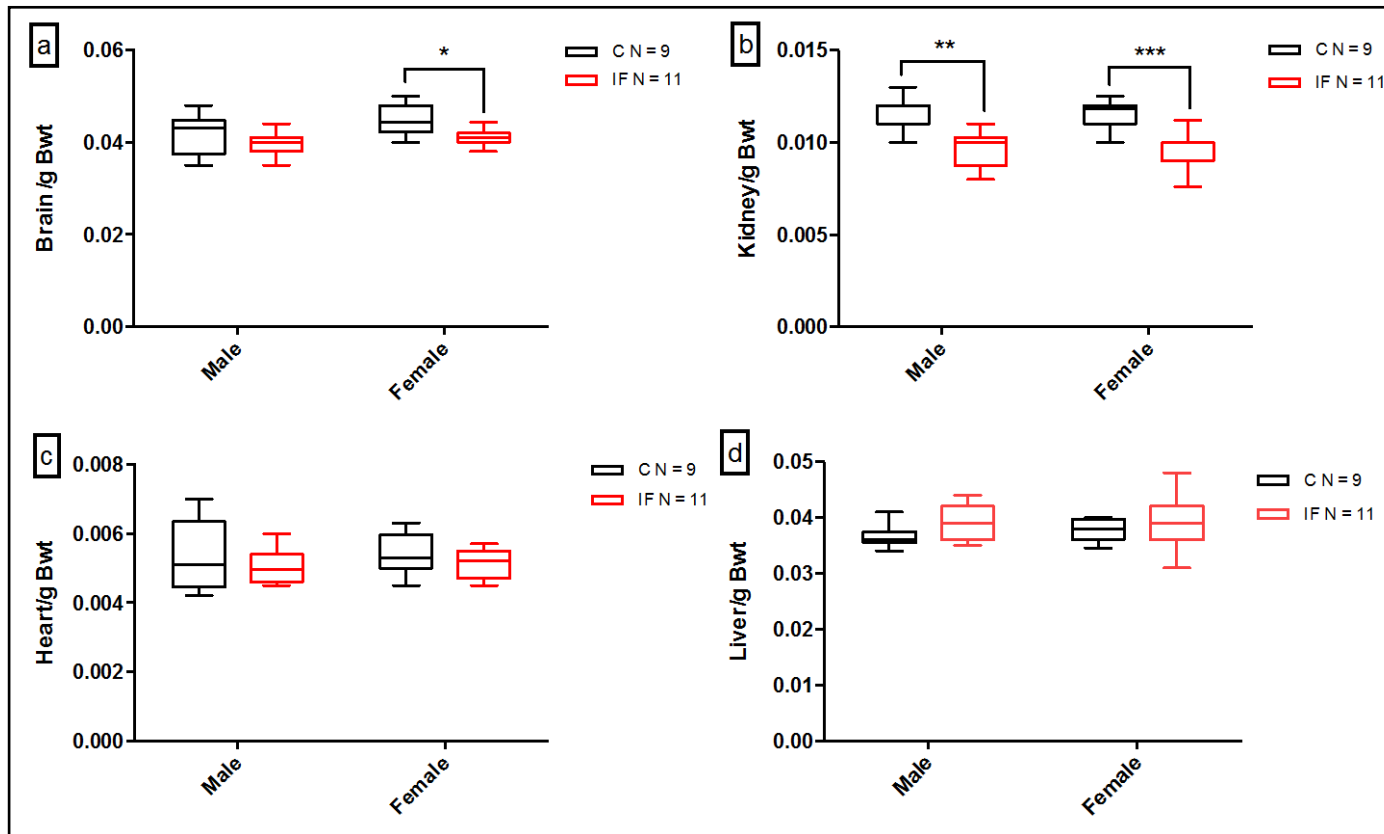
Figure 3.3.6 Neonatal organ weights relative to bodyweight (Bwt) at PD 1. **a.** brain, **b.** kidney, **c.** heart and **d.** liver. There was a significant decrease in relative kidney weights of both sexes in the IF group (red) and a significant decrease in relative brain weight for IF female neonates only compared to controls (black). There were no significant differences in the relative heart and liver weights in both sexes or relative brain weight of neonatal males between both dietary groups. Data were analysed as mean/litter and expressed as box and whisker plots. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ IF versus control neonates (two-way ANOVA followed by Tukey's post hoc test).
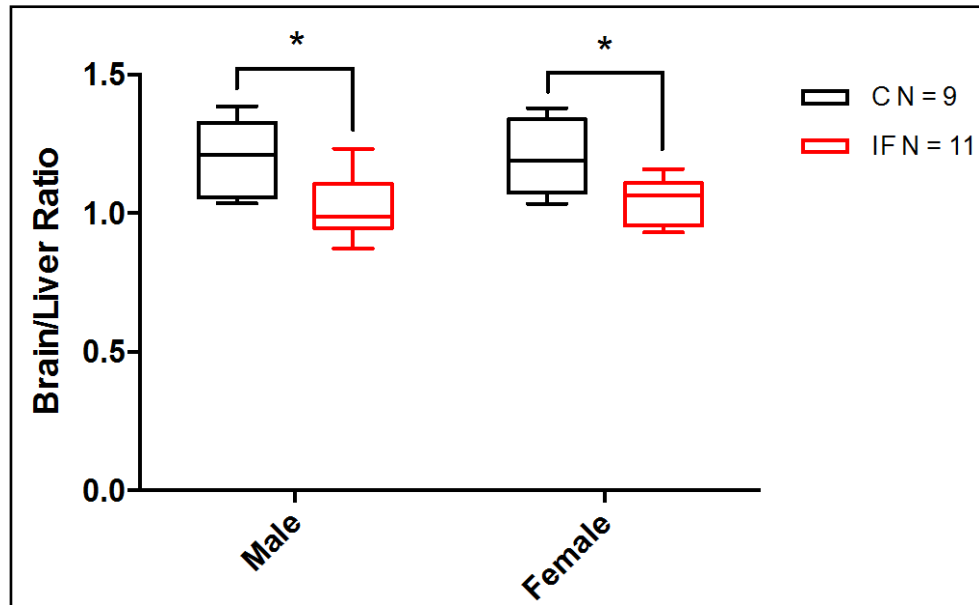
Figure 3.3.7 The effect of maternal intermittent fasting on the neonatal brain/liver weight ratio at birth. The fetal brain/liver weight ratio decreased in the IF (red) compared to the control (black) group. Data were analysed as mean/litter and expressed as box and whisker plots. * P < 0.05 IF versus control group (two-way ANOVA followed by Tukey's post hoc test).
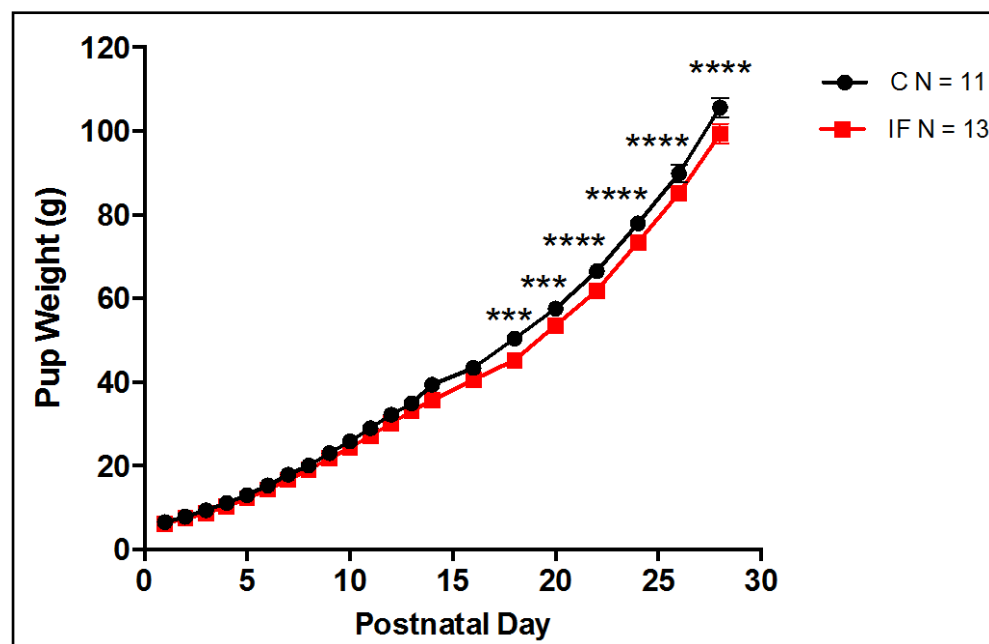
Figure 3.3.8 The effect of maternal intermittent fasting on postnatal weight gain in offspring. The pups were weighed daily to PD 14 and then every two days until 4 weeks of age. Data are presented as mean ± SEM. Offspring bodyweight was significantly lower in the IF group (■) compared to controls (●) from PD 18 onwards. $P_{Day} < 0.0001$, *** $P_{Diet} < 0.001$ at PD 18 and 20, **** $P_{Diet} < 0.0001$ from PD 22 onwards. Statistical analysis was carried out using repeated-measures two-way ANOVA followed by Tukey's post hoc test.
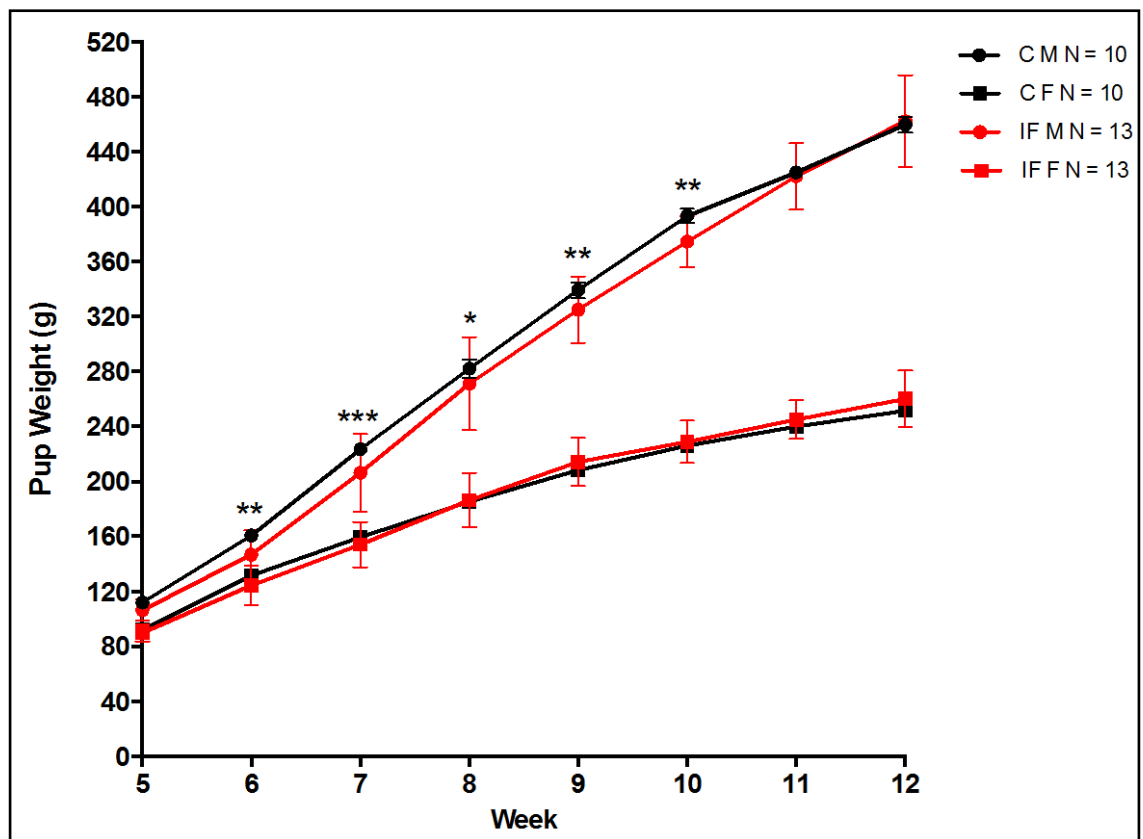
Figure 3.3.9 The effect of maternal intermittent fasting on postnatal weight gain in offspring over 5 - 12 weeks of age, separated according to offspring sex. Offspring were weighed weekly. IF male offspring (●) were significantly lighter than controls (●) from 6 weeks until 10 weeks of age, but following this at 11 and 12 weeks of age, IF male offspring weight was comparable to control. In contrast, IF female offspring (■) were of similar weight to controls (■) over 5 - 12 weeks of age, with no differences observed. Date are presented as mean ± SEM. $P_{Day} < 0.0001$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ IF males versus control males. Statistical analysis was carried out using repeated-measures two-way ANOVA followed by Tukey's post hoc test.

3.3.7 Systolic blood pressure in post-weaning offspring

Hypertension is one of the documented hallmarks of prenatal undernutrition in rats (Woodall et al., 1996; Vickers et al., 2000; Sahajpal and Ashton, 2003; Ashton, 2000). Therefore, systolic blood pressure and heart rate were measured in control and IF offspring at 5, 7 and 10 weeks of age using tail-cuff plethysmography. As shown in Figures 3.3.9 a, b and c, in both dietary groups the systolic blood pressure tended to rise from week 5 until week 10 of age by ~8 ± 0.9 mmHg; however there were no significant differences between the two dietary groups and sexes. Offspring heart rate (bpm) was comparable between the two dietary groups over the same period; no sex-specific effects were observed (P > 0.05, Table 3.3.2).

Table 3.3.2 Heart rate in control and IF offspring measured using tail-cuff plethysmography

Experimental group		Control N = 5		IF N = 6	
		Male	Female	Male	Female
Heart rate (bpm)	Week 5	431 ± 8	430 ± 6	435 ± 10	433 ± 6
	Week 7	440 ± 3	434 ± 4	439 ± 2	434 ± 4
	Week 10	432 ± 3	429 ± 6	438 ± 2	434 ± 1

Values are mean ± SEM with P > 0.05 (Kruskal-Wallis test followed by Dunn's multiple comparison test).

3.3.8 Offspring glucose and insulin tolerance

An intraperitoneal glucose tolerance test (GTT) was performed in offspring at 12 weeks of age. The glucose response to GTT did not differ significantly between the control group and IF offspring or between the sexes of each group as reflected by the area under the curve (P > 0.05, Figures 3.3.11 a, b, c and d). Likewise, the glucose response to insulin administration was similar in both dietary groups and between sexes (P > 0.05, Figures 3.3.12 a, b, c and d).
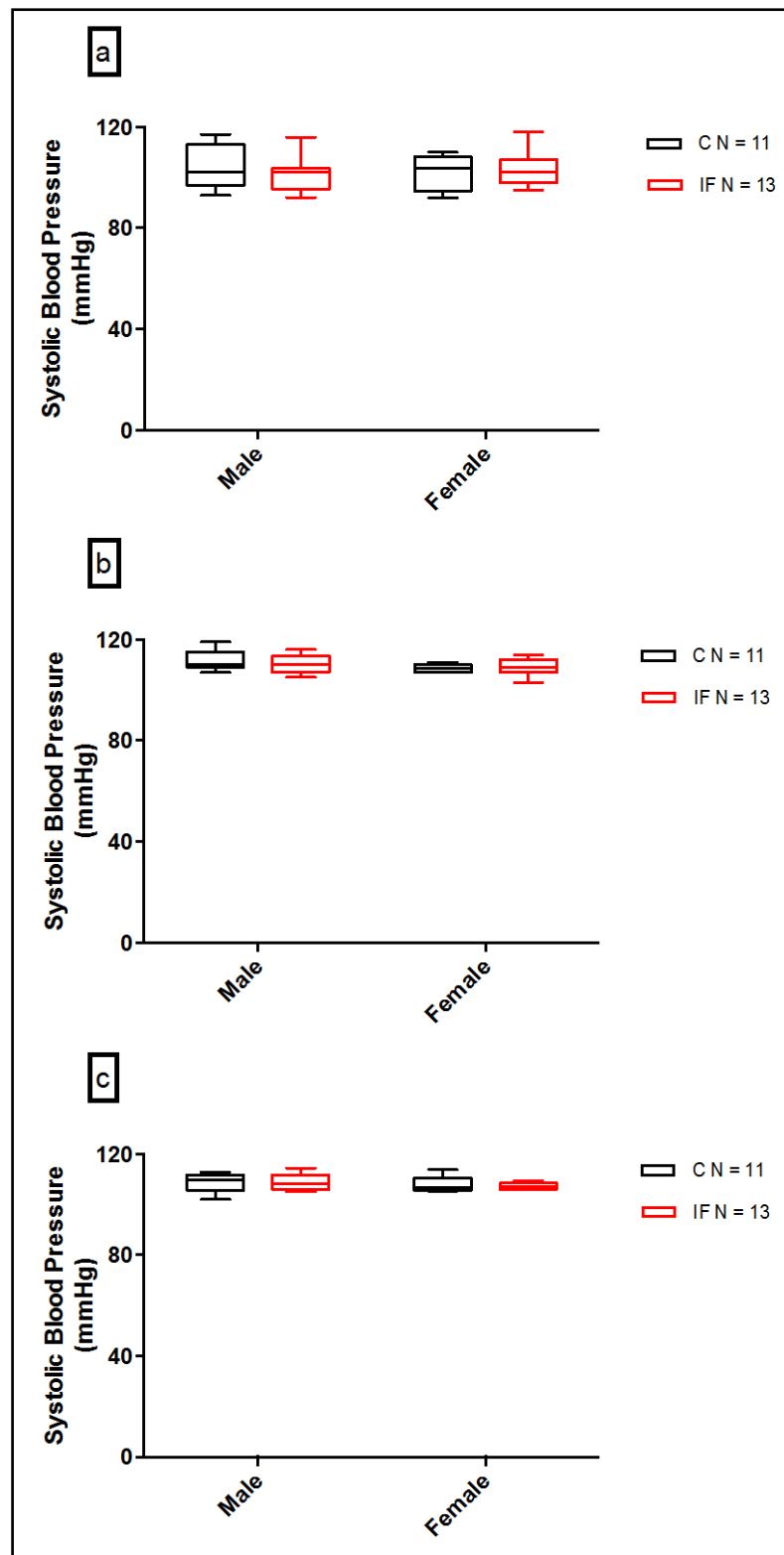
Figure 3.3.10 Comparison of systolic blood pressure of control (black) and IF (red) offspring. Blood pressure was measured at postnatal **a.** week 5, **b.** week 7 and **c.** week 10. The systolic blood pressure was similar between dietary groups and sexes. Data are analysed as mean/litter and expressed as box and whisker plots. P > 0.05 (two-way ANOVA followed by Tukey's post hoc test).
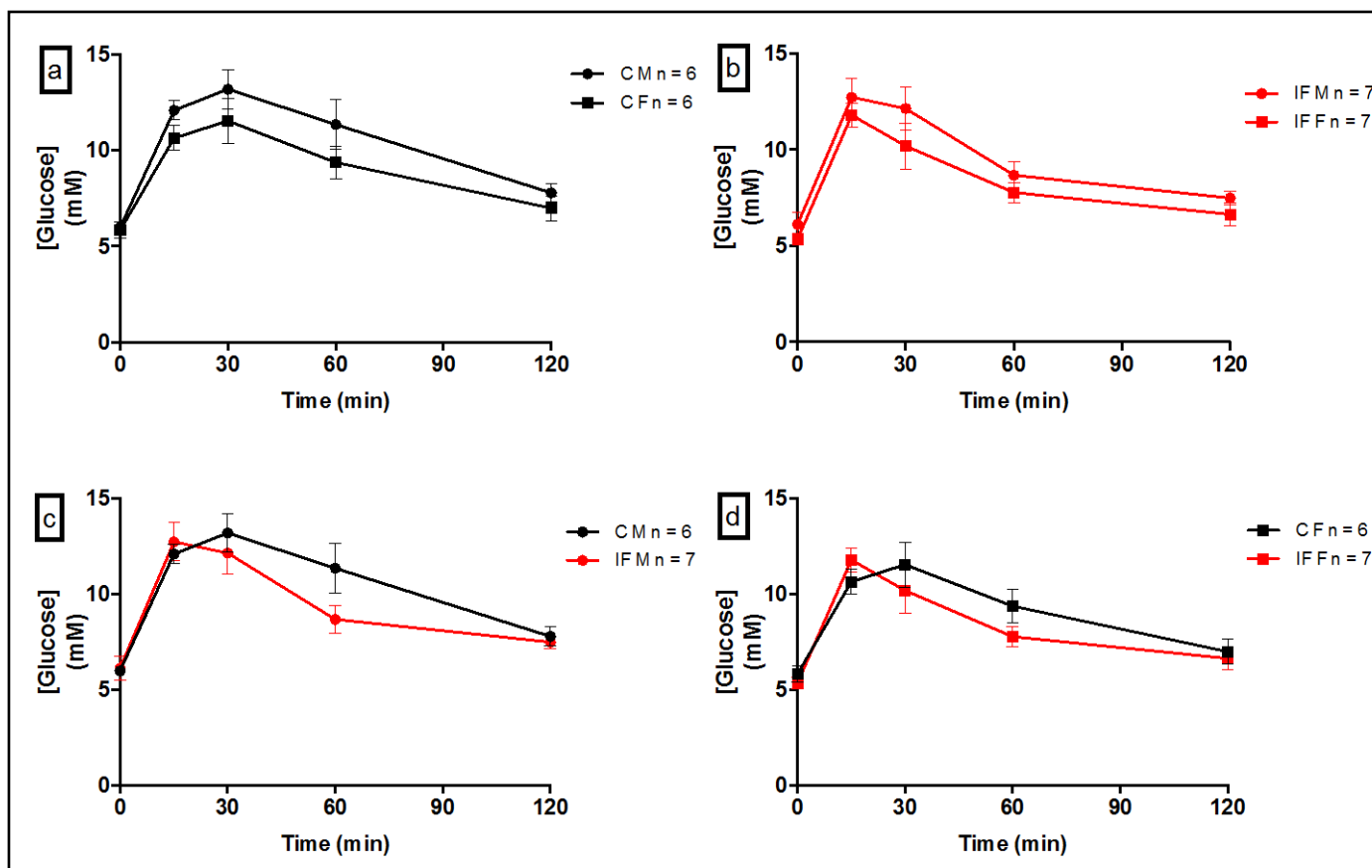
Figure 3.3.11 Comparison of glucose tolerance test profiles of control (black) and IF (red) offspring at 12 weeks of age. The offspring were fasted overnight for 16 h and administered with 1 g glucose/kg bodyweight by intraperitoneal injection. Serial blood samples were taken between 0 and 120 min. The area under the curve showed no difference between the two groups, or between the sexes of each group. Data are presented as mean ± SEM. $P > 0.05$ (two-way ANOVA followed by Tukey's post hoc test was used to assess area under the curve).
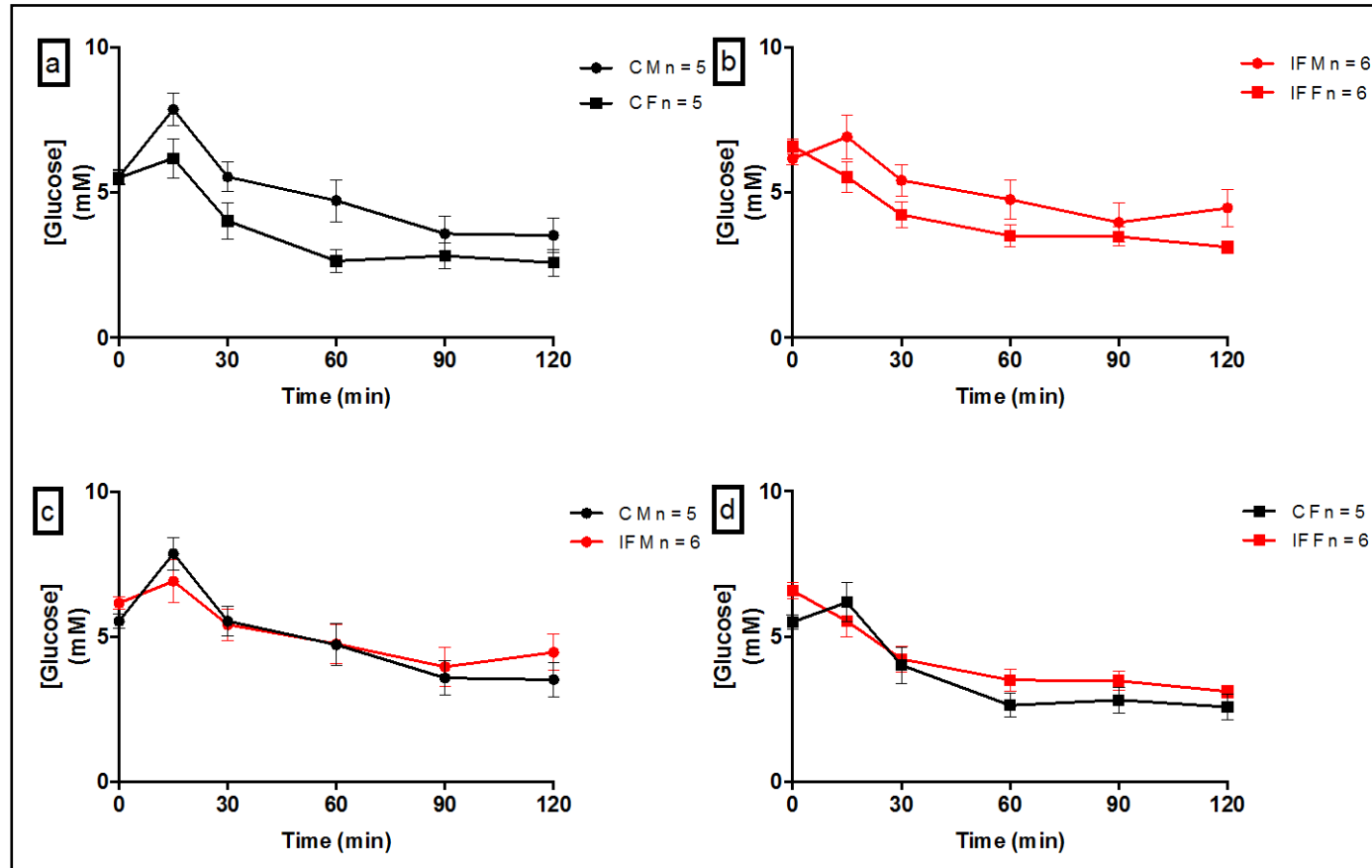
Figure 3.3.12 Comparison of insulin tolerance test profiles of control (black) and IF (red) offspring at 12 weeks of age. The offspring were fasted overnight for 16 h and injected with 0.75 unit human insulin/kg bodyweight. Serial blood samples were taken between 0 and 120 min. The area under the curve showed no difference between the two dietary groups, or between the sexes of each group. Data are presented as mean ± SEM. $P > 0.05$ (Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to assess area under the curve).

3.3.9 Offspring bodyweight and terminal organ weights at 14 weeks of age

At 14 weeks of age, the offspring from controls and IF were weighed before the renal clearance studies. Both sexes of IF offspring were of equivalent weight to their control counterparts (P > 0.05, Figure 3.3.13).

The terminal organ weights were expressed relative to g bodyweight. There was no significant difference among the offspring groups regarding heart, lung, kidney and liver weights (P > 0.05, Figures 3.3.14 a, b, c and d). However, there was a sex-specific difference in that the females of both IF and controls had significantly heavier heart (P < 0.01, Figure 3.3.14 a) and lung ($P_{Control}$< 0.0001, P_{IF}< 0.01, Figure 3.3.14 b) weights compared to their male counterparts. In contrast, liver weight was significantly heavier only in control males compared to their littermate females (P < 0.05, Figure 3.3.14 d).
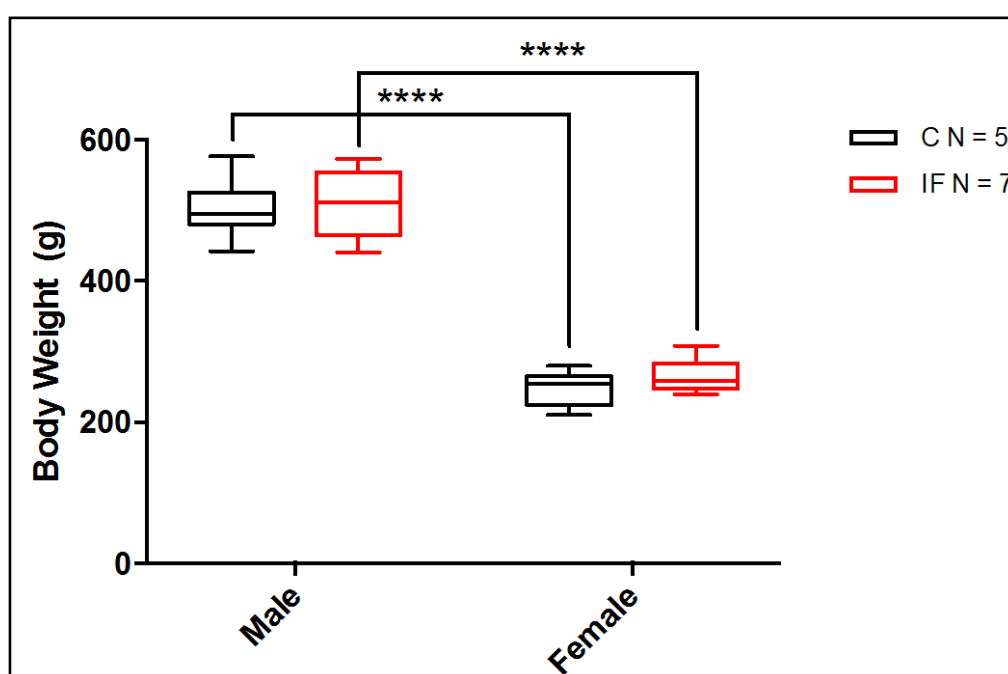
Figure 3.3.13 The effect of maternal intermittent fasting on offspring bodyweight at 14 weeks of age. Offspring bodyweight was similar between the two dietary groups (P > 0.05). Males in both control and IF dietary groups were heavier than female littermates (**** P < 0.0001). Data were analysed as mean/litter and expressed as box and whisker plots (two-way ANOVA followed by Tukey's post hoc test).
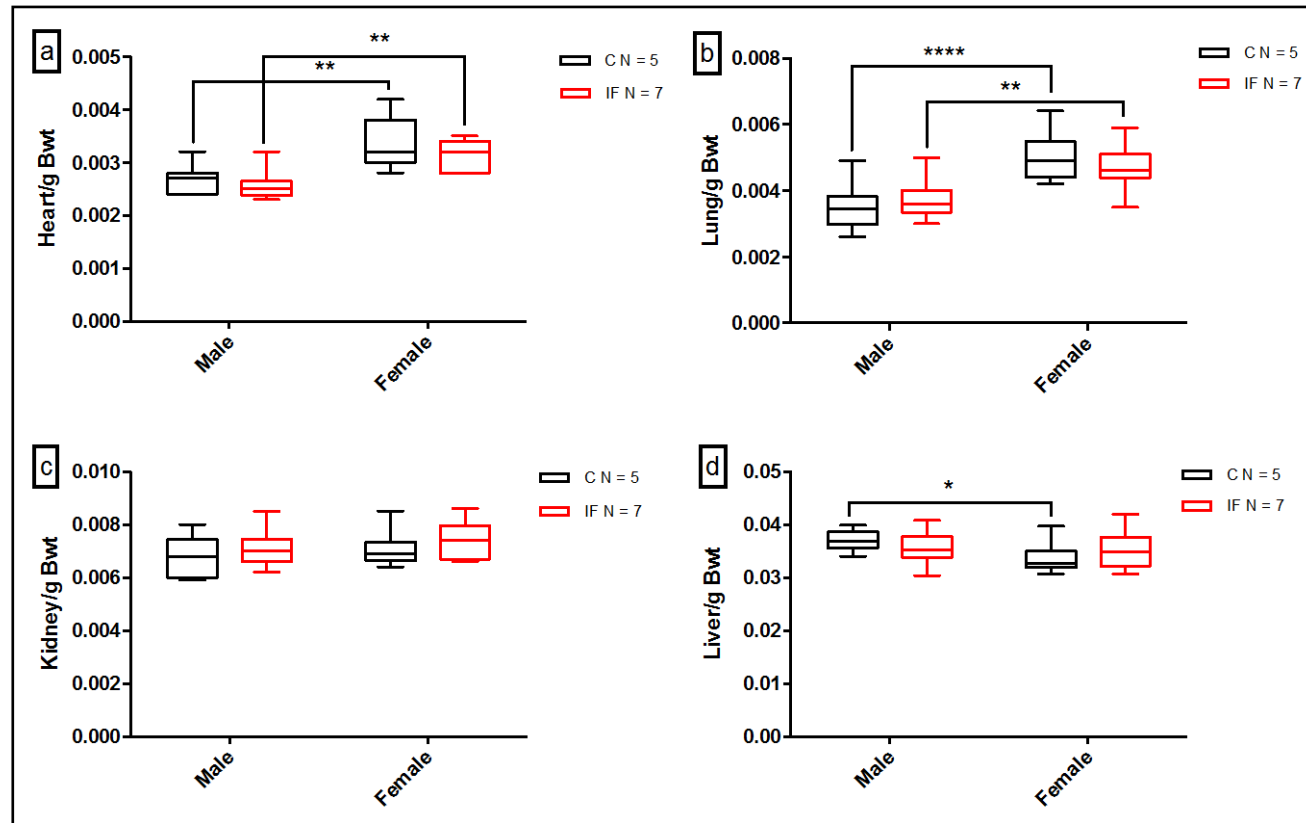
Figure 3.3.14 The terminal organ weights relative to bodyweight (Bwt) at 14 weeks of age. **a.** heart, **b.** lung, **c.** kidney and **d.** liver. Relative organ weights were similar between IF (red) and control (black) groups. Females in both dietary groups had significantly heavier relative heart and lung weights compared to male counterparts. Male offspring in the control group had heavier relative liver weights compared to female counterparts. Data were analysed as mean/litter and expressed as box and whisker plots. * P < 0.05 control male versus control female, ** P < 0.01, **** P < 0.0001 female versus male littermates (two-way ANOVA followed by Tukey's post hoc test).

3.3.10 Blood pressure in anaesthetised rats

The mean arterial pressure was measured in rats used in the renal clearance studies. This was done under Inactin anaesthesia. Blood pressure recordings were collected during the 3 h post-equilibration phase over 15 min periods. Blood pressure was stable in both control and IF groups throughout the experiments ($P_{time} > 0.05$, Figure 3.3.15 a). Intra-arterial blood pressure recordings revealed no differences in mean arterial pressure (MAP) between control and IF offspring nor between sexes of each group ($P > 0.05$, Figure 3.3.15 b).

3.3.11 Renal function in anaesthetised rats

Renal clearance studies were performed to measure kidney function in offspring at 14 weeks of age. Following an equilibration period, urine samples were collected every 15 min and plasma at the mid-point of each hour for the 3 h post-equilibration periods. All values were standardised to 100 g bodyweight.

3.3.11.1 Plasma electrolytes

From the renal clearance studies, plasma concentrations of sodium, potassium, chloride and protein were measured in arterial blood samples. Plasma potassium concentration was significantly greater in male offspring from the IF group compared to control males ($P = 0.048$, Table 3.3.3) and to female littermates ($P = 0.02$, Table 3.3.3). Plasma chloride, sodium and osmolality were comparable among dietary groups and sexes ($P > 0.05$, Table 3.3.3). Similarly, plasma protein concentration was equivalent between the two dietary groups as well as the sexes ($P > 0.05$, Table 3.3.3).

The haematocrit of arterial blood samples did not differ between controls and IF offspring; however there was an effect of offspring sex with males in both dietary groups having a significantly higher haematocrit than their female littermates ($P_{Control} < 0.05$, $P_{IF} < 0.0001$, Figure 3.3.16).
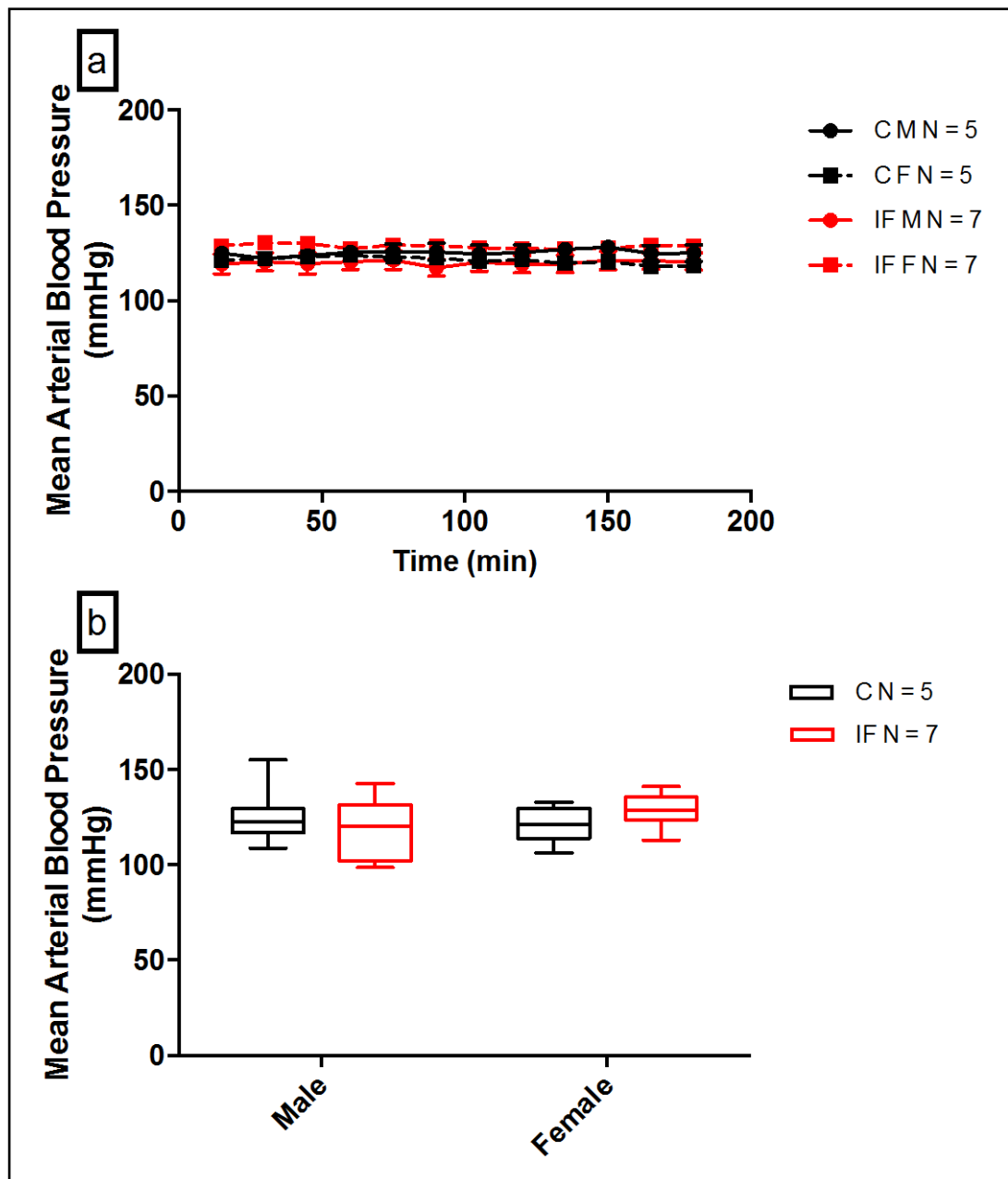
Figure 3.3.15 Mean arterial blood pressure (MAP) in anaesthetised offspring at 14 weeks of age. **a.** Measurements taken over 15 min periods during the post-equilibration phase of the renal experiments. **b.** Overall mean of 3 h experimental period. **a.** MAP was stable over 3 h experimental period. Data are presented as mean ± SEM. $P > 0.05$ IF versus control group (repeated-measures two-way ANOVA followed by Tukey's post hoc test). **b.** MAP was similar among dietary groups and sexes. The data are expressed as box and whisker plots. $P > 0.05$ IF versus control group (two-way ANOVA followed by Tukey's post hoc test).

Table 3.3.3 Plasma electrolytes, osmolality and protein concentration measured in anaesthetised offspring at 14 weeks of age

	Control N = 5		IF N = 7	
	Male	Female	Male	Female
Na$^+$ (mmol/L)	141 ± 3	134 ± 4	144 ± 3	141 ± 3
K$^+$ (mmol/L)	3.0 ± 0.2	3.1 ± 0.2	3.7 ± 0.1*	3.0 ± 0.1†
Cl$^-$ (mmol/L)	105 ± 1	107 ± 2	107 ± 1	104 ± 1
Osmolality (mOsm/kg H$_2$O)	357 ± 11	357 ± 9	363 ± 8	361 ± 6
Protein (g/100 mL)	4.5 ± 0.1	4.1 ± 0.1	4.3 ± 0.2	3.9 ± 0.2

Values are mean ± SEM with P > 0.05 IF versus control, * P < 0.05 IF male versus control male, † P < 0.05 IF female versus male littermates. (For K$^+$ and osmolality which did not conform to a normal distribution, Kruskal-Wallis test followed by Dunn's multiple comparisons test were used; for Na$^+$, Cl$^-$ and protein concentrations that were normally distributed, two-way ANOVA followed by Tukey's post hoc test were used).
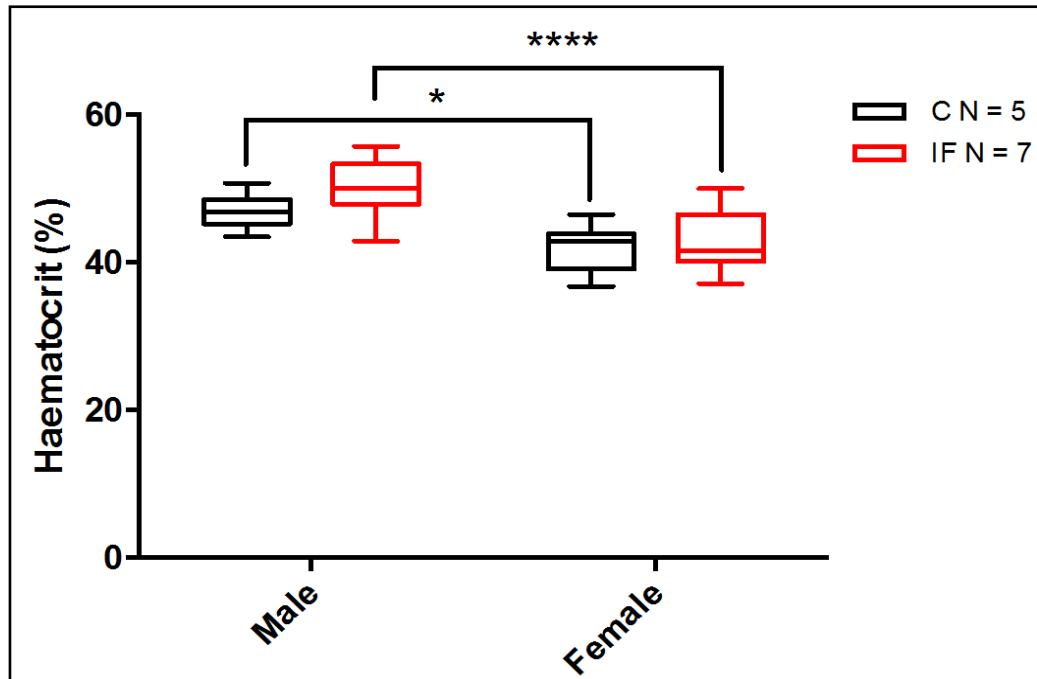
Figure 3.3.16 Haematocrit (%) measured in anaesthetised offspring at 14 weeks of age. Haematocrit was similar between the two dietary groups with males in both dietary groups having a significantly higher haematocrit compared to their female littermates. Data were analysed as mean/litter and are expressed as box and whisker plots. * P < 0.05 control male versus female, **** P < 0.0001 IF male versus female (two-way ANOVA followed by Tukey's post hoc test).

3.3.11.2 Renal haemodynamics

A renal haemodynamic analysis showed that effective renal blood flow (ERBF) and glomerular filtration rate (GFR) were stable throughout the experiment (P_{time} > 0.05, data are not shown). Maternal intermittent fasting did not have an impact on the offspring ERBF, GFR or filtration fraction (FF) (Figures 3.3.17 a, b and c). GFR and FF were similar between the two dietary groups and sexes (P > 0.05, Figures 3.3.17 b and c). ERBF, on the other hand, showed an effect of offspring sex: females in both dietary groups had significantly higher ERBF than their male counterparts (P < 0.01, Figure 3.3.17 a).

3.3.11.3 Arteriole resistance

Afferent and efferent arteriole resistances were also determined in this study. Afferent arteriole resistance was not affected by either diet or sex (P > 0.05, Figure 3.3.18 a). Efferent arteriole resistance did not differ between the diet groups. However, IF male offspring had greater resistance compared to their female counterparts (P_{Male} < 0.01, Figure 3.3.18 b).

3.3.11.4 Urine electrolytes

Urine concentrations of sodium, potassium and chloride were also measured. As shown in Table 3.3.4, all electrolyte concentrations were similar between the two dietary groups (P > 0.05). In the control group, sex divergence was apparent with regards to K^+ urine concentration and osmolality. Female offspring had a significantly lower K^+ concentration and thus osmolality compared to their male counterparts (P < 0.01, Table 3.3.4). The urine anion gap, calculated as $[Na^+]+[K^+]-[Cl^-]$, was similar between dietary groups. However, there was an effect of offspring sex with males in both dietary groups having a significantly higher urine anion gap than their female littermates ($P_{Control}$ < 0.01, P_{IF} < 0.05, Table 3.3.4).
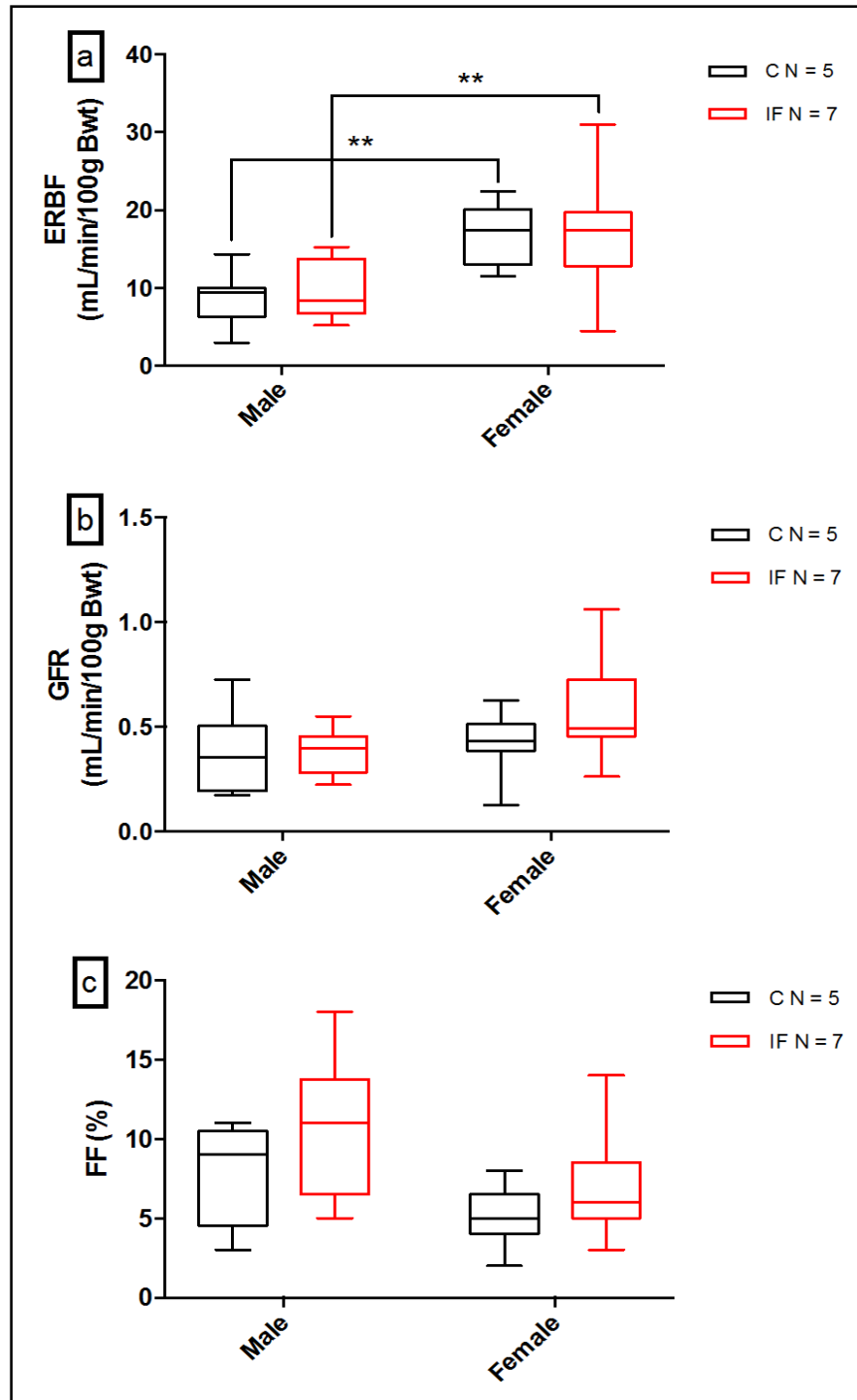
Figure 3.3.17 The effect of maternal intermittent fasting on renal haemodynamics of offspring at 14 weeks of age. **a.** Effective renal blood flow (ERBF) **b.** glomerular filtration rate (GFR) and **c.** filtration fraction (FF) determined in anaesthetised offspring at 14 weeks of age. ERBF, GFR and FF did not differ between control and IF offspring. For ERBF, females had higher ERBF than male counterparts. Data are expressed as box and whisker plots. P > 0.05 IF versus control group, ** P < 0.01 female versus male counterparts (two-way ANOVA followed by Tukey's post hoc test).
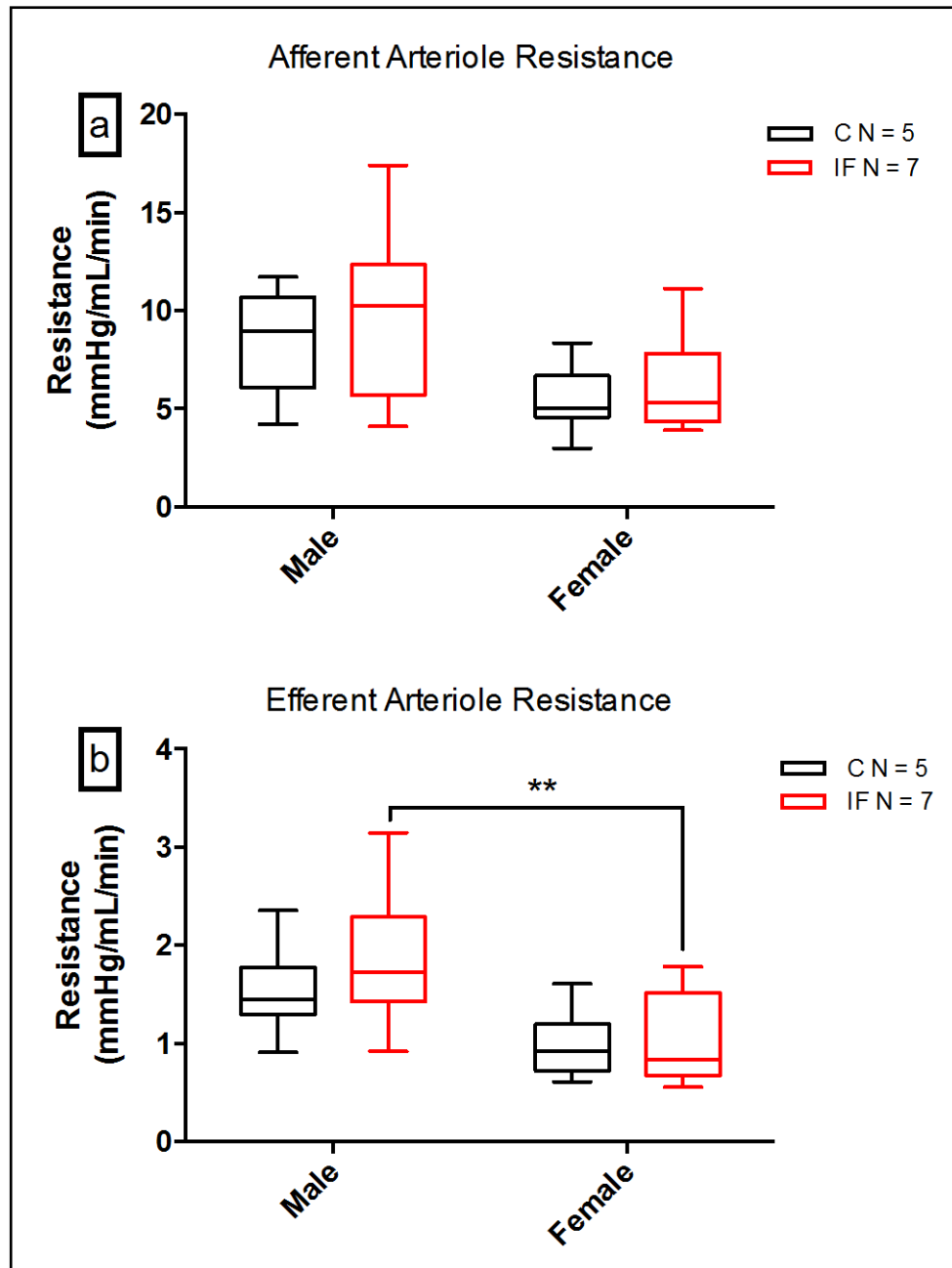
Figure 3.3.18 The effect of maternal intermittent fasting on **a.** afferent and **b.** efferent arteriole resistance of offspring at 14 weeks of age. Afferent and efferent arteriole resistances were similar between dietary groups. IF males had higher efferent arteriole resistance than their female counterparts. Data are expressed as box and whisker plots. P > 0.05 IF versus control group, ** P < 0.01 IF male versus IF female. For efferent arteriole resistance, two-way ANOVA followed by Tukey's post hoc test was used, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to analyse afferent arteriole resistance.

Table 3.3.4 Urine electrolyte concentration, urine anion gap and osmolality measured in anaesthetised offspring at 14 weeks of age

	Control N = 5		IF N = 7	
	Male	Female	Male	Female
Na$^+$ (mmol/L)	105 ± 9	108 ± 6	98 ± 11	106 ± 5
K$^+$ (mmol/L)	71 ± 4	48 ± 4**	67 ± 5	55 ± 4
Cl$^-$ (mmol/L)	164 ± 9	167 ± 7	138 ± 11	162 ± 6
Urine anion gap (mmol/L)	18 ± 3	-7 ± 7**	22 ± 6	1 ± 4*
Osmolality (mOsm/kg H$_2$O)	778 ± 53	535 ± 54**	736 ± 68	524 ± 25

Values are mean ± SEM with $P > 0.05$ IF versus control, * $P < 0.05$ and ** $P < 0.01$ female versus male littermates. (For osmolality which was not normally distributed, Kruskal-Wallis test followed by Dunn's multiple comparisons test were used; for Na$^+$, K$^+$, Cl$^-$ and urine anion gap which were normally distributed, two-way ANOVA followed by Tukey's post hoc test were used).

3.3.11.5 Urine flow rate, osmolar excretion and free water clearance

The urine flow rate was determined by weighing the urine collected in a defined time period. The urine flow rate was not affected by time and remained constant in both sexes of both dietary groups throughout the experiment ($P_{time} > 0.05$, Figure 3.3.19 a). The overall urine flow rate calculated during the 3 h post-equilibration period did not differ between the two dietary groups. However, sex differences were observed: females had a higher urine flow rate than males in both groups ($P_{IF} < 0.001$, $P_{Control} < 0.0001$, Figure 3.3.19 b). This is consistent with the fixed infusion rate and lighter bodyweight of the female offspring (Figure 3.3.13).

Osmolar excretion followed the same trend as that of the urine flow rate. Measurements were similar between the two dietary regimes ($P > 0.05$, Figure 3.3.20 a) and the females had significantly higher osmolar excretion than their male counterparts ($P > 0.0001$, Figure 3.3.20 a). For the two diet groups, there was a negative value for free water clearance (C_{H2O}), which showed that the excreted urine was hyperosmotic to plasma. C_{H2O} did not differ between the dietary groups or the sexes ($P > 0.05$, Figure 3.3.20 b).

3.3.11.6 Renal handling of electrolytes

To assess kidney handling of electrolytes, electrolyte excretion and fractional excretion of Na^+, Cl^- and K^+ were measured. Maternal intermittent fasting had no effect on renal handling of electrolytes. There were no differences in Na^+ (Figure 3.3.21 a), Cl^- (Figure 3.3.22 a), and K^+ (Figure 3.3.23 a) excretion rates between the dietary groups. Likewise, the fractional excretion (FE) of these electrolytes was similar between the two different dietary regimes ($P > 0.05$, Figures 3.3.21 b, 3.3.22 b and 3.3.23 b). However, a sex-difference was observed, as females in both diet groups had significantly higher electrolyte excretion rates than their male counterparts (Figures 3.3.21 a, 3.3.22 a and 3.3.23 a).

For fractional excretions, some differences were observed between electrolytes. No differences were observed between the sexes in terms of FE_K ($P > 0.05$, Figure 3.3.23 b). In contrast, FE_{Na} was higher only in females of the control group compared to their male counterparts ($P < 0.05$, Figure 3.3.21 b), whereas for FE_{Cl}, the females of both dietary groups had significantly higher FE_{Cl} than their male counterparts ($P < 0.05$, Figure 3.3.22 b).
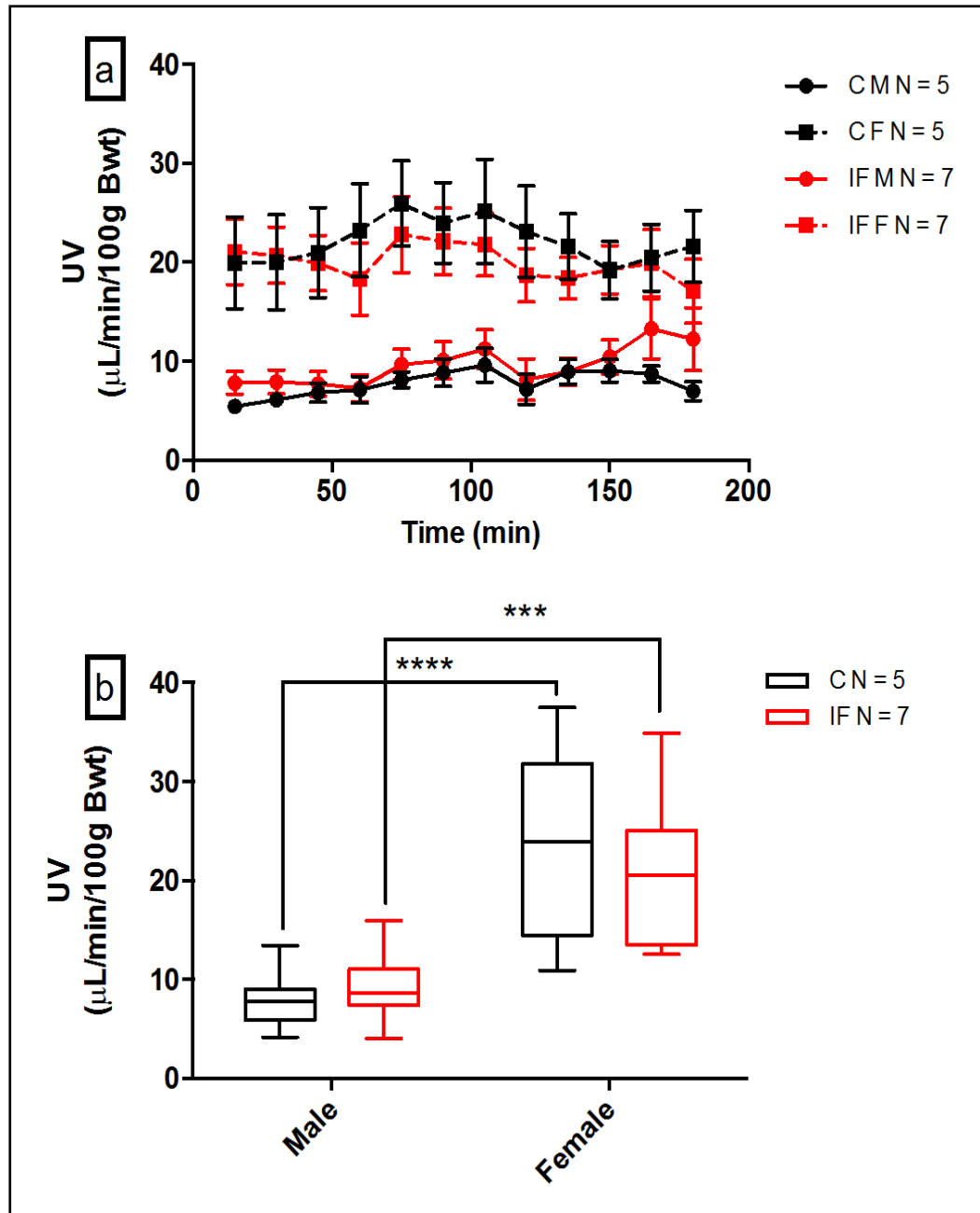
Figure 3.3.19 Urine flow rate (UV) in anaesthetised offspring at 14 weeks of age. **a.** Measurements taken over 15 min periods during the post-equilibration phase of the renal experiments showed that UV was stable over the 3 h experimental period. Date are presented as mean ± SEM. P > 0.05 IF versus control group (repeated-measures two-way ANOVA followed by Tukey's post hoc test). **b.** Overall mean of 3 h experimental period. UV was similar between dietary groups, but females had higher UV than males in both groups. Data are expressed as box and whisker plots. P > 0.05 IF versus control group, *** $P_{IF} < 0.001$, **** $P_{Control} < 0.0001$ for male versus female comparisons (two-way ANOVA followed by Tukey's post hoc test).
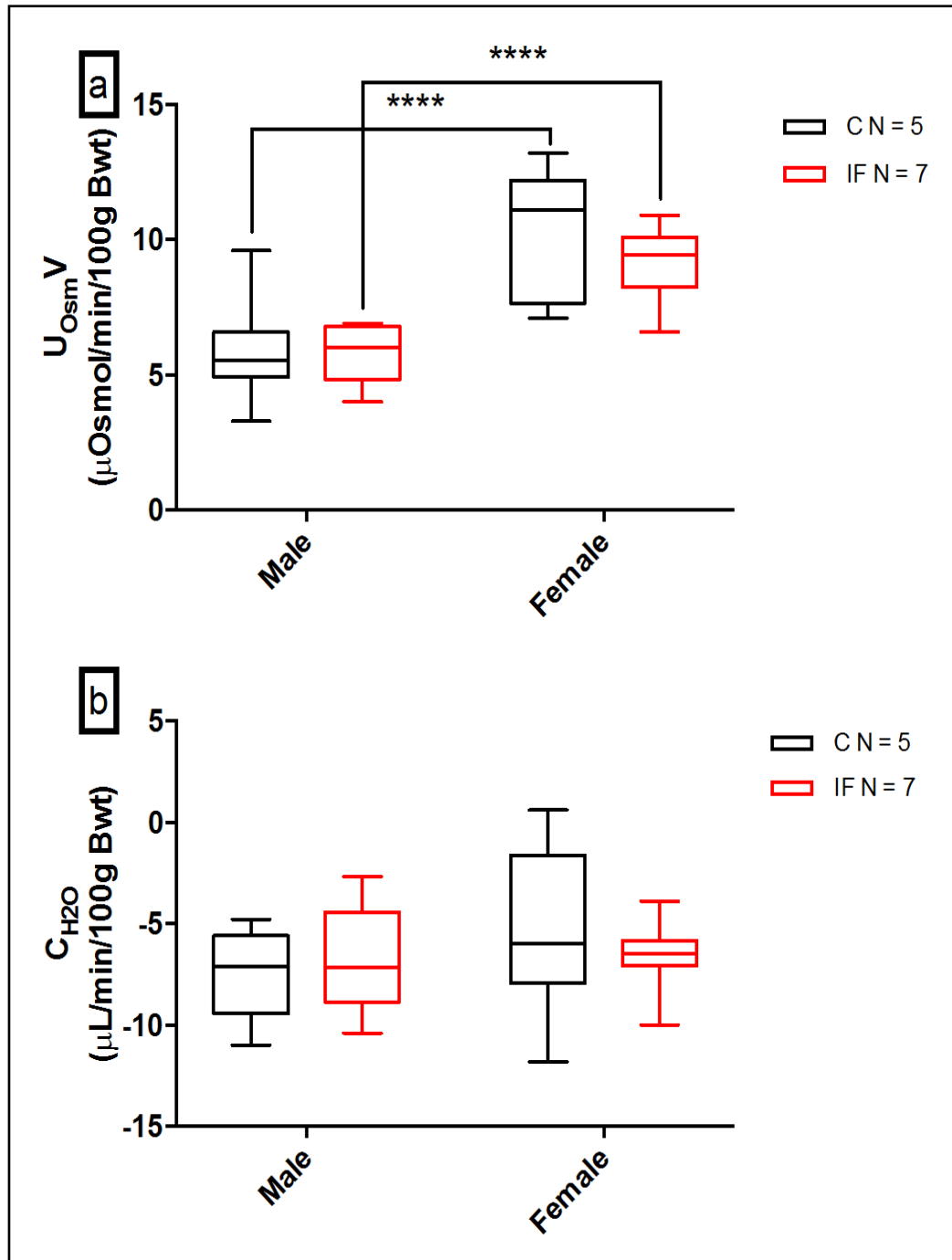
Figure 3.3.20 The effect of maternal intermittent fasting on **a.** osmolar excretion and **b.** free water clearance of offspring at 14 weeks of age. Osmolar excretion was similar between the dietary groups with females having higher osmolar excretion than the males in both groups. Free water clearance (C_{H2O}) did not differ between the dietary groups or sexes. P > 0.05 dietary groups and sexes, **** $P_{Osmolar\ excretion}$ < 0.0001 female versus male counterparts (two-way ANOVA followed by Tukey's post hoc test).
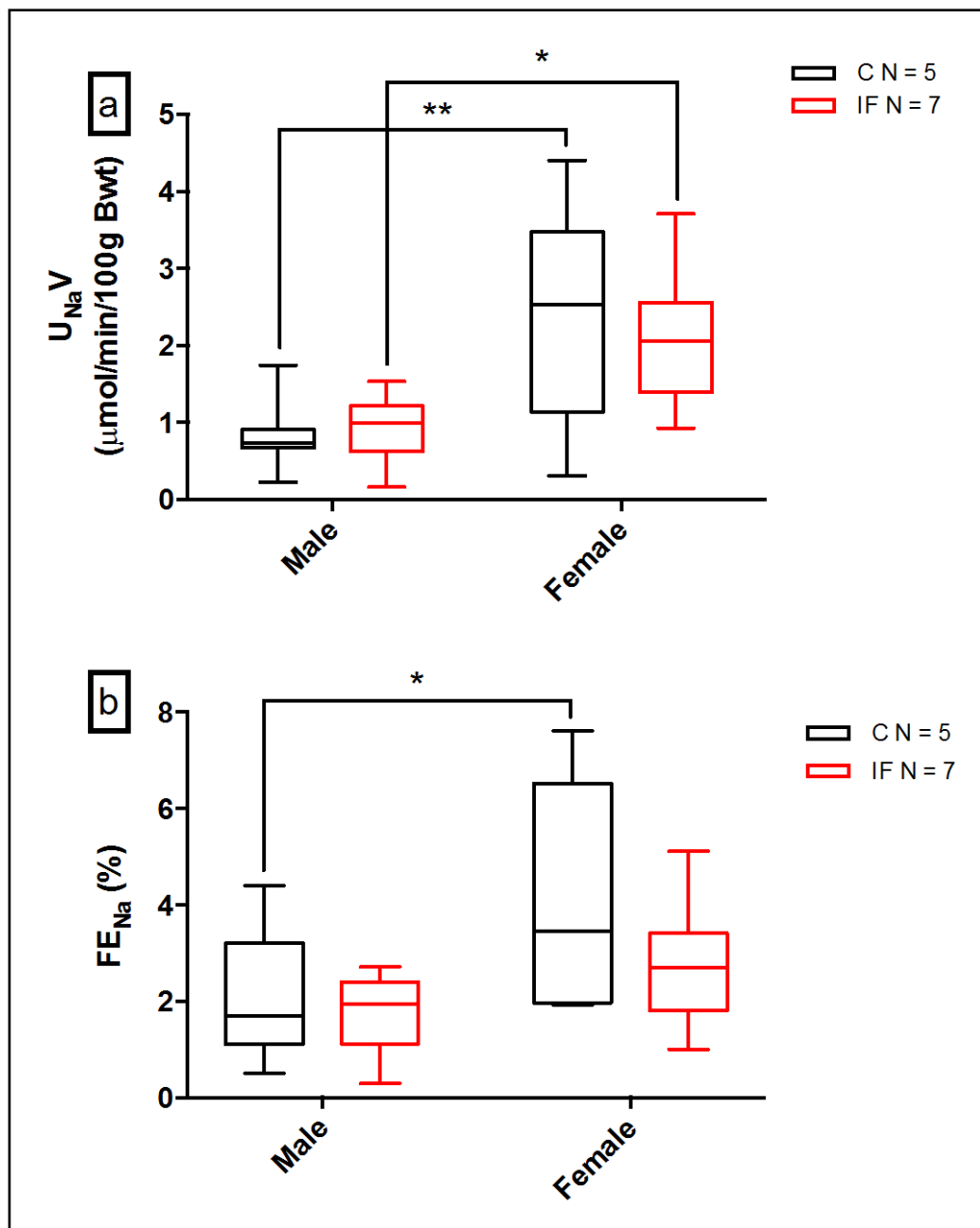
Figure 3.3.21 The effect of maternal intermittent fasting on **a.** sodium excretion and **b.** fractional excretion of sodium of offspring at 14 weeks of age. Sodium excretion and fractional excretion were similar between dietary groups. Females in both dietary groups had higher sodium excretion than their male counterparts, yet only control females showed higher FE_{Na} than males. Data are expressed as box and whisker plots. $P > 0.05$ IF versus control group, * $P < 0.05$, ** $P < 0.01$ female versus male counterparts. For sodium excretion, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used and two-way ANOVA followed by Tukey's post hoc test was used to analyse fractional excretion of sodium.
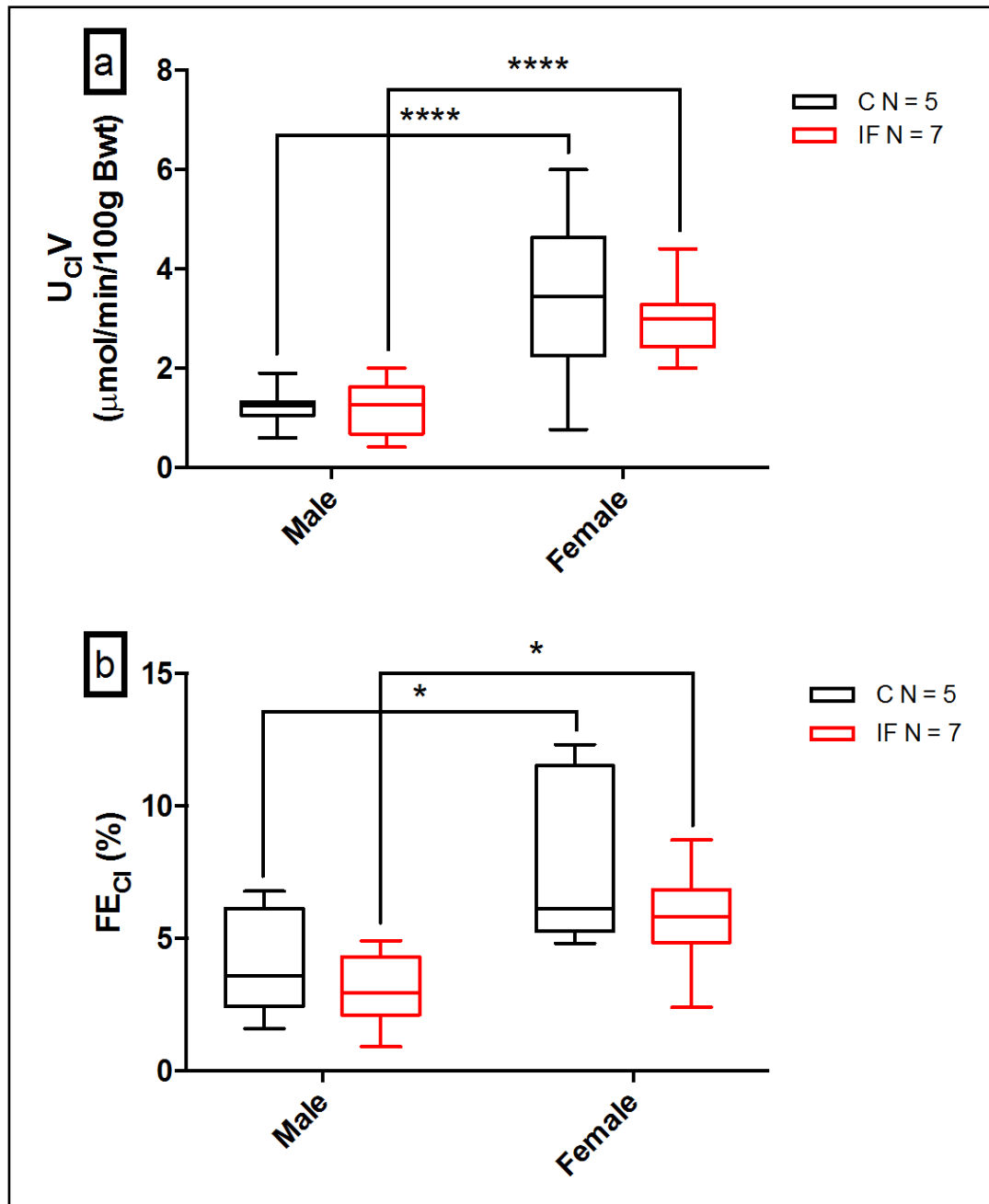
Figure 3.3.22 The effect of maternal intermittent fasting on **a.** chloride excretion and **b.** fractional excretion of chloride of offspring at 14 weeks of age. Chloride excretion and fractional excretion were similar between dietary groups. Females in both dietary groups had higher chloride excretion and FE_{Cl} respectively than their male counterparts. Data are expressed as box and whisker plots. $P > 0.05$ IF versus control group, * $P < 0.05$, **** $P < 0.0001$ female versus male counterparts. For chloride excretion, two-way ANOVA followed by Tukey's post hoc test was used, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to analyse fractional excretion of chloride.
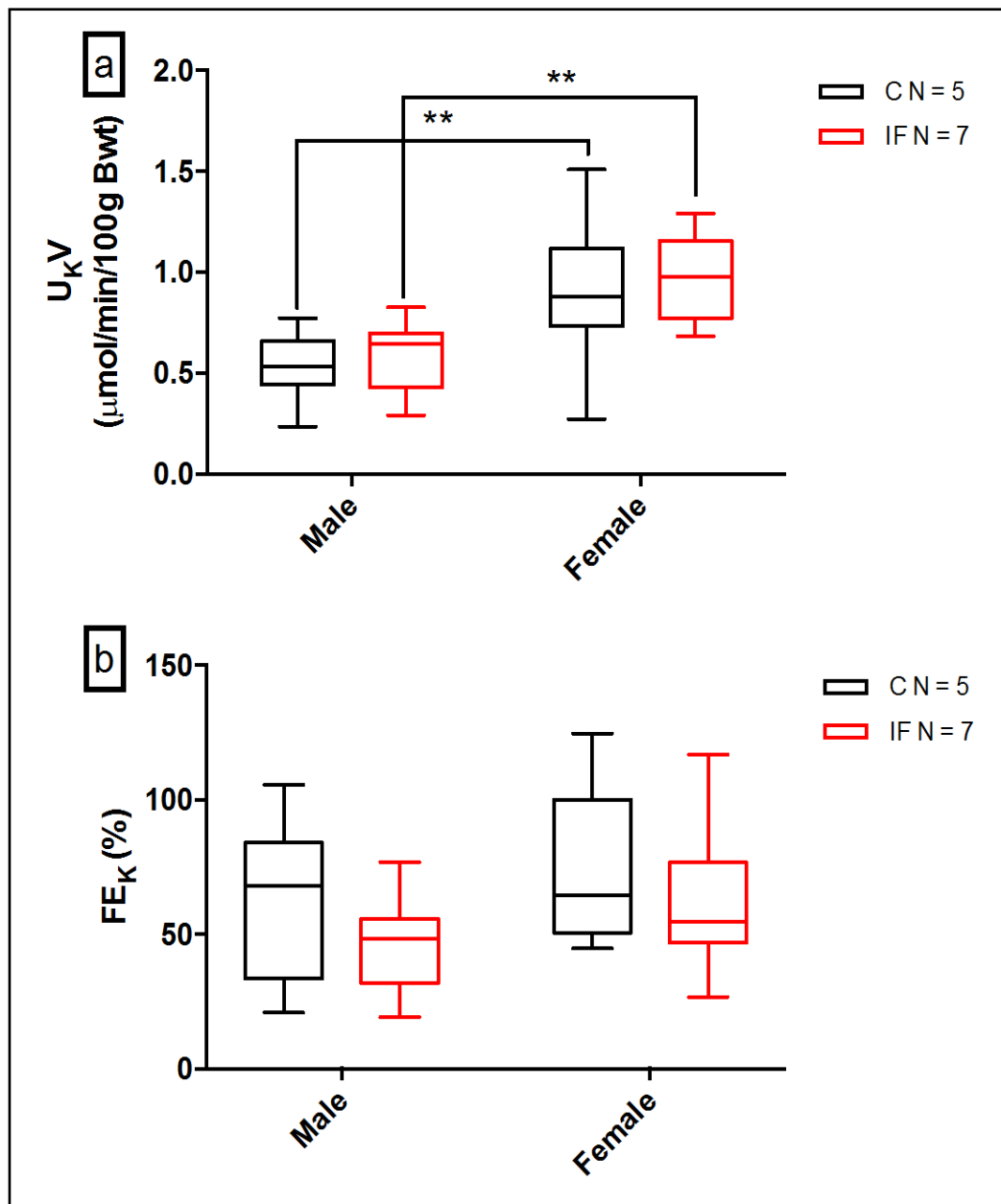
Figure 3.3.23 The effect of maternal intermittent fasting on **a.** potassium excretion and **b.** fractional excretion of potassium of offspring at 14 weeks of age. Potassium excretion and fractional excretion were similar between the dietary groups. Females in both dietary groups had higher potassium excretion than their male counterparts but with comparable FE_K between sexes. Data are expressed as box and whisker plots. P > 0.05 IF versus control group, ** P < 0.01 female versus male counterparts (two-way ANOVA followed by Tukey's post hoc test).

3.4 DISCUSSION

It has been documented previously that sub-optimal maternal nutrition leads to pre- and postnatal changes that affect the growth efficiency and health of the offspring. This study set out with four main objectives to investigate the impact of maternal intermittent fasting during pregnancy on offspring development and health. First, to assess whether rats that are growth restricted *in utero*, due to maternal intermittent fasting, remain growth restricted at birth and if this was associated with postnatal catch-up growth. Second, to examine whether maternal intermittent fasting leads to impaired metabolic function and hypertension of offspring in adulthood. Third, to assess whether a model of maternal intermittent fasting, induced throughout gestation, had detrimental effects on the renal function of adult offspring. Fourth, to determine whether effects on measured cardio-renal outcomes were dependent on the sex of offspring.

In summary, in this chapter the following observations have been made: prenatal exposure to maternal intermittent fasting, despite it shortening gestational length (all IF dams delivered early by ~ half-a-day), did not affect the birth weight or litter size. However growth of the organs was affected: the relative kidney weight was reduced in both sexes of the IF neonates with smaller relative brain weight in IF females only, despite the similarity in the bodyweight at birth (PD 1) between the groups. In both sexes of the IF cohort, neonates demonstrated a significant reduction in brain/liver weight ratio, suggesting that brain growth had been preferentially compromised.

At PD 18, there was slower growth mainly in male offspring, which faltered at 10 weeks of age. However, in contrast to other models of developmental programming, this was not associated with the onset of hypertension at 5, 7 or 10 weeks of age, as measured by tail-cuff plethysmography. In addition, IF offspring showed similar glucose and insulin tolerance profiles compared to the control offspring.

At 14 weeks of age, the bodyweight was similar between IF and control offspring, and males were heavier than females in both groups as anticipated and as previously reported (Kwong et al., 2000; Gugusheff et al., 2013). During that stage, the relative kidney weight of IF offspring became similar to controls, whereas the relative heart and lung weights were greater in females compared to males. Interestingly, despite the smaller relative kidney weight at birth, this was not associated with any changes in renal function or MAP in adult IF offspring. Renal haemodynamics, renal handling of electrolytes (Na^+, K^+ and Cl^-), plasma and urine electrolyte concentrations were similar between IF and control offspring. There were, however, sex differences in renal function measured using a constant infusion protocol.

3.4.1 Effect of intermittent fasting on maternal food intake and bodyweight

As discussed in Chapter 2 (Section 2.4.1), IF dams consumed 30% less food than the control group throughout the gestation period. This is despite being given the opportunity to compensate food intake during daytime hours, the normal period of sleep for rats. Both dietary groups gradually increased their food consumption over the course of pregnancy to reach its peak at GD 21. However, during the last day before parturition, both control and IF dams decreased their food intake by approximately $15 \pm 3\%$ compared to GD 21. This finding concurs with previous studies (Kristal and Wampler, 1973; Shirley, 1984; Parimi et al., 2004). Changes in the dams' feeding behaviour are controlled by alterations in neurotransmitter levels. Hypothalamic norepinephrine increases as gestation progresses, stimulating an increase in food intake. Before parturition, serotonin levels increase mediating a fall in food intake (Morgan and Winick, 1981). This pattern of food intake was mirrored by water consumption which gradually increased as pregnancy progressed and then fell in the last two days before birth, consistent with water intake being strongly linked to food consumption in rodents (Ellacott et al., 2010). A similar result was also reported in a study by Kirstal and Wampler (1973).

Maternal bodyweight gain was similar in control and IF dams until GD 17 (which corresponds with the onset of exponential fetal growth); after which the IF dams' weight gain was significantly lower over the last six days of gestation (see Section 2.4.1). As litter size was comparable between control and IF groups (Table 3.3.1), the reduced weight gain is likely to reflect diminished litter weight. In contrast, other studies have shown that an overall decrease in the dams' consumption of food led to an abrupt reduction in weight, but that the weight slowly rose in the latter gestational phases, in conjunction with a rapid increase in feto-placental mass (Woodall et al., 1996; Leizea et al., 1999; Langley-Evans, 2000; Vickers et al., 2000; Brennan et al., 2008; Agale et al., 2010). The observation that the IF dietary regime produced a different pattern of maternal weight gain compared with other dietary models aroused interest as to how this might impact on the offspring's growth characteristics.

3.4.2 Growth trajectories of control and IF offspring

Maternal intermittent fasting caused an alteration in the rate of the growth of the offspring. Changes in the growth trajectory of the fetal and neonatal rats occurred as follows. At GD 21, the IF fetuses were growth restricted due in part to a reduction in system A transport activity in the placenta *in vivo* (Section 2.3.15). This was mirrored by a decreased concentration of certain essential and non-essential amino acids in the maternal and fetal plasma of the IF dietary group compared to the control group (Section 2.3.9). Between GD 21 and birth (PD 1), rat fetuses underwent a phase of rapid growth, as judged by the degree of weight gain and as also reported by Knopp et al. (1973) and Langley-Evans et al. (1996a) for other models of developmental programming. IF fetuses showed a $97 \pm 5\%$ increase in their body mass over

the last two days of gestation, compared to 79 ± 6% in the control group, ultimately achieving a birth weight similar to that of the control group.

It is also important to note that all IF dams had preterm deliveries by half a day. Nevertheless, neither birth weight nor litter size was affected, suggesting that maximal catch-up of fetal growth had been achieved by this stage. Previous maternal nutrition perturbation (LP or calorific restriction) studies did not show any tendency towards preterm delivery, but instead indicated growth restriction (Lucas et al., 1997; Vickers et al., 2000; Almeida and Mandarim-de-Lacerda, 2005; Desai et al., 2005a; Alwasel et al., 2010b). Similar growth restriction was also observed at GD 21 following maternal intermittent fasting; but, as outlined above, growth retardation was no longer apparent at birth. Hence, the IF model displays an unusual phenotype compared to other dietary restriction models.

The outcome suggests that despite reduced maternal food intake by the IF group and preterm delivery, there was capacity for an adaptive response in late gestation to accelerate body growth. This adaptation may be achieved through an increase in SNATs mRNA expression seen at GD 21 (Section 2.3.13), which could plausibly be translated to an up-regulation in system A activity. However, it is important to note that the up-regulation in SNATs mRNA expression was only significant in IF male placentas. An alternative interpretation is that IF mothers undergo a rapid catabolic phase, breaking down their protein and fat stores to support the fetal growth demands in late pregnancy. Knopp et al. (1973) identified that normal pregnant dams have two distinct metabolic phases. At mid-gestation, the maternal metabolism is characterised by an increase in food intake, plasma insulin concentrations and hepatic conversion of glucose to glycogen and adipose tissue fatty acids. Hence, the ingested fuels are directed to maternal stores, as feto-placental needs are minimal at this stage. However, near term, fetuses undergo rapid growth and a second phase of maternal metabolic adaptation begins. This phase is characterised by continued hyperinsulinaemia with decreased tissue responsiveness to insulin and a decline in hepatic conversion of glucose to fatty acids (Knopp et al., 1973; Zammit 1985). It allows substrates to be shifted from the mother to the fetus to achieve resource requirements for fetal growth whereas lipolysis provides the energy source for the mother (Knopp et al., 1973). Therefore, for IF dams to overcome FGR seen at GD 21 and reach a weight similar to that of control fetuses at PD 1, we speculated that a depletion in the IF maternal body reserves was coupled with a more effective utilisation of substrates in fetal tissues. The results seen in the previous chapter (Sections 2.3.4 and 2.3.8), in which IF dams showed significantly lower plasma glucose and amino acid concentrations and diminished heart and liver weights relative to bodyweight as compared to control dams, while maintaining plasma insulin concentrations similar to those of the controls at GD 21, lend support to this notion.

In addition, the day before parturition food was not restricted to avoid the potential for cannibalism of pups by hungry dams. The question that arose was whether the dams of the IF group consumed more food on that day and whether the sudden food provision was rapidly transported to the fetus as system A activity increased toward the end of gestation: this is still unclear. Coan et al. (2010) reported that 20% food restricted mothers had lower weights than control dams at GD 16 and GD 19. Although the dams in both groups gained weight between GD 16 and GD 19, the undernourished mothers had significantly less weight at GD 19 than GD 16 after undergoing hysterectomy (maternal weight minus the contests of the uterus). This points towards mobilisation of maternal stores during the catabolic phase to sustain fetal growth at late gestation. Moreover, the undernourished fetuses underwent twice the weight gain between GD 19 and birth than that seen in control fetuses, which supports what was observed in the current study.

Looking at growth trajectory, a different pattern of growth was reported in previous studies in connection with exposure to low-protein diets *in utero*. Langley-Evans et al. (1996a) found that LP-restricted fetal rats were heavier than controls from GD 18 to 20. The authors proposed that this weight gain was due to an increased maternofetal substrate transfer via a larger placenta as an adaptive response to a low protein diet from mid- to late gestation (Langley-Evans et al., 1996a). However, in this model, such a late gestational adaptive response may have been relatively suppressed, as the LP group achieved a birth weight similar to that of the controls. In contrast, the IF dietary regimen induced a different pattern: IF placentas were similar in weight to controls at GD 21, but late gestational accelerated fetal weight gain was promoted. Alwasel et al. (2010b) also reported that fetuses exposed to an LP diet *in utero* became heavier between GD 18 and 21, but in this study they were lighter than the control group at birth. Similarly, a 50% - 70% calorific restriction resulted in growth restriction at birth (Lucas et al., 1997; Vickers et al., 2000; Almeida and Mandarim-de-Lacerda, 2005; Desai et al., 2005a). These outcomes highlight that growth trajectory and growth phenotype, as determined by birth weight, appear to be dependent on diet type, duration and gestational stage.

One of the main objectives of the present study was to compare growth outcomes between fetal sexes. However, no sexually dimorphic responses to maternal intermittent fasting were observed in terms of birth weight. In contrast other studies imposing a moderate LP diet either during the pre-implantation period (Kwong et al., 2000) or throughout gestation (Zambrano et al., 2006) showed that only females had reduced birth weight. In mice, exposing female dams to a LP diet 3.5 days pre-mating showed that LP males had a tendency towards increased birth weight (P = 0.073; Watkins et al., 2008b), whereas in another study imposing protein restriction during pre-implantation caused significant increases in birth weight of both males and females (Watkins et al., 2008a). Thus, it appears that the type of maternal insult and timing of manipulation, as well as animal strains, modulate the effect of sexual dimorphic responses.

Consistent with this study's findings, several studies in humans have found that neonatal birth weight is unaffected by exposure to Ramadan fasting (Cross et al., 1990; Arab and Nasrollahi, 2001; Kavehmanesh and Abolghasemi, 2004; Alwasel et al., 2010a; Barker et al., 2010a; Ozturk et al., 2011; Seckin et al., 2014). However, other studies in Michigan (Almond and Mazumder, 2011) and the Netherlands (Savitri et al., 2014) reported the converse result that prenatal exposure to Ramadan fasting during early pregnancy negatively affected birth weight. These conflicting observations made regarding birth weight among human studies could be related to the season (day length) in which Ramadan fell and trimester of exposure. Studies also investigated the correlation between preterm delivery and Ramadan fasting. The authors reported that Ramadan fasting at any stage of pregnancy does not affect the rate of preterm delivery (Kavehmanesh and Abolghasemi, 2004; Ozturk et al., 2011; Awwad et al., 2012; Petherick et al., 2014). In contrast, Herrmann et al. (2001) reported that pregnant women fasting for 13 h or longer are at threefold higher risk of delivering prematurely at week 34 of pregnancy than those fasting for less than 13 h. Prentice et al. (1983) also reported that mothers who observed Ramadan fasting had a raised occurrence of admission of their newborn babies to special care baby units. Nevertheless, there is a lack of well-conducted human studies addressing the relationship between Ramadan fasting and birth outcomes in humans. In most of the existing studies, the numbers of enrolled subjects were low, thereby reducing statistical validity.

The offspring of the IF group demonstrated similar growth rates to those of control pups during the early suckling period, but had significantly reduced weight at PD 18 and remained significantly lighter than the controls until the end of 4 weeks. It is well established that for the first 15 to 17 days after birth, the dam's milk is the only source of nutrients for the pups. Thereafter, they begin to eat solid food, as well as milk, before weaning from 16 to 18 days of age (Thiels et al., 1990). It was from PD 18 that IF offspring started to show a reduction in their growth rates, which lasted until week 10 in males; females did not differ from controls. This is consistent with a preliminary study in our laboratory (Wilkinson et al., 2010), which also showed that IF pups were lighter at 4 weeks of age but had caught up by 10 weeks after birth. It is likely that the reduced body growth in IF males resulted from reduced food intake, consistent with the evidence shown in Chapter 4 (Section 4.3.1) that food intake relative to bodyweight was significantly higher in control males compared to IF males at postnatal weeks 5 and 6. One of the factors that plays a role in regulating appetite and energy expenditure is leptin (Friedman and Halaas, 1998). In rodents, an increase in leptin generally occurs in the second week following birth (Ahima et al., 1998; Yura et al., 2005). A study by Yura et al. (2005) demonstrated that maternal undernutrition induced a premature neonatal leptin surge in mice, leading to altered hypothalamic development. Accordingly, the underfed mice gained more weight when weaned on a high-fat diet. The significance of this early upsurge as a basis for future obesity has been shown via induction of an artificially premature leptin increase in young that were nourished normally, giving rise to the development of a phenotype identical to that of

the underfed pups. Consequently, metabolic system development seems to require a postnatal increase in leptin, and this can be substantially influenced by prenatal diet. Vickers et al. (2005) also demonstrated that prenatal undernutrition programmed rat offspring appetite and reduced energy expenditure; however, leptin administration during the first 10 postnatal days reversed these outcomes. Another study by Desai et al. (2005b) found that offspring subjected to a 50% food restriction during pregnancy were growth restricted at birth with reduced leptin concentrations. Cross-fostering those offspring to control-fed dams during the lactation period resulted in a rapid catch-up growth at 3 weeks of age that was sustained, with weight gain increased into adulthood that was associated with offspring becoming hyperleptinaemic. Furthermore, LP offspring displayed higher plasma leptin concentration at 4 and 12 weeks of age associated with leptin resistance compared to controls (Song et al., 2007). The overall findings of these analyses indicate that early concentrations of leptin cue the development of the hypothalamic circuitry. In IF offspring the reduction in growth coincides with the normal postnatal leptin surge; hence intermittent fasting may further stimulate leptin secretion or/and sensitivity at this stage, leading to a reduction in food intake and increased energy expenditure. With higher leptin concentrations, hypothalamic leptin resistance can occur which increases bodyweight gain in adulthood. This topic should be a priority for forthcoming investigations.

There are no comparable published reports on the effect of exposure to Ramadan fasting *in utero* on growth trajectories of children and young adults. In human populations and other animal dietary models, changes in growth trajectory seem to be subtle and dependent on the nature of the maternal nutrition perturbation. As an example, in the Dutch famine, a reduction in maternal nutritional intake at any stage of pregnancy presented with increased rates of obesity among female offspring (Ravelli et al., 1999). On other hand, males exposed to famine during the first two trimesters of pregnancy developed obesity at 19 years of age with about two-fold higher prevalence of obesity than that of controls (Lumey et al., 1993; Ravelli et al., 1999; Selassie and Sinha, 2011). In some animal studies, a prenatal calorific restriction and a low-protein diet resulted in postnatal catch-up growth in offspring and the probability of developing obesity in later life (Manning and Vehaskari, 2001; Ozaki et al., 2001; Almeida and Mandarim-de-Lacerda, 2005; Desai et al., 2005a, b; Parlee and MacDougald, 2014), while other calorific-restricted offspring studies had low birth weight neonates that remained consistently lighter than the controls during the entire experimental period (Wlodek et al., 2008; Chen and Chou, 2009; Ellis-Hutchings et al., 2010; Akitake et al., 2015).

Accelerated postnatal growth, or "catch-up growth", following fetal growth restriction has been shown in both human and animal studies to be an important factor in the programming of later cardiovascular and metabolic disease risks (Eriksson et al., 1999; Ong et al., 2000; Langley-Evans et al., 2005; Ibáñez et al., 2006; Berends et al., 2013) and to be a separate risk factor for becoming overweight even as early as in childhood (Stettler et al., 2002). In severe food restriction, the undernourished offspring developed hyperphagia, obesity and hypertension in

adulthood (Vickers et al., 2000), in which these detrimental effects were exacerbated by excessive postnatal nutrition (Vickers et al., 2003). The processes implicit in the phenotypic responses in Vickers et al. (2000) study have not yet been explained, but evidence indicates that attenuation of sensitivity to leptin's appetite-depressing and metabolic influences may be a key factor (Krechowec et al., 2006). Ozanne and Hale (2004) also reported that "catch-up growth" in male mice reduced longevity compared with animals that do not show "catch-up growth". In the current study there was a trend towards catch-up growth in IF male offspring over the post-weaning period, but, interestingly, these offspring failed to develop cardio-renal disease at 14 weeks of age. Jimenez-Chillaron et al. (2006) evaluated how nutrition in the early postnatal period can attenuate catch-up growth and prevent glucose intolerance and obesity in low birth weight mice. In their study, growth-restricted mice were suckled either by dams fed 50% less than *ad libitum* during lactation or by dams on an *ad libitum* diet. Normal birth weight mice were suckled by dams on a restricted diet during the lactation period. The study came to the conclusion that low birth weight male mice that underwent early postnatal catch-up growth developed obesity and glucose intolerance by the age of six months. However, blunting catch-up growth during the lactation period or delaying it to the post-weaning period prevented adverse metabolic outcomes. Accordingly, the rate and time window of catch-up growth are critical determinants of the degree of metabolic programming in growth-restricted mice (Jimenez-Chillaron et al., 2006). It follows that impaired fetal development correlates with a greater risk of future metabolic disorders, and this risk is exaggerated by either increased early postnatal growth, increased nutritional intake later in life or both.

3.4.3 Effect of IF on neonatal and adult organ weights

In this study, neonatal kidney, liver, heart and brain weights were recorded to determine whether intermittent fasting during pregnancy has an influence on organ development. It was revealed that maternal intermittent fasting caused a change in offspring organ weights, with some selectively affected in a sex-dependent manner.

Both absolute and relative (adjusted for bodyweight) kidney weights were significantly reduced in both sexes of IF neonates at birth. This accords with previous studies that have described similar effects following exposure to 30% and 50% calorific restriction (Lucas et al., 1997; Vickers et al., 2000; Brennan et al., 2008) and protein restriction in rats (Woods et al., 2001a; Vehaskari et al., 2001; Courrèges et al., 2002). Previous data from our laboratory have demonstrated that kidney weights increase over the first 12 postnatal days in IF rats (at which point nephrogenesis has ceased (Kavlock and Gray, 1982)) such that they catch up with those of controls (Mallett, unpublished observations). However this recovery in kidney weight may not necessarily reflect a recovery in kidney structure and function. For example, the smaller kidney sizes seen in 50% calorific-restricted animals only persisted for the first two weeks after birth, yet the nephron number was reduced into adulthood (Brennan et al., 2008). Lucas et al. (1997)

showed that the kidneys of calorific-restricted neonates not only exhibited reductions in absolute and relative weight but also alterations in structure, such as glomerular hypertrophy and increased medulla/cortex ratio, along with nephron deficits that were present in 3 month-old offspring. Similar outcomes were reported as a result of low-protein diets during pregnancy by Courrèges et al. (2002), Sahajpal and Ashton (2003), Pires et al. (2006) and Alwasel et al. (2010b). These studies highlight that maternal undernutrition when imposed during gestation results in a permanent impairment in renal development of the offspring.

It would have been interesting to determine the nephron number in kidneys of control and IF offspring, but due to time constraints, this was not possible as part of this study. However it would be an interesting objective to determine in the future, to characterise more fully the renal phenotype in this model. Nonetheless, it is hypothesized that nephron number would be reduced, at least at birth if not by the end of nephrogenesis. A study by Hughson et al. (2003) that determined the nephron number in normal postnatal human kidneys revealed a linear correlation between birth weight and nephron number. Furthermore, Gubhaju and Black (2005) demonstrated the presence of a tight, linear relationship between the nephron number and the weight of fetal baboon kidneys during nephrogenesis. This informative observation suggests the possibility of reduced nephron endowment in the kidneys of IF offspring, although it is important to acknowledge that, unlike humans, nephrogenesis is not complete in the rat at birth.

Further support for this notion is provided in a recent PhD thesis by Mohamed Elwan from our collaborator's laboratory at King Saud University in Saudi Arabia. He studied the impact of maternal intermittent fasting throughout pregnancy with both food and water deprivation for 16 h/day on kidney structure in male offspring. IF kidney weight was reduced at birth and showed a recovery in terms of organ weight, yet altered kidney structure and morphology lasted until adulthood. At birth, neonatal kidneys from this model showed a 30% deficit in the nephron number that was associated with increased apoptosis and connective tissue deposition. In the adult kidneys from this group, mild glomerulosclerosis with thickening in Bowman's capsule in the renal cortex and tubular dilation in the renal medulla were found. Thus, maternal intermittent fasting impaired renal development and may program the offspring for hypertension and renal disease (Saleh Alwasel, personal communication), although in the present study, no impact of maternal intermittent fasting was seen on cardio-renal function of offspring at the ages studied. However, the possibility of disease onset later in life cannot be excluded.

Absolute and relative liver weights were unaffected in the present study. This contrasts with studies of rats in which the offspring of dams fed a low protein diet had significantly reduced liver weights (Langley-Evans et al., 1996a; Zhang and Byrne, 2000) with altered metabolic zonation in which the activity of hepatic enzymes in different liver zones were altered differentially in response to the maternal nutritional insult (Desai et al., 1995). Maternal diet had

no effect on heart weight; however, there was a sex divergence with females of both the IF and control groups having significantly heavier relative heart and lung weights compared to the males at 14 weeks of age. Such sex differences in organ weights have been reported previously in rodent studies by Wallen et al. (2000), Massaro and Massaro (2006) and Carey et al. (2007). The difference is postulated to be related to the oestrogen hormone that binds to the intracellular oestrogen receptor and induces an increase in the expression of certain genes that consequently have an effect on heart mass (Pelzer et al., 1996; Wallen et al., 2000; Babiker et al., 2002) and lung volume (Massaro and Massaro, 2006; Carey et al., 2007). Other studies, however, such as Desai et al. (2005a), showed a permanent reduction in the relative heart and kidney weights in calorific-restricted offspring up to nine months, contrary to the present study. Collectively, studies by Desai et al. (1996; 2005a) and Joshi et al. (2003) have documented differing impacts of nutritional perturbation during pregnancy or/and lactation on the growth of vital organs at different points in life.

The development of the brain is highly sensitive to nutrient provision. The absolute weight of the brain was significantly reduced in both sexes of IF neonates. The relative weight of the brain, on the other hand, was lower only in IF females. However, both fetal sexes of the IF group exhibited ~15 ± 2% reduction in the brain/liver weight ratio, indicating that the brain had been preferentially affected relative to other organs. This finding is in contrast with the phenomenon of 'brain sparing' (where the brain/liver weight ratio would be expected to increase if brain growth was relatively protected), which has been reported in other studies of offspring exposed to undernutrition *in utero* (Desai et al., 1996; Saito et al., 2009; Agale et al., 2010).

The consequences of this observed reduction in brain weight were followed up in a separate study by two MRes students in our laboratory, Ivana and Olivera Rajkovic. They investigated the impact of exposure to intermittent fasting *in utero* on cognitive behaviour, particularly recognition memory, in juvenile, adolescent and adult offspring by applying the novel object recognition (NOR) test. The NOR test is based on the spontaneous tendency of rats to spend more time exploring a novel object than a familiar one (Antunes and Biala, 2012). They found that maternal intermittent fasting had sex-specific effects. A permanent cognitive deficit was observed in female offspring starting at the juvenile stage, whereas males did not develop a cognitive deficit until adulthood (Rajkovic and Rajkovic, unpublished observations). These results suggest that maternal intermittent fasting could be a risk factor for the developmental progression of impaired cognitive function and learning disabilities in the offspring and that female offspring might be especially susceptible. This may also explain the greater vulnerability of females in the IF group to seizure attack. The results captured the interest of MRes student Emma Scott of our laboratory, leading her to explore the effect of maternal intermittent fasting during the pre-implantation period on the cognitive behaviour in adolescent and adult offspring, by mimicking Ramadan fasting in humans during the early stage of pregnancy. The study used

three groups of dams: the controls fed *ad libitum*, the IF group were food restricted overnight for only the first three days of gestation and a pair-fed calorific-restricted group that was determined by quantification of food intake in the IF rats over the first 3 days of pregnancy. The research revealed sex-specific cognitive deficits, whereby IF females were severely affected starting at adolescence until adulthood, while IF males showed memory deficits only at adult age. The remarkable finding was that female offspring of pair-fed calorific-restricted dams showed a cognitive deficit only in adolescence which was abolished at adulthood. The effect could be related to the novelty of this dietary regimen and to the imposed regular cyclical pattern of maternal eating-fasting, rather than the amount of food consumed. The next chapter will highlight this relationship in more detail.

All of these data add to the wealth of literature showing that prenatal exposure to a nutritional insult can affect the offspring's cognitive behaviour. In Jaiswal and Bhattacharya's (1994) study, a 50% food restriction during gestation resulted in impaired learning acquisition in the rat offspring. As well, baboon offspring exposed to a 30% calorific restriction throughout gestation exhibited impaired working memory and decreased motivation (Rodriguez et al., 2012). Likewise, mouse offspring that were subjected to further moderate calorific restriction in the early lactation period demonstrated mental disabilities such as anxiety and poor cognitive function (Akitake et al., 2015). Watkins et al. (2008b) found that an LP diet during the periconceptional period induced behavioural dysfunction observed by increased anxiety-related behaviour in open field activities in both adult male and female offspring. Together, the data from these studies show that cognitive function can be influenced by maternal diet during pregnancy and lactation.

It is interesting to note in this context that human census data from Iraq and Uganda showed that in children exposed to Ramadan fasting *in utero*, especially during the first trimester when organogenesis occurs, there was a 20% increase in the incidence of mental and learning disabilities (Almond and Mazumder, 2011). Furthermore, Alwasel et al. (2011) reported that there were sex-differences in body size among neonates in Saudi Arabia who were exposed to Ramadan fasting in the second trimester. Male babies had larger head circumferences with thinner chests while female babies had larger chests and smaller head circumferences. Hence, the authors concluded that somatic and brain sparing may confer an immediate protective advantage to boys, while the growth of visceral and soft tissue is compromised (Alwasel et al., 2011). This would also accord with the notion that fetal sex influences adaptive developmental responses that occur *in utero* in response to an altered nutrient environment.

3.4.4 Systolic blood pressure in IF and control offspring using tail-cuff plethysmography

In the present study, systolic blood pressure was measured by myself and Dr Heather Eyre using tail-cuff plethysmography. This non-invasive technique is widely used to measure SBP in

rats. However, it is prone to introducing measurement artefacts as the conscious rat is restrained, and it requires preheating to increase the blood flow in the caudal artery to enhance the amplitude of pulsation for pulse signalling (Borg and Viberg, 1980). Another approach is radio-telemetry, where a monitoring device is implanted intraperitoneally with a catheter inserted in the abdominal aorta of the rat, which allows for direct high-fidelity blood pressure readings that are free from the effects of stress on the rat (Van Vliet et al., 2000). This approach is considered the gold standard for measuring blood pressure (Van Vliet et al., 2000); however, the high cost of the device and the large number of enrolled animals in this experiment prohibited its use. In the future, it would be of value to determine blood pressure in the IF model using radio-telemetry in order to obtain a more complete picture in free-moving rats over a 24-hour cycle.

The rats in this study were either placed on the arm in a cradle position or in a restraint tube placed on a Thermopad heated mat (only for 5 min during the acclimation period before placing the tail-cuff device). The SBP readings obtained using either method were similar and consistent, which demonstrates that the readings were unaffected by preheating or restraint and were also highly reproducible between operators. Based on the extensive literature showing that poor maternal nutrition during pregnancy is associated with raised blood pressure in the offspring (Langley-Evans and Jackson, 1996; Langley-Evans et al., 1996b; Woodall et al., 1996; Manning and Vehaskari, 2001; Vehaskari et al., 2001; Ozaki et al., 2001; Sahajpal and Ashton, 2003; Alwasel et al., 2010b; Swali et al., 2010) and the observation that fetal weight and the fetal-to-placental weight ratio were significantly reduced in the IF group at GD 21 with disproportionate growth between late gestation and birth (Section 2.4), we hypothesised that maternal intermittent fasting during pregnancy may increase the risk of IF offspring developing hypertension. Therefore, SBP was measured in offspring at postnatal weeks 5, 7 and 10 (Figure 3.3.10) as the animals progressed through puberty into young adulthood.

Contrary to expectations, there was no difference in SBP between the dietary groups or between the sexes. The SBP tended to rise from week 5 until week 10 of age by approximately 8 ± 0.9 mmHg in both IF and control animals. Offspring heart rate was also recorded and was similar over the same period between control and IF groups and between sexes (~ 430 bpm). MAP was also measured directly via a carotid artery catheter in anaesthetised offspring at postnatal week 14. No differences between control and IF offspring or between the sexes of each group were observed (Figure 3.3.15 b), although it is acknowledged that the anaesthetic used in this study depresses the cardiovascular system which may have affected the results (Elmer et al., 1972). Interestingly, an MRes student in the laboratory, Grace Mallett, observed increased MAP in anaesthetised IF rats at 4 weeks of age. Though it is difficult to explain why differences in blood pressure were not manifest one week later at 5 weeks when measured by the tail cuff method, a possible explanation is that hypertension programmed by prenatal

intermittent fasting in 4 week-old IF offspring may be compensated for in adult life. Nonetheless, measurement of blood pressure by two different methods shows that exposure to intermittent fasting has no effect on offspring blood pressure from 5 weeks up to 14 weeks of age. This observation is contrary to the reported effects of dietary manipulation *in utero* both in humans and in animal models, where the manifestation of hypertension persisted into adulthood. Hence, the offspring of the IF dietary model present with a distinct cardio-renal phenotype compared to other dietary restriction models, as referenced below.

Numerous epidemiological studies have shown that the incidence of adult hypertension is strongly linked to exposure to a sub-optimal intrauterine environment (Barker and Martyn, 1992). A substantial amount of research indicates a strong relationship between low birth weight and systolic blood pressure and the occurrence of hypertension in adulthood (Law and Shiell, 1996; Curhan et al., 1996; Huxley et al., 2000). Moreover, studies by Barker et al. (1990) and Hemachandra et al. (2006) indicate that placental size and placental-to-birth weight ratio can predict hypertension development in offspring more accurately than lower birth weight.

In rat studies, maternal dietary protein or calorific restriction during pregnancy causes hypertension in the offspring (Langley and Jackson, 1995; Langley-Evans et al., 1996b; Woodall et al., 1996; Manning and Vehaskari, 2001; Vehaskari et al., 2001; Ozaki et al., 2001; Sahajpal and Ashton, 2003; Alwasel et al., 2010b). The Langley-Evans group showed that moderate protein restriction, starting before conception and continuing during gestation, led to hypertension in the offspring at 4 weeks of age in both sexes (Langley-Evans et al., 1994). In a later study, the same authors described the effect of protein restriction at discrete periods of pregnancy (early, mid and late gestation) on initiating hypertension in weaning offspring (Langley-Evans et al., 1996b). SBP was elevated by 26 - 28 mmHg (similar to the magnitude found in their earlier study) in 4 week-old offspring exposed to an LP diet at each stage of gestation. However, the exposure to an LP diet in early gestation induced hypertension only in the male offspring. Moreover, a study by Kwong et al. (2000) showed that imposition of an LP diet during the preimplantation period alone was sufficient to increase SBP by 12 mmHg in male rat offspring at 4 weeks of age. These data are all based on measurements obtained by tail-cuff plethysmography while the rat is under restraint in a Plexiglas tube. Similar studies in mice employing an LP diet during the pre-implantation period, also showed cardiovascular disorders in adult mice. These included relative hypertension in both sexes, increased lung angiotensin-converting enzyme activity and reduced capacity for arterial dilatation in response to isoprenaline as well as reduced size of the heart in females only, factors that may play a role in increasing blood pressure (Watkins et al., 2008a; 2010). In Woodall et al. (1996), offspring exposed to severe calorific restriction throughout pregnancy were also examined for blood pressure using plethysmography. A modest elevation of SBP was observed (5 to 8 mmHg) between 30 and 56 weeks of age.

Another study looked at the effect of two windows of prenatal 50% undernutrition (GD 1 - 15 or GD 10 - 21) on adult offspring blood pressure in two rat strains (Wistar and Sprague-Dawley) using the tail-cuff technique (Ellis-Hutchings et al., 2010). They observed elevated SBP in Sprague-Dawley offspring at postnatal week 10, much earlier than Wistar offspring, which did not show elevated blood pressure until postnatal week 26. Therefore, the strain of rat can have an impact on the timing of the onset of hypertension. Since Wistar rats were used in the current study, it is possible that blood pressure had not begun to diverge at 14 weeks of age and that a longer follow-up period is necessary to detect changes in the blood pressure of the offspring. However, it is worthy of comment that previous studies in our laboratory have showed an increase in blood pressure at 4 weeks of age in Wistar rats exposed to protein restriction during pregnancy (Sahajpal and Ashton, 2003; Alwasel and Ashton, 2012). Alternatively, exposure to intermittent fasting may not affect offspring blood pressure, as other studies have shown that dietary manipulation during gestation does not always result in raised blood pressure. Brennan et al. (2008) reported that the offspring of 50% calorific-restricted dams, even though they were growth restricted at birth with smaller kidneys up to 14 days of age and a lower nephron number that persisted into adulthood, failed to show raised blood pressure between 10 and 11 weeks of age.

The method used to measure blood pressure may also have affected the outcome. Using radio-telemetry, Tonkiss et al. (1998) reported only a minor elevation of diastolic blood pressure (DBP; 4 mmHg) and heart rate (25 bpm) in the waking phase of unstressed offspring of rats fed a 6% casein (LP) diet 5 weeks before mating and throughout pregnancy. However, when control and LP rats were stressed via exposure to ammonia the difference in MAP between the two groups increased significantly. This observation led the authors to conclude that differences in blood pressure between LP and control rats measured by the tail-cuff method were stress artefacts rather than differences in basal blood pressure. Ozaki et al. (2001) recorded blood pressure via a femoral artery catheter in 30% calorific-restricted offspring at 60, 100 and 200 postnatal days. The findings demonstrated a subtle but significant increase in SBP (~ 20 mmHg), DBP (~ 10 mmHg) and MAP (~ 11 mmHg) from PD 60 onward in restricted male offspring and in restricted female offspring from PD 100 onward. The authors ascribed the difference in the elevated blood pressure between the telemetric method and femoral artery catheter technique to 'the different dietary protocols used or the result of an altered response to stress' (Ozaki et al., 2001). Yet other authors (Sahajpal and Ashton, 2003) have shown that MAP is elevated in LP rats when measured directly via an arterial catheter under anaesthesia. However, Swali et al. (2010) rebutted the disparity when they established a direct comparison of blood pressure measurements between 12 week-old male control and LP offspring using radio-telemetry and tail-cuff plethysmography simultaneously under baseline conditions and in response to a stressor. At 8 weeks and under baseline conditions, LP offspring showed a higher SBP compared to controls but failed to reach statistically significant differences at 12 weeks of age using tail-cuff plethysmography. In contrast, at 12 weeks, LP offspring were

hypotensive compared to controls using the radio-telemetric method, whereas the control offspring showed similar blood pressure readings with either method. In response to a stressor, all of the parameters (SBP, DBP and HR) were elevated in both control and LP offspring to a similar degree in both methods. Hence, rather than the methods having varying outcomes, the study confirms that the impact of a prenatal low-protein diet can differ between the central and peripheral vessels in terms of vascular function and blood pressure.

An important point that many studies, including this one, have focused on are the sex differences in the onset of hypertension in response to a prenatal insult. According to Kwong et al. (2000), maternal undernutrition during the preimplantation period in rats causes hypertension in male offspring only. The authors furthermore asserted that during the preimplantation period male offspring exhibit a greater propensity to show a response to the environment of the mother and therefore show more susceptibility to particular programming-related impacts. Conversely, female embryos seem to be shielded from these influences and show lower levels of susceptibility to regulated insults during their development. 30% food restriction during pregnancy also results in sex-specific hypertension in which there was an earlier onset of high blood pressure among young male offspring than females in association with abnormalities in their vascular function in isolated peripheral arteries (Ozaki et al., 2001). Woods et al. (2005) reported a marked increase in blood pressure in male offspring which were exposed to moderate protein restriction *in utero* coupled with a decrease in nephron number. However, only when the dams were exposed to more severe protein restriction, were hypertension and changes in renal structure observed in both male and female offspring (Woods et al., 2004). Therefore, research on fetal programming using animal models shows that male offspring are more susceptible to the programming of diseases due to insults during their fetal development than female offspring, and that female offspring may be less sensitive (or adapt differently) to the impacts of such insults. A series of studies from Alexander et al. (2003), as well as Ojeda et al. (2007a, b) suggested that sex hormones play a role in the sex-differences that occur in response to fetal insult. The study by Alexander (2003) reported increases in blood pressure in both pre-pubertal male and female growth-restricted offspring induced by placental insufficiency during late gestation. Post-puberty, however, the growth-restricted females' blood pressure normalized, while the males remained hypertensive (Alexander, 2003; Ojeda et al., 2007a, b). This finding is in contrast with the earlier mention that both sexes of IF offspring were hypertensive at 4 weeks of age but then appeared to have normalised their blood pressure at 5 weeks onwards. A noteworthy point in the placental insufficiency model is that the growth-restricted male offspring's hypertension can be abolished through castration (Ojeda et al., 2007b) whereas in females, ovariectomy induces hypertension (Ojeda et al., 2007a). Hence, sex hormones, which modify regulatory pathways that are key to the control of blood pressure may have an influence on the sexual dimorphism that is found in this example of programming of adult disease.

Hence the IF dietary model, which does not exhibit hypertension at early life, may be useful to compare against others which appear to program hypertension in the offspring. Comparing the impact of intrauterine programming on the developing cardiovascular system in the IF and other dietary models may aid in understanding how hypertension is programmed, particularly as there appears to be divergence from a common point of altered fetal growth. McMullen et al. (2012) introduced the concept of the 'gatekeeper hypothesis' which explains how different maternal nutritional insults (under- or overnutrition) can produce similar phenotypes: programmed hypertension and obesity. The hypothesis postulates that there are a number of common genes or gene pathways that are altered by various nutritional insults (Figure 3.4.1). Therefore, the identification of any alteration in the gatekeepers in the target organs of IF offspring should make it possible to predict the manifestation of hypertension at a later stage of life beyond 14 weeks of age.
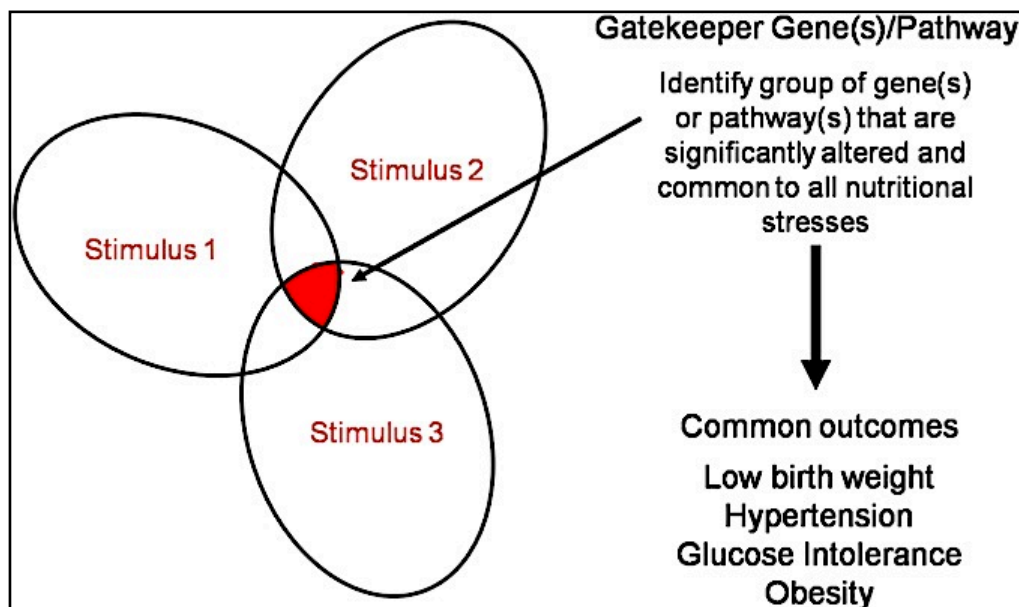
Figure 3.4.1 The gatekeeper hypothesis. Within the differences in applied nutritional stressors, common genes or gene pathways (the shaded area) participate in generating similar phenotype outcomes from divergent nutritional stimuli. Taken from McMullen et al. (2012).

3.4.5 Glucose and insulin tolerance tests in IF and control offspring

Glucose and insulin tolerance tests were carried out to determine whether exposure to an IF diet *in utero* leads to insulin resistance in the offspring. Insulin resistance has been linked to poor maternal diet during pregnancy in a number of epidemiological studies (Leger et al., 1997; Ravelli et al., 1998) and similar observations have been made in a number of animal models (Vickers et al., 2000; Ozanne et al., 2003; Zambrano et al. 2006). However, no such effect was observed in IF rats. Both sexes of IF offspring at 12 weeks of age responded to glucose and insulin challenges in the same way as the offspring from the control group, with no differences

in the area under the curve (AUC) between either the dietary groups or sexes (Figures 3.3.11 and 3.3.12).

The age of the animals used in this study may have had an effect on the outcome; the literature has documented changes in glucose and insulin tolerance of offspring exposed *in utero* to maternal malnutrition during the later stages of life. Hence, the possibility is raised that IF offspring may have exhibited glucose and insulin intolerance at a later stage of life, beyond 12 weeks of age. A number of studies by Ozanne and colleagues support this concept. Ozanne et al. (1996) initially found that LP offspring were not glucose intolerant but rather developed an improved glucose tolerance with increased skeletal muscle insulin sensitivity at 3 months of age. However in a follow-up study Ozanne et al. (2003) showed a remarkable impairment in glucose tolerance of male LP offspring by the age of 15 months compared to controls. The mechanism(s) behind the initial improvement in glucose tolerance and insulin sensitivity could be explained by an increase in the number of insulin receptors expressed in insulin-sensitive tissue (Ozanne and Hales, 1999), whereas later there was a tendency for a raised concentration of plasma insulin, with insulin resistance prevailing rather than insulin deficiency. Interestingly, female LP offspring were able to maintain similar levels of glucose and insulin concentrations as controls, even up to 15 months of age (Hales et al., 1996; Sugden and Holness, 2002). Nevertheless, Fernandez-Twinn et al. (2005) demonstrated that female LP offspring at the age of 21 months developed insulin resistance associated with decreased insulin-signalling protein expression resulting in an increased risk of type 2 diabetes.

Contrasting with the observations made in the present study, Langley et al. (1994) and Dahri et al. (1991) showed an impairment of glucose tolerance even among young adult offspring exposed to an LP diet *in utero,* at 9 and 10 weeks of age respectively. In addition, a study by Zambrano et al. (2006) demonstrated a sex-specific effect of an LP diet *in utero* where only LP restricted males from PD 110 (16 weeks of age) became insulin resistant as compared to controls, but with no effect on LP females.

The collective evidence above emphasises the importance of further exploring insulin resistance in older IF offspring at different ages, particularly as there appears to be divergence between the sexes in response to nutritional insults during development. There is also value in determining whether prenatal intermittent fasting can cause any distinct modifications in the microRNA profiles of IF offspring, which are considered to be detectable biomarkers of disease even before the metabolic disorder manifests. For example, Ferland-McCollough et al. (2012) demonstrated that changes in the expression of certain microRNAs in adipose tissue of LBW adult humans, as well as young adult LP-exposed rats, could be used to predict insulin resistance and the susceptibility to metabolic disease later in life.

3.4.6 Renal function in IF and control offspring

Many studies using a variety of animal paradigms have shown that subjecting fetuses to nutritional perturbations has long-term effects on renal function that are linked to reductions in nephron numbers and high blood pressure (see Table 1.3). It was this substantial body of evidence that provided the rationale for establishing whether maternal intermittent fasting throughout pregnancy predisposed the offspring to impaired renal function, especially as kidney weights were observed to be significantly lighter in IF neonates. However, contrary to expectations there was no apparent effect of maternal intermittent fasting on renal function in the adult offspring at 14 weeks of age. There was a sex divergence within both dietary regimes in which renal parameters were higher in females than males. However, this difference was attributable to the approach that was used to assess renal function, which did not take into account the weight differences between the sexes.

3.4.6.1 Renal experimental approach

In this study, a renal clearance experiment was performed under Inactin anaesthesia using a constant infusion protocol that is well established in the laboratory. All of the renal parameters were measured after a 3 h equilibration period and were standardised to bodyweight. Renal clearance measurements can be performed in conscious or anaesthetised rats (Walter et al., 1989). Anaesthetics can induce changes in cardiovascular and renal function, such as depressed heart rate, arterial pressure and cardiac output (Bailie et al., 1979; Walker et al., 1983), as well as minimal alterations in kidney performance (Bailie et al., 1979). Inactin is considered to have the most minimal effect on cardio-renal function in the rat out of all anaesthetics available (Buelke-Sam et al., 1978; Walker et al., 1983); hence, it is the anaesthetic agent of choice for such studies.

Anaesthesia and surgery are known to decrease GFR in the early stages of the anaesthesia; this effect is gradually reversed over a relatively short time. A second effect is a reduction in electrolyte excretion following both intravenous and intraperitoneal anaesthetic administration (Walker et al., 1983). Thus, to circumvent the effects of surgery and anaesthesia, some researchers tend to assess renal function in conscious rat models. One of the common methods used to study renal function in conscious rats involves chronic vascular and urinary bladder cannulations, which have several limitations (Thomsen and Olsen, 1981; Walter et al., 1989). For instance, in Walker et al. (1983), the femoral vessels of anaesthetised rats were catheterised for intravenous infusion, blood sampling and blood pressure measurement and the urinary bladder for urine collection. Renal clearance was then assessed after a few hours of surgical recovery, and it was concluded that there was a severe depression of renal haemodynamics and function.

To overcome this, rats should be left for at least one week after surgery for full recovery until they have surpassed their pre-operative bodyweight before renal functional assessment (Garland et al., 1999). Restraint, which is necessary during urine collection, significantly increased urine flow rate in the rat as a physiological reaction response (Thomsen and Olsen, 1981). Pre-medications and antibiotics are also necessary in this type of surgery to avoid a risk of infection (Garland et al., 1999), which in turn may affect renal function. Interestingly, Walter et al. (1989) assessed renal function in rats both in the conscious and anaesthetised state during active and inactive periods. The study showed that renal parameters were similar between conscious and anaesthetised rats in their active phase. In the current study we therefore chose to assess renal function in rats under non-recovery anaesthesia, while acknowledging the limitations of this approach.

To study renal function, tracers and markers are infused into the anaesthetised rat in one of two main ways: constant or servo-controlled infusion, both of which have been used in our laboratory.

Renal blood flow, GFR and sodium excretion can be sensitive to small changes in body fluid balance; therefore, some investigators have used servo-controlled fluid replacement systems in order to study renal function. This technique maintains extracellular fluid volume at a constant level throughout the experiment, despite variations in excretion rates. By means of a computer-driven, servo-controlled replacement of spontaneous urinary output, fluid balance is maintained (Burgess et al., 1993). The advantage of controlled fluid replacement was demonstrated by Garland et al. (1999) who compared renal function in conscious diabetic rats using constant and servo-controlled infusion. They were able to observe both hyperfiltration and polyuria in the servo-controlled diabetic rats, but not in the constantly infused diabetic rats.

However, the servo-controlled method also has some disadvantages. As the system employs a positive feedback loop, in which fluid infusion is matched to urine output, there is a tendency to establish an expansion of extracellular fluid that is maintained as a new 'set point'. Furthermore, it does not compensate for any changes in the plasma electrolyte concentration and is sometimes associated with increases in proximal tubular fluid output and excretion rates (Thomsen and Shirley, 2007). Thus while it is of value in certain circumstances, it is perhaps less useful for initial investigation of new models.

Most *in vivo* studies, including this one, have employed a constant rate of infusion as the gold standard (Garland et al., 1999; Sahajpal and Ashton, 2003; Huang et al., 2016). This type of infusion causes expansion of extracellular fluid volume which ultimately leads to an increase in urine flow and sodium excretion rates (Thomsen and Shirley, 2007). Thus, over time the urine flow and sodium excretion rates steadily increase and reach a value similar to that of the infusion rate. The time taken to reach equilibration varies between animals; hence, a 3 h

stabilisation period was chosen for this study, based on our extensive previous experience in the laboratory, prior to collecting any samples. The effectiveness of this approach is illustrated by the stability of the urine flow rate and GFR throughout the experiment (Figure 3.3.19), which indicates that the plasma volume was in a steady state throughout the experimental period.

As renal function is dependent, in part, upon the mass of the kidneys, and thus the size of the animal, laboratories have adopted differing methods with which to standardise constant infusion protocols. The two main approaches are either to adjust the rate of infusion according to the bodyweight of the rat and apply no correction to the measured outputs or to infuse all rats at the same rate and then correct the measured outputs for bodyweight or kidney weight. The former method has the advantage that the degree of extracellular fluid volume expansion is kept to a similar magnitude, relative to bodyweight, between animals. However the disadvantage is that unless experimental animals are of the same weight, each preparation requires a separate pump, which limits the number of experiments that can be conducted. The latter method has the advantage that all corrections are made after the data have been collected, maximising efficiency; however, the disadvantage is that where bodyweights vary greatly between animals, as may be the case between male and female rats, the relative degree of extracellular fluid expansion is greater in the smaller animals. Many laboratories, including ours, use the second approach, correcting for differences in kidney mass by adjusting measured outputs for body or kidney weight. Hence the data generated in this study can be compared readily with those published by other groups. Since the bodyweights, as well as the organ weights, between both sexes of the IF group and the control group were equivalent, normalising the renal outputs to 100 g Bwt will allow appropriate comparison and interpretation of the data between the two experimental groups.

3.4.6.2 Renal clearance outcomes

Plasma electrolyte (Na^+, K^+, Cl^-) and protein concentrations (Table 3.3.3) were stable over the course of the experiment and were within the normal physiological range. Similarly, MAP was stable over the experiment and did not differ between the dietary groups or sexes. These observations imply that the animals were in a steady state during the experimental period when renal function was assessed, and thus that any observed differences in urinary excretion are not attributable to variation in plasma composition or systemic blood pressure. These findings are comparable to those of earlier studies using constant infusion in Sprague-Dawley (Garland et al., 1999) and Wistar rats (Garland et al., 1999; Sahajpal and Ashton, 2003) as well as servo-controlled infusion (Ashton et al., 2007; Alwasel and Ashton, 2009; 2012).

Haematocrit was recorded at the end of the experiment and did not differ between dietary groups (Figure 3.3.16). However there was a sex divergence between males and females, where males in both dietary groups had a significantly higher haematocrit than their female

littermates. Probst et al. (2006) and Murphy (2014) previously examined haematocrit sex differences in rats and in other animal species (Murphy, 2014). They concluded that the sex hormones (androgens and oestrogen) influenced haematocrit through their actions on the kidney, rather than affecting red blood cell production by the bone marrow (Murphy, 2014). These hormones constrict the microvasculature of the kidney. Vasoconstriction and dilation in vessels that have a diameter of less than 300 µm raise or lower the haematocrit in blood in venules, capillaries and arterioles, changing the oxygen supply per unit of red cell mass, which offers a way to change the red cell mass while leaving erythropoiesis unaltered (Murphy, 2014). In addition to the influence of sex hormones on haematocrit, the experimental protocol may also have exerted a sex-dependent effect. Renal function was assessed using a constant infusion approach in which a fluid load was applied independent of bodyweight. Thus the lighter females would have experienced a relatively greater degree of volume expansion through application of this technique. Therefore, they would have a greater ratio of plasma to red blood cells and consequently, lower haematocrit.

The renal haemodynamic variables measured during the experimental procedure were stable and did not differ between control and IF offspring. ERBF was approximately 9.4 ± 1.1 mL/min/100 g Bwt in males and 16.7 ± 1.8 mL/min/100 g Bwt in females, whereas GFR was more tightly controlled, ranging from 0.4 ± 0.1 to 0.6 ± 0.1 mL/min/100 g Bwt. Filtration fraction ranged from 5.2 ± 0.6% to 10.6 ± 1.2%, reflecting the variations in ERBF seen between males and females. The recorded values for ERBF, GFR and FF are consistent with numerous previous reports using both constant (Alexander, 2003; Hoppe et al., 2007) and servo-controlled infusion techniques (Bogzil et al., 2005; Alwasel and Ashton, 2009; 2012). Sahajpal and Ashton (2003) and Woods et al. (2001a) arrived at higher values for the renal variables measured in this study; however, they normalised their data to kidney weight. When adjusted instead to bodyweight, these studies too are in agreement with the range of values reported in the current study.

Urine flow rate did not differ between dietary groups; however, it was significantly greater in females (24.1 ± 3.1 µL/min/100 g Bwt) compared to males (9.3 ± 1.0 µL/min/100 g Bwt). This difference is largely attributable to the administration of the infusate to both sexes at a standard rate of 50 µL/min. Outputs were subsequently adjusted to 100 g Bwt; however, because the female offspring were lighter in weight compared to males, their corresponding values were almost two-fold higher. A similar outcome was obtained if the renal outputs were normalised to kidney weight, as female kidney weights were lighter than those of males. It is acknowledged that through the use of a standard constant infusion, potential physiological differences between the sexes may have been obscured; in the future, a more detailed study using servo-controlled fluid delivery may be warranted.

Although the differences in urine output between sexes in the present study can be attributed to the approach applied, there are other previous studies which have investigated sex differences in the renal function of 'normal' rats and showed that urine flow rate is different between males and females (Wang et al., 1993; 1996). Male rats are more sensitive to vasopressin than females (Wang et al., 1993); this is due to the inhibitory action of oestrogen on the activity of vasopressin (Wang et al., 1996). Sex-related differences in renal function have also been reported when the renal system is challenged. Following uninephrectomy males had a significantly higher urine flow rate than females due to gonadal steroids (Mulroney et al., 1999), whereas in response to salt loading the opposite was seen (Dickinson et al., 2013). Limited studies have explored sex-differences in renal function in rodents exposed to maternal insult, but there were no observed differences in urine output (Nwagwu et al., 2000; Alwasel and Ashton, 2009).

In terms of renal handling of electrolytes, no significant differences were seen between control and IF offspring. Osmolar and electrolyte excretion rates as well as electrolyte fractional excretion values were consistent between control and IF offspring and were within a range similar to that of previous studies conducted in this laboratory using the servo-controlled infusion method (Alwasel and Ashton, 2009; Bogzil et al., 2005). Hence these data do not provide any evidence to suggest that IF rats retain or lose electrolytes, relative to controls, under basal conditions. This observation is consistent with the lack of difference in blood pressure between IF and control rats. However, sex-differences were apparent again: females had two-fold higher electrolyte excretion and fractional excretion than males in both dietary groups, but this is most likely due to the constant fluid infusion method used in this study.

The urine anion gap was also calculated as an indicator of the kidney's ability to acidify urine appropriately. A value near zero or slightly positive is taken to be in the normal range. With the exception of the control females, all calculated values were positive. In the control female group, the urine anion gap was -7 ± 7 mmol/L. A negative value is indicative of gastrointestinal loss of bicarbonate, usually due to diarrhoea. Although a systematic study was not undertaken, daily observation of the animals as part of normal husbandry did not point towards the control females suffering from diarrhoea. Therefore, given the magnitude of variance in the calculated data, a more cautious interpretation is that the ability of control females to acidify their urine is at the lower end of the normal range. It is worth noting, however, that males in both groups had higher urine anion gap values than their female littermates. This divergence is accounted for, in part, by the urine potassium concentration, which was higher in males than females of both dietary groups (Table 3.3.4).

Despite growing evidence that the pathways regulating cardiovascular and renal function differ between males and females (Grigore et al., 2008; Ojeda et al., 2014), the majority of developmental programming studies investigating the influence of perinatal sub-optimal

nutrition on renal function have focused on outcomes in males only (Woods et al., 2001a; Sahajpal and Ashton, 2003; Hoppe et al., 2007; Ashton et al., 2007; Lim et al., 2011; Alwasel and Ashton, 2012). Only a few studies have concentrated on females (Langley-Evans et al., 1999) or included both sexes (Nwagwu et al., 2000; Alwasel and Ashton, 2009). One of the studies that took both sexes into consideration is that of Nwagwu et al. (2000) who investigated urine output and creatinine clearance, but not renal handling of electrolytes, in young and adult LP rats. Although there are some methodological flaws in this study, the authors found no sex differences in any of the measured variables; hence the data from the two sexes were not analysed separately. Alwasel and Ashton (2009) reported that while 4 week-old LP male and female rats exhibited altered renal Na^+ handling compared with controls, there were no further differences between the sexes when function was assessed using the servo-controlled method.

Despite the consistent observation of a reduced nephron endowment in a number of different developmental programming models (Celsi et al., 1998; Langley-Evan et al., 1999; Burrow, 2000; Ortiz et al., 2001; Woods et al., 2001a; Almeida and Mandarim-de-Lacerda, 2005; Alwasel et al. 2010), there are several inconsistencies in the reported renal function of offspring from dams exposed to various dietary manipulations during pregnancy. For example, Zimanyi et al. (2004; 2006) showed that adult rats (24 weeks of age) exposed *in utero* to maternal protein restriction failed to develop hypertension or renal dysfunction despite a nephron deficit. Langley-Evans et al. (1999) found that whilst 4-week old LP offspring had 13% fewer nephrons accompanied by a 13 mmHg increase in blood pressure compared to controls, GFR was normal. One explanation for this apparent inconsistency is that the increase in blood pressure was sufficient to promote hyperfiltration and thus an increase in single nephron GFR (SNGFR) without having an impact on total GFR. However, evidence for this type of adaptation was not found in the present study with GFR, ERBF, FF and renal vascular resistance remaining unaltered, and offspring from IF dams being normotensive over the period of the study. In a different programming model in which low birth weight was induced by reduced uterine perfusion, Alexander (2003) reported that the offspring were hypertensive at 12 weeks of age but that GFR was unaltered. In contrast, 50% calorific restriction either during the first or second half of, or throughout, pregnancy induced a nephron deficit and reductions in GFR and renal plasma flow in offspring aged 3 months (Lucas et al., 1989; 1991; 1997). Interestingly, urine flow rates were also decreased, but only in rats exposed to calorific restriction during the first half or the whole of gestation (Lucas et al., 1989). Since the kidney does not begin to develop until GD 12 in the rat (Paixão and Alexander, 2013), this observation implies that other regulatory systems were influenced by the intrauterine insult.

The renin-angiotensin system (RAS) has been implicated in the development of the kidney and hypertension. RAS components are expressed in the developing kidney starting from GD 12 and are more abundant in fetal rats until birth than in adult rats (Yosypiv and El-Dahr, 2005). Several studies have shown that maternal nutritional perturbations, such as protein restriction,

altered RAS components. Alwasel et al. (2010) showed that rats exposed to a LP diet had significant reductions in renal AT_1 and AT_2 receptor protein levels from GD 18 to PD 10; thus, the reduction continued throughout the period of nephrogenesis. At birth, renal tissue angiotensin II levels and renin protein and mRNA were reduced in LP newborns (Woods et al., 2001a). Cooke et al. (2014) demonstrated that protein restriction *in utero* stimulated sex-specific changes of the RAS system in which angiotensin converting enzyme (ACE) and renin mRNA expressions were upregulated in the fetal kidneys in males but not females, as early as GD 19, compared to controls. At PD 21, male offspring kidneys showed elevation in AT_1 receptor, indicating that altered expression of the RAS system in response to protein restriction during pregnancy may change nephron numbers in developing kidneys, specifically in male offspring (Cooke et al., 2014). At 4 weeks of age, LP offspring exhibited a 35% nephron deficit along with a reduction in glomerular volume (Alwasel et al., 2012). However, renal angiotensin II receptor (AT_1 and AT_2) protein expression was significantly upregulated compared with control offspring (Sahajpal and Ashton, 2003; 2005). By 16 weeks, LP offspring showed a decrease in AT_2 receptor protein expression (Mesquita et al., 2010a). This was confirmed by immunohistochemical analysis which demonstrated a total absence of this receptor in the glomeruli with intense expression only in the intercalated cells of the distal convoluted tubules and collecting ducts (Mesquita et al., 2010a). Moreover, administration of losartan, an AT_1 receptor antagonist, in normal rats for the first 12 days postnatally resulted in a nephron deficit and hypertension development in adulthood (Woods and Rasch, 1998). These data show collectively that RAS components are altered during fetal and postnatal periods and play a role in the reduction of nephron number, development of hypertension and alteration in renal function in LP offspring.

A review by Mesquita et al. (2010b) outlined the interaction of the RAS with renal function in the LP rat model (Figure 3.4.2), explaining that even though the nephron number is reduced in LP offspring, renal function is maintained by changes in renal morphology such as enlargement of the filtration area and glomerular volume. Furthermore expression of the AT_2 receptor, which is localised in efferent arterioles, is reduced in the protein-restriction model causing vasoconstriction which in turn increases the intraglomerular pressure to maintain normal GFR. This was not the case in the current study as arteriole resistances were unchanged in the offspring of the IF group. However, the RAS was not assessed in IF rats in the current study; it is worthy of further investigation in future, particularly if a nephron deficit is identified.

Cardio-renal disease is dynamic, often progressing through different stages as the condition develops and adaptations develop in order to mitigate changes. Hence the timing of experimental measurements in offspring can influence functional readouts. In this context, it is of interest to note that an MRes student, Grace Mallett, who worked in our laboratory observed that IF offspring of both sexes had increased MAP under anaesthesia at 4 weeks of age (Section 3.4.4). She also found sex differences between the 4 week-old offspring. Thus, while

ERBF and FF did not differ between control and IF offspring, GFR was significantly reduced in IF females. Osmolar excretion was reduced overall; but fractional excretion of water, Na^+ and K^+ were significantly higher in IF females. The results suggest that renal function, in particular reabsorption of electrolytes, is impaired in a sex-dependent manner with the IF female offspring being more vulnerable than males at 4 weeks of age. However, an interesting question arises from these observations: what is responsible for the natriuretic phenomenon apparent in IF female offspring against the background of a reduced GFR? One explanation is an alteration in tubular Na^+ reabsorption downstream of the proximal tubule; decreased Na^+/K^+ ATPase activity has been reported previously in rats exposed to an LP diet (Alwasel and Ashton, 2009; 2012) and high fat diet (Armitage et al., 2005) *in utero*. As a result, the animals had raised urinary sodium loss against a background of elevated blood pressure. Remaining questions that need to be explored in more detail are what drives the marked sex-specific changes in renal function of the IF offspring at this developmental stage, and are there any underlying mechanisms that are common between the IF and LP dietary models? LP offspring of both sexes are hypertensive and exhibit a significant increase in Na^+ excretion and fractional excretion which appear to be driven by altered salt appetite and regulation of total body sodium (Alwasel and Ashton, 2009; 2012).
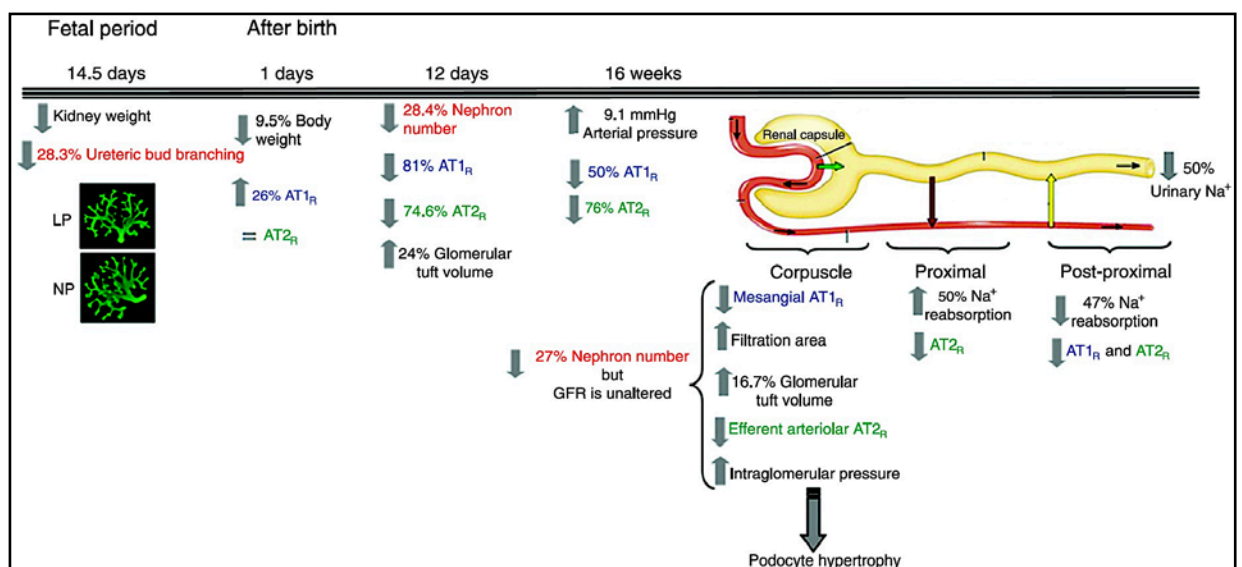
Figure 3.4.2 The interaction of RAS with kidney development and function from fetal to postnatal periods in a LP diet model (rat). Ages are specified on the line. AT_{1R}, type 1 angiotensin II receptor; AT_{2R}, type 2 angiotensin II receptor; GFR, glomerular filtration rate; LP, low protein; NP, normal protein. ↑, ↓, and = represent an increase, decrease and no change respectively. Adapted from Mesquita et al. (2010b).

The differences in blood pressure and renal function between IF and control rats reported by Mallett at 4 weeks of age did not appear to persist, as by 14 weeks of age such differences were no longer observed (Results, Section 3.3.11). Such an improvement in cardio-renal

function with offspring age is perhaps unexpected; however, there have been some similar reports in other programming models. Nwagwu et al. (2000) observed a significant reduction in creatinine clearance in 4 week-old rats exposed to LP diets *in utero* compared to control offspring, but this difference between the two dietary groups later disappeared at 12 and 20 weeks of age. Hall and Zeman (1968) offer one explanation for such a change: a GFR impairment that is apparent in newborn offspring may be corrected by physiological adjustments as a normal part of maturation. Pathological changes may also appear to correct and restore impaired renal function with increasing age. Young spontaneously hypertensive rats (SHR) have reduced GFRs (Uyehara and Gellai, 1993; Gouldsborough and Ashton, 2001), whereas the adult SHR does not show altered GFR or other renal outcomes (Beirewaltes and Arendshorst, 1978; Arendshorst and Beirewaltes, 1979). In this instance, it is the rapid increase in systemic pressure and thus renal perfusion pressure that restores GFR, but at the expense of chronic raised blood pressure.

Several dietary models characterised by a fetal growth restriction phenotype exhibit altered nephrogenesis and diminished nephron number in the offspring. It is anticipated that such a reduction in nephron endowment would be associated with a decrease in GFR (Courrèges et al., 2002; Sahajpal and Ashton, 2003; Pires et al., 2006; Alwasel et al., 2009); indeed, a reduction in nephron number and GFR has been observed in male offspring in a more severe model of IF than that used in the current study (PhD thesis by Mohamed Elwan). If nephron number is indeed reduced in IF offspring the lack of difference in total GFR in IF adult rats in this study could be explained by an increase in single nephron GFR, associated with a larger glomerular volume. Another possibility, based on previous studies, is that the alteration in renal Na$^+$ handling present in IF offspring at 4 weeks of age, which was restored later at 14 weeks old, may be due to an increase in the renal medulla/cortex ratio as a protective mechanism for the deepest nephrons against the effects of undernutrition. Lucas et al. (1997) demonstrated that a 50% food restriction during rat pregnancy resulted in a reduced number of nephrons at birth and in 3 month-old offspring, yet tubular function was preserved with adequate handling of Na$^+$. Moreover, Bertram et al. (2001) showed increased renal mRNA expression of the α_1 and β_1 subunits of Na$^+$/K$^+$ ATPase in LP rats at 12 weeks of age. Likewise, an induction in Na$^+$/K$^+$ ATPase activity was observed by De Assis et al. (2003) who demonstrated that rats exposed to a high-cholesterol (HF) diet *in utero* had diminished Na$^+$/K$^+$ ATPase activity in the outer medulla at weaning, whereas pump activity in adult HF rats was similar to control. Therefore, IF offspring may be able to compensate for a speculated nephron deficit and maintain normal function through an increase in filtration area and thus hyperfiltration of the remaining individual nephrons. A second interpretation is that increased expression of sodium transporters leads to increased sodium reabsorption in the proximal tubule or loop of Henle and thus decreased sodium delivery to the distal nephron and macula densa. As a consequence, tubulogomerular feedback will be decreased and GFR increased in the remaining nephrons.

Although no differences in renal function were observed in offspring exposed to intermittent fasting *in utero* in this study (as measured at 14 weeks of age), there may still be a deficit which is masked or not overt but which may become manifest upon a second additional stressor challenge. Nenov et al. (2000) and Moritz et al. (2009) propose that a primary insult such as a decreased nephron number or reduced filtration surface area might have insufficient impact to create overt hypertension or nephropathies on its own, but that it may lead to an increased vulnerability to a second hit. Woods et al. (2004) demonstrated salt-sensitive hypertension in rats exposed *in utero* to protein restriction, and considered that this effect was driven through the impairment of renal development. In our laboratory, Alwasel and Ashton (2012) demonstrated that when LP rat fetuses are programmed to elicit sodium wasting, they compensate for this with a raised appetite for salt. Therefore, it was hypothesized that if IF rats have a low nephron number, but do not develop hypertension and renal disease under normal dietary control conditions, such changes may become apparent when these rats are challenged with a high-salt diet. This hypothesis will be tested in the next chapter.

3.5 SUMMARY

The most important findings of this study are that prenatal exposure to maternal intermittent fasting altered the growth trajectory of the offspring which was associated with disproportionate organ growth. Unlike IF females, IF male offspring underwent late postnatal catch up growth. In contrast to other models of developmental programming, both sexes of IF offspring failed to show any metabolic disorders at 12 weeks of age (normal glucose and insulin tolerance) and were normotensive at 5, 7, 10 and 14 weeks of age. These data are in contrast to the results of studies of humans where offspring exposed to Ramadan fasting *in utero* were growth restricted at birth (Almond and Mazumder, 2011) and more likely to develop cardiovascular disease and type 2 diabetes in adulthood (van Ewijk, 2011; Savitri et al., 2014).

Surprisingly, despite the observation that absolute and relative kidney weights were lighter (smaller) at birth in IF animals than controls, basal renal function was unaltered at 14 weeks of age. Overall, exposure to intermittent fasting *in utero* does not appear to program offspring for cardio-renal disease in later life, at least as revealed by this age. This is particularly intriguing based on comparisons with other dietary models that also show altered fetal and neonatal growth trajectories whilst programming cardio-renal disease later in life. In order to determine whether the IF kidney is able to respond appropriately when challenged, the next chapter describes the impact of dietary salt loading on cardio-renal function.

CHAPTER 4

**THE IMPACT OF DIETARY SALT CHALLENGE
ON CARDIO-RENAL FUNCTION**

4.1 INTRODUCTION

Various animal models employing diverse sets of nutritional insults *in utero* have resulted in remarkably consistent and narrow phenotypic endpoints, such as hypertension, glucose intolerance, insulin resistance and obesity in later life (Langley-Evans, 2006). With regard to the maternal intermittent fasting model, the data presented in Chapter 3 suggest that prenatal maternal intermittent fasting leads to an altered growth trajectory of the IF fetus, resulting in a lower kidney weight in relation to bodyweight at birth. Yet, adult IF offspring did not demonstrate any subsequent rise in blood pressure nor altered renal function at 14 weeks of age.

Several studies in humans and animals have documented that blood pressure is directly related to sodium intake (Friedman et al., 1990; Meneton et al., 2005). Studies have also proposed that reduced nephron endowment may induce salt-sensitive hypertension, which may manifest as renal disease in later life (Brenner et al., 1988). Nenov et al. (2000) have argued that a congenital decrease in nephron endowment as a first developmental hit may predispose individuals to overt renal disease and that an acquired renal insult as a second hit raises the chance of overt and progressive renal disease. Therefore, challenging the kidney of IF offspring with a high-salt diet may allow for identification of potential deficiencies in renal excretory capacity and also provide insights into any aberrant underlying mechanisms. To put these studies into context, the development of thirst and salt appetite will be described together with the consequences of maternal undernutrition as regards the regulation and programming of fluid intake and salt sensitivity in offspring during adult life.

4.1.1 Development of thirst and salt appetite

The early developmental period is considered a vital stage for the establishment of the offspring's thirst and sodium appetite behaviour. In neonatal rats, thirst behaviour could be evoked via cellular dehydration two days after birth or via hypovolaemia four days after birth (Wirth and Epstein, 1976). Intracranial angiotensin II (Ang II) and renin play a role in inducing thirst: Ellis et al. (1984) have demonstrated that the administration of intracranial Ang II stimulates a thirst response at postnatal day 2. However, pups at this stage cannot distinguish between milk and water. At postnatal day 8, the pups were able to differentiate between water and milk and displayed a preference for water, a behaviour characteristic of adults (Ellis et al., 1984). Activating the brain renin-angiotensin system (RAS) in newborn rats via renin administration also induces thirst, with a preference for water at postnatal day 15 (Leshem et al., 1988).

Salt preference is a hedonistic response; most mammals consume salt even in the absence of a physiological requirement related to the concentration of NaCl being offered (Moe, 1986). In

rats, salt appetite is an age-dependent ingestive behaviour based on the development and maturation of the gustatory system. A preference for hypertonic NaCl solutions (2% or greater) has been reported to emerge between postnatal days 10 and 15; thereafter salt preference approaches isotonic concentrations (0.6% - 0.9% NaCl) after weaning and during adulthood (Moe, 1986). Leshem et al. (1994) have demonstrated that intracranial renin administration in newborn rats stimulates the brain RAS and encourages an increased intake of salt as early as postnatal day 3. The study demonstrated that renin-induced salt appetite is unrelated to thirst during this crucial developmental window for the following reasons: (1) the rats favoured hypertonic NaCl over water, (2) appetite develops more quickly than thirst, and (3) renin-stimulated rats enthusiastically licked dry salt on postnatal day 3. The activation of brain Ang II in rats at postnatal day 3 thus elicits an early and specific sodium appetite (Leshem et al., 1994).

4.1.2 Programming of thirst and salt appetite

A study by Smart and Dobbing (1977) has shown that 50% calorific restriction during pregnancy and after birth during lactation and post-weaning until the offspring reached 42 days of age induces thirst in calorific-restricted adult offspring (16 to 20 weeks of age). When subjected to a Skinner box test for ingestive behaviour, such offspring drank more frequently in response to water deprivation and ran toward water more quickly than did control rats. Offspring from mothers fed a low-protein diet during pregnancy also showed an increase in water ingestion compared with the control group at postnatal week 4 when water was the only available fluid (Alwasel et al., 2012). Such thirst could be driven by hyperphagia, which was observed among those offspring. Similar patterns have also been documented by Vickers et al. (2000), who have demonstrated that adult offspring exposed to a hypocalorific diet in early life display growth restriction and hyperphagia as well as the development of hypertension.

As mentioned earlier, sodium intake is correlated with hypertension development. Meneton et al. (2005) have reported that human hypertension and cardiovascular diseases largely result from chronic exposure to a diet high in salt and that a decrease in salt consumption causes a decrease in arterial blood pressure among hypertensive individuals. An understanding of the factors driving preference for salt and stimulating its consumption is therefore relevant, especially as evidence has implied that early developmental events may determine salt preference and intake.

Several studies in humans have revealed that moderate to severe maternal emesis can lead to enhanced salt preference in offspring (Crystal and Bernstein 1995; 1998; Leshem et al., 1988). Research studies have also found that pregnant women who fast during the month of Ramadan suffered from excessive vomiting, that was worst during the first month of pregnancy (Rabinerson et al., 2000; Joosoph et al., 2004), which can programme salt preference in the

offspring. As well, studies in children and adults have also outlined an inverse relationship between salt sensitivity and birth weight (de Boer et al., 2008; Simonetti et al. 2008). Accordingly, Stein et al. (2006) have shown that salt appetite is inversely associated with birth weight over the first seven years of life, though Shirazki et al. (2007) have found that this correlation is absent from 10 to 15 years of age. In summary, undernutrition such as from food restriction or constraint of protein intake, associated with decreased birth weight, may result in alterations to renal function and sodium excretion, which in turn may be responsible for the expression of a natriophilic phenotype.

In animal studies, Alwasel et al. (2012) have demonstrated that among rats offered a choice between water and isotonic saline solutions, protein-restricted offspring exhibit an enhanced preference for saline as a consequence of a renal sodium wasting phenotype (Alwasel and Ashton, 2009). Langley-Evans and Jackson (1996), on the other hand, have reported that LP rat offspring, who developed hypertension due to exposure to maternal insult *in utero*, were unable to further raise their blood pressure in response to salt overload over a seven-day period. This was despite an initial increase in their intake of hypertonic saline (1.5% NaCl) compared both with their previous water intake and compared with control rats, which avoided obligatory saline solution intake during the first day of salt overload and showed an increased in blood pressure, probably due to renal dysfunction.

In this context, Harrap et al. (1984) have shown that enhanced salt appetite results in an increase in extracellular fluid volume (ECFV) among spontaneously hypertensive rats (SHR), an important factor for hypertension development. One main factor affecting salt appetite, thirst and hypertension is the components of the RAS. De Lima et al. (2013) have shown that adult protein-restricted offspring exhibit reduced expression of type 1 Ang II receptors in the hypothalamus as well as a reduced thirst response following Ang II administration. Such rats also demonstrate lower fractional sodium excretion than control rats, related to increased systolic blood pressure. Overall, maternal undernutrition during pregnancy is associated with altered offspring sodium excretion and sensitivity, which contribute to the onset of hypertension in later life.

Therefore, the aims of this particular study were to:

(1) Investigate the effect of maternal intermittent fasting *in utero* on adult offspring saline preference and saline aversion.
(2) Challenge the IF offspring kidney with a high-salt diet in order to identify potential deficiencies in renal excretory capacity.
(3) Test if this high-salt challenge will induce hypertension in IF offspring.
(4) Determine whether prenatal intermittent fasting was accompanied by changes in the ECFV.
(5) Examine the sex-dependency of elicited responses.

4.2 MATERIALS AND METHODS

4.2.1 Animals and diet

Control (N = 5) and IF (N = 6) female rats were mated and subjected to dietary manipulation during pregnancy as described previously (Section 3.2.1). Maternal food intake reverted to *ad libitum* following delivery of the litter. Following weaning at 4 weeks of age, two males and two females per litter per group (C, N = 5 litters; IF, N = 6 litters), were randomly allocated to receive either a high-salt (4% NaCl) diet (BK001 (E) 4% NaCl SDS Rodent Breeder and Grower, LBS Biotech, Redhill, UK) or continued to receive standard rat chow diet (1% NaCl, Rodent Breeder and Grower, BK001 (E) SDS, LBS Biotech, Redhill, UK). The latter group acted as time controls until they were 14 weeks of age.

4.2.2 Postnatal measurements

Offspring body weights and food and water intakes were measured weekly from week 5 until week 12 and finally at week 14 when the experiment was terminated. The offspring were assigned to different experimental groups as shown schematically in Figure 4.2.1.

4.2.3 Effect of dietary NaCl intake on systolic blood pressure determined by tail-cuff plethysmography

Systolic blood pressure (SBP) measurement was undertaken by Dr Heather Eyre in conscious offspring using the non-invasive blood pressure system (model LE5001, PanLab, Spain) at 5, 7 and 10 weeks of age as described previously in section 3.2.4. SBP was measured in offspring maintained on both the high-salt (4% NaCl; n = 24, in which n represents individual pups) diet and the control standard rat chow diet (normal salt diet of 1% NaCl; n = 20).

Control rats
5 litters
40 offspring

IF rats
6 litters
48 offspring

4% high salt diet
n= 2 M & F/litter
(Total 20) Week 4

1% Normal salt diet
n= 2 M & F/litter
(Total 20) Week 4

4% high salt diet
n= 2 M & F/litter
(Total 24) Week 4

1% Normal salt diet
n= 2 M & F/litter
(Total 24) Week 4

Renal function
n=2/litter/sex
(Total 20) Week 14

Saline preference
n=1/litter/sex
(Total 10) Week 7

ECFV
n=1/litter/sex
(Total 10) Week 12

Renal function
n=2/litter/sex
(Total 24)
Week 14

Saline preference
n=1/litter/sex
(Total 12) Week 7

ECFV
n=1/litter/sex
(Total 12) Week 12

SBP was measured at week 5, 7 and 10 in offspring from control and 4% high-salt diet groups

Figure 4.2.1 Study design for investigation of responses to high-salt challenge in control and IF offspring. The number of litters and offspring in each treatment group are indicated. All offspring were used for systolic blood pressure (SBP) measurements at weeks 5, 7 and 10. Abbreviations: ECFV, extracellular fluid volume; M, male; F, female.

4.2.4 Determination of saline preference and water intake

At 7 weeks of age, saline preference and total fluid intake were evaluated in two-bottle choice tests as described previously (Alwasel et al., 2012). Before the start of the experiment, 250 mL bottles were filled with water and placed into empty cages for two days in order to assess any leakage from the bottle. Males and females on a standard chow diet (1% NaCl) from both control (N = 5) and IF (N = 6) groups were then housed individually in standard cages and given access to two bottles containing either sterile water or 0.9% saline (0.154 M NaCl) for 2 days prior to data collection to monitor adaptation of drinking modality. After 2 days, the intake of each solution and bodyweight were recorded daily for 5 consecutive days. The bottles were washed, replenished with fresh drinking solutions and repositioned daily to avoid positional preference. Total fluid intake was determined (mL/100 g Bwt/day). Saline preference was calculated as the amount of saline consumed per day relative to bodyweight (mL/100 g Bwt).

4.2.5 Determination of saline aversion threshold

To establish the concentration threshold for saline aversion, saline solutions of increasing concentration were employed in a series of two-bottle choice tests. 7 days after the saline preference test, rats were offered water and saline with increasing concentrations from 0.9% to 2.1% in 0.3% increments every 3 days, over the course of 2 weeks. The water and saline intake and bodyweight were recorded daily; fresh saline solution was refilled and the positions of the bottles were alternated daily. Total fluid intake per day and saline preference per 100 g bodyweight were calculated.

4.2.6 Extracellular fluid volume measurements

To measure extracellular fluid volume (ECFV), ^{3}H-inulin was used as a marker of inulin space as described previously (Alwasel et al., 2012). At 12 weeks of age, offspring from the control (N = 5 per sex) and IF (N = 6 per sex) groups were anaesthetised with isoflurane by inhalation (4% in oxygen at 2 L/min), and then with an intra-peritoneal injection of sodium thiobutabarbital (Inactin, 100 mg/kg Bwt). The anaesthetised rats were then placed on an electrical hotplate to maintain body temperature at 37 °C. Following a small incision in the neck, the right external jugular vein was cannulated with 0.58 mm ID and 0.96 mm OD Portex polythene tubing (Portex Ltd, Nottingham, UK) tightly secured for the administration of ^{3}H-inulin. The left carotid artery was also cannulated in the same manner to allow blood sampling and was connected to a pressure transducer (Power Lab, ADIntruments Ltd, Chalgrove, Oxford shire, UK) to determine mean arterial blood pressure. A tracheotomy was then performed to ensure a patent airway. An abdominal laparotomy was performed; both pairs of renal arteries and veins were exposed and tied off using sterile 3-0 mersilk sutures to prevent ^{3}H-inulin excretion. The abdominal wall was then closed. Figure 4.2.2 depicts the schematic layout of the ECFV experiment.

A bolus dose (0.222 MBq (6 µCi)) of ^{3}H-inulin in 350 µL 0.9% saline was injected into the external jugular vein at time zero. The isotope syringe was weighed before (with isotope) and after injection to determine the exact volume of isotope injected into each rat. Saline was used to flush the jugular vein catheter. The volume of the flushing saline was calculated for rats to achieve a 500 µL volume of injectate per 100 g Bwt. The formula used in doing so was:

Flushing saline volume (µL) =

$$(bodyweight (g) \times 500 (µL) / 100) – inulin\ infusate\ (µL)$$

In order to determine the equilibration period, the time point at which ^{3}H-inulin was equally distributed in the body, blood samples (50 µL) from 5 rats were collected from the carotid artery into 1.5 mL eppendorf tubes at 10 min intervals over 2.5 h. Following ^{3}H-inulin counting, plasma ^{3}H inulin concentration was found to be stable beyond 60 min post-injection ($P > 0.05$) as illustrated in Figure 4.2.3. Therefore, plasma samples were collected for one hour after a 1.5 h equilibration period for the remaining experiments (n = 17).

Approximately 50 µL blood sample was taken from the carotid artery (in duplicates) into non-heparinised 1.5 mL eppendorf tubes every 10 min for the remaining one-hour experiment to measure ^{3}H-inulin activity. The blood samples were immediately centrifuged at 14000 xg for 1 min and the plasma recovered for radioactivity analysis. The animals were then sacrificed by cervical dislocation and urine samples collected following urinary bladder puncture to confirm occlusion of renal vessels and that the kidneys had not filtered any ^{3}H-inulin. One rat was excluded from the experiment due to the failure of renal vessel occlusion.
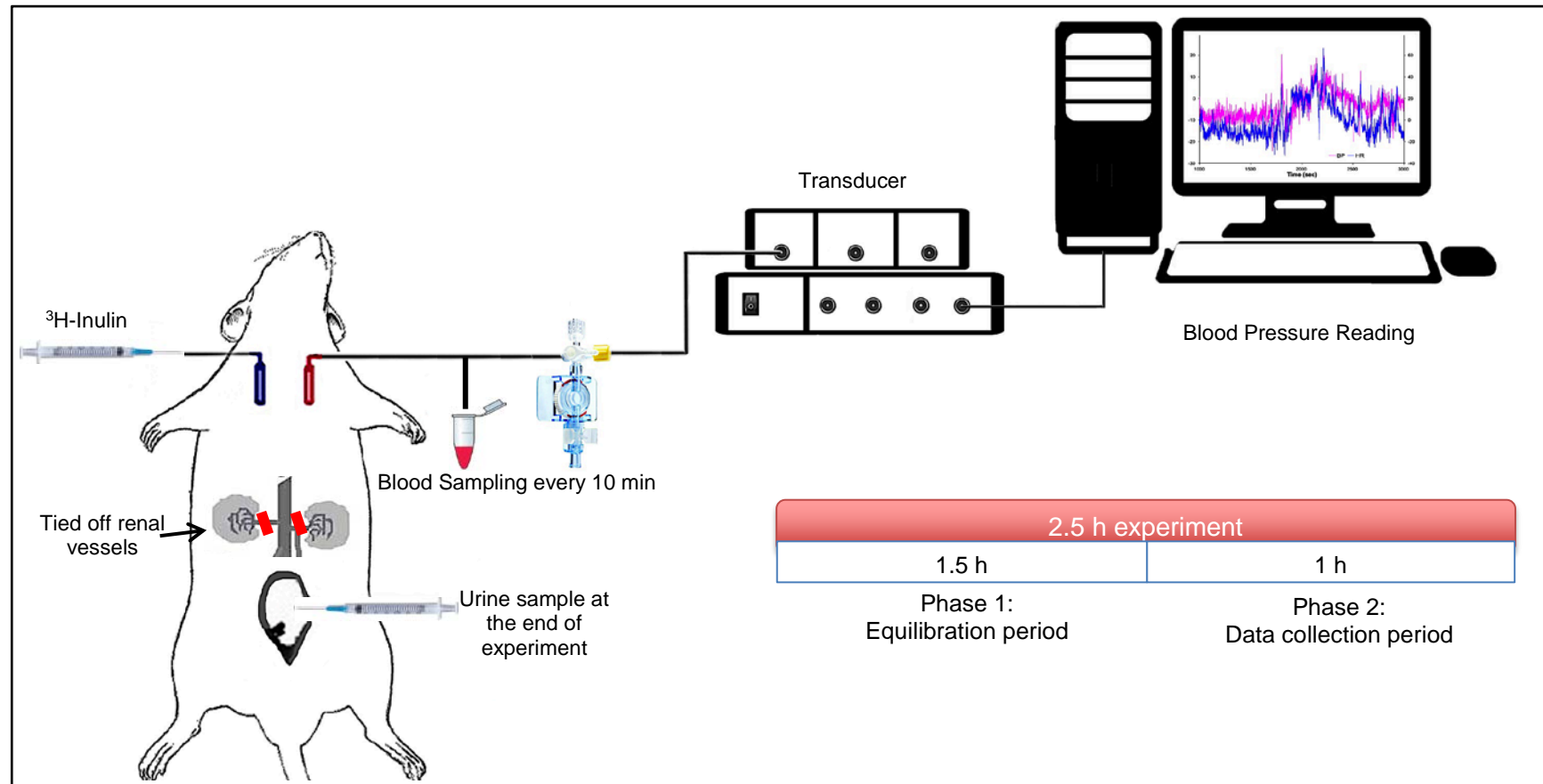
Figure 4.2.2 A diagram illustrating the measurement of ECFV. ^{3}H-inulin was injected into the jugular vein (blue-coloured vessel) and blood samples collected from the carotid artery (red-coloured vessel) which was connected to a transducer for arterial blood pressure recording. The renal vessels were occluded to prevent ^{3}H-inulin excretion, allowing plasma equilibration of ^{3}H-inulin concentration and measurement of ECFV.
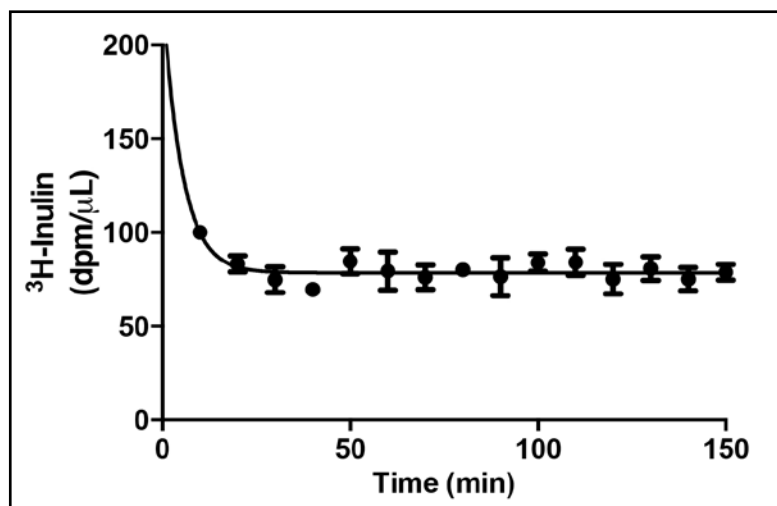
Figure 4.2.3 A curve depicting plasma ^{3}H-inulin concentration. ^{3}H-inulin distribution curve was constructed from radioactivity measured in plasma from 5 rats and fitted to a one-phase exponential decay model ($r^2 = 0.87$). Distribution of plasma inulin radioactivity was stable from 60 min post-injection onwards ($P > 0.05$, repeated-measures one-way ANOVA). Data are shown as mean ± SEM.

4.2.6.1 Isotope counting and ECFV calculation

For radioactivity analysis, 10 µL plasma (to determine ^{3}H-inulin concentration), urine and isotope stock (to determine mean ^{3}H-inulin count / µL) were taken in triplicates followed by the addition of 4 mL liquid scintillation fluid (Optiphase Hisafe II, Fisher Scientific Ltd, Leicestershire, UK). The vials were then counted in a liquid scintillation counter (Packard 2000CA) using standard windows for ^{3}H with water blanks (background count) for 5 min per sample. All the sample counts were adjusted for background counts.

Extracellular fluid volume was calculated by dividing total ^{3}H-inulin counts (per µL) injected into the body by the mean plasma inulin count (per µL). The dilution ratio was then multiplied by the volume of ^{3}H-inulin solution injected (mL) and corrected to 100 g bodyweight as follows:

$$\text{ECFV (mL / 100 g Bwt)} = (I_{In}C / P_{In}C) \times (I_{In}V / 1000) \times (100 / \text{Bwt})$$

where, $I_{In}C$ is the mean injected ^{3}H-inulin count per µL, $P_{In}C$ is the mean plasma ^{3}H-inulin count per µL, $I_{In}V$ is the injected inulin volume (µL) and Bwt is the bodyweight.

4.2.7 Renal function in conscious rats: metabolism cages

At 12 weeks of age, two male and two female offspring per litter from the control (N = 5) and IF groups (N = 6) which had been fed a high-salt diet (4% NaCl) or normal salt diet (1% NaCl) since 5 weeks of age, were transferred to individual metabolism cages with stainless steel

mesh bottoms in a temperature and ventilation controlled environment. Food and water were provided *ad libitium*. Rats were held in cages until they had voided sufficient urine for subsequent analysis (at least 3 mL); typically 3 h. Urine samples were obtained and stored at -80 °C for the following analyses.

4.2.7.1 Urine creatinine concentration

Creatinine concentrations in urine samples (C, N = 5 litters; IF N, = 6 litters) were measured using a colorimetric assay (DetectX® urinary creatinine kit, Arbor Assays, MI, USA), according to manufacturer's instructions. 50 µL standards, blank or samples (diluted 1:20) were added to wells of a 96 well plate, followed by addition of 100 µL DetectX® Creatinine Reagent at room temperature for 30 min. Absorbance was then measured at 490 nm (FLUOstar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Buckinghamshire, UK). A standard curve of creatinine (mg/dL) vs absorbance at 490 nm was plotted by linear regression, r^2 = 0.99 (Prism® software, Graphpad, UK) and used to interpolate sample creatinine concentration. Interpolated values were multiplied by the dilution factor to obtain concentration (mg/dL) in urine samples. The sensitivity of the kit as stated by the manufacturer is 0.019 mg/dL and the intra-assay CV was 2.34% (from n = 3 replicates).

4.2.7.2 Urine albumin concentration

Urinary albumin concentration was determined using a rat albumin ELISA kit (Bethyl Laboratories, Inc. TX, USA) according to manufacturer's instructions. 100 µL standards or samples (diluted 1:1000 – 1:3000) were added to wells of a 96 well plate and incubated at room temperature for 1 h. After washing 4 times with 1x Wash Buffer, 100 µL Detection Antibody was added to each well, mixed and incubated at room temperature for 1 h. This was followed by washing, the addition of 100 µL / well horseradish peroxidase (HRP) and incubation at room temperature for 30 min. Following washing, 100 µL TMB Substrate Solution was added for 30 min protected from light, before stopping the reaction with 100 µL / well Stop Solution and absorbance read at 450 nm. A standard curve of albumin concentration (ng/mL) against absorbance was fitted using nonlinear-regression, fitting to a hyperbolic function (Michaelis-Menten analysis fit, r^2 = 0.99). Urine albumin concentration was interpolated from the standard curve and multiplied by the dilution factor. The intra-assay coefficient of variance was 2.75% (from n = 3 replicates). Urinary albumin:creatinine concentration ratio (UACR) was calculated using the matched data for each urine sample.

4.2.7.3 Neutrophil gelatinase-associated lipocalin (NGAL) measurement

NGAL is a protein that belongs to the lipocalin superfamily which is secreted by renal tubular cells in response to renal injury. It is considered an early marker that predicts kidney

impairment (Bolignano et al., 2008). Urinary NGAL concentration was determined using a rat Lcn2 ELISA kit (Sigma Aldrich; RAB0906, UK) according to manufacturer's instructions. 100 μL standards or samples (diluted 1:300) were added to wells of a 96 well plate and incubated at room temperature for 2.5 h with gentle shaking. After washing 4 times with 1x Wash Buffer, 100 μL Biotinylated Rat Lipocalin-2 Detection Antibody was added to each well, mixed and incubated for 1 h at room temperature with gentle shaking. This was followed by washing, the addition of 100 μL / well horseradish peroxidase (HRP)-Streptavidin and incubation at room temperature for 45 min. Washing was repeated, then 100 μL TMB Substrate Solution was added for 30 min at room temperature protected from light. The reaction was stopped by adding 100 μL / well Stop Solution and then read at 450 nm. A standard curve of NGAL concentration (pg/mL) against absorbance was fitted using nonlinear-regression, fitting to a hyperbolic function (Michaelis-Menten analysis fit, r^2 = 0.98). Urine NGAL concentration was interpolated from the standard curve and multiplied by the dilution factor. The intra-assay coefficient of variance as stated by the manufacturer is CV < 10%.

4.2.8 Renal function in anaesthetised rats: renal clearance

At 14 weeks of age, 2 male and 2 female rats from the control (N = 5 litters) and IF (N = 6 litters) groups were used to determine the impact of exposure to a high-salt diet on renal function. Under terminal anaesthesia, blood pressure was recorded directly and samples were collected and treated as described previously in Section 3.2.7. Effective renal blood flow, glomerular filtration rate, urine flow rate, electrolyte excretion rates and electrolyte fractional excretion were then determined as described previously in Section 3.2.7.4. Furthermore, organs including the heart, lung, liver and kidney were harvested and weighed. Four animals were excluded from this experiment due to blood loss from either the jugular vein (n = 2) or the carotid artery (n = 2).

4.2.9 Statistical analysis

The data were analysed using the IBM SPSS statistical package (version 22, New York, US) and GraphPad Prism® software (La Jolla, USA). After applying Shapiro-Wilks normality distribution tests, the data are presented as mean ± SEM and parametric analyses were used. Where data did not conform to a normal distribution, non-parametric analyses were used. The data are expressed as box and whisker plots unless stated otherwise. The boxes mark the interval between the 25th and 75th percentile, the lines inside the box represent the median, and the whiskers denote the interval between the 5th and 95th percentiles. For all analyses, N = number of litters. P < 0.05 was considered statistically significant.

4.2.9.1 Offspring postnatal weekly food and water intake and bodyweight

Offspring food and water intake and bodyweight were assessed by repeated-measures two-

way ANOVA with Tukey's post hoc test.

4.2.9.2 Offspring organ weights, blood pressure and heart rate

Offspring (14 weeks of age) organ weights were expressed relative to bodyweights. Offspring organ weights, blood pressure and heart rate were analysed by two-way ANOVA with a Tukey's post hoc test.

4.2.9.3 Salt preference and aversion tests and extracellular fluid volume

All values were adjusted to 100 g bodyweight. Salt preference tests and extracellular fluid volume between the dietary groups and sexes were assessed by a two-way ANOVA with Tukey's post hoc test. In the salt aversion test, in which the amount of different saline concentrations the offspring drank was compared against 0.9% saline, intake was assessed by one-way ANOVA followed by Dunnett's post hoc test.

4.2.9.4 Urinary analysis

Urine creatinine and NGAL concentrations and albumin:creatinine concentration ratio were assessed by two-way ANOVA with Tukey's post hoc test. Urine albumin concentration was assessed by Kruskal-Wallis followed by Dunn's multiple comparison test.

4.2.9.5 Renal function

Differences in renal function between the dietary groups were assessed by a two-way ANOVA with Tukey's post hoc test. The effect of time on renal function within dietary groups was analysed using a repeated measures two-way ANOVA with Tukey's post hoc test. The Kruskal-Wallis test followed by Dunn's multiple comparison test was used to analyse all plasma electrolytes and urine K^+ concentration and osmolality and fractional excretions of chloride and sodium.

4.3 RESULTS

4.3.1 Food intake in response to a high-salt diet in control and IF offspring

Food intake was measured weekly after weaning for eight consecutive weeks in both control and IF rats fed on either a standard chow diet or a high-salt diet. Prenatal dietary manipulation had no effect on standard chow intake (Figure 4.3.1 a). Male and female offspring exposed to intermittent fasting *in utero* behaved in a similar manner, as did control rats. However, in control male offspring, food intake increased significantly for the first week after weaning (week 6 compared to week 5) ($P < 0.01$, Figure 4.3.1 a), whereas IF male offspring consumed almost a constant amount of food over the duration of the experiment (Figure 4.3.1 a).

Exposure to a high-salt diet did not alter the feeding behaviour of the IF offspring, which tended to consume a similar amount of food compared to the salt-loaded controls and to their counterparts fed a standard chow diet (Figures 4.3.1 b, c and d). The exception to this pattern was that salt-loaded IF male offspring had higher food consumption for two consecutive weeks, after which their consumption remained stable for the rest of the experiment ($P < 0.0001$, Figures 4.3.1 b and d), with a significant difference observed at postnatal week 7, when compared to standard-diet IF male rats ($P < 0.0001$, Figure 4.3.1 d). Furthermore, the food intake of salt-loaded control males diverged at week 12 compared to that of standard-diet controls ($P < 0.05$, Figure 4.3.1 c). In females, food consumption was stable during the experiment, independent of dietary exposure before birth or after weaning (Figure 4.3.1 a and b), and was significantly lower than that of male littermates from postnatal week 6 onward ($P < 0.0001$, Figures 4.3.1 a, b, c and d).

When food intake (standard chow and high-salt) was expressed relative to bodyweight, no difference between the control and IF groups or between the sexes was detected (Figures 4.3.2 a, b, c and d). However, control group males ingested significantly more food (standard chow and high-salt) than IF males and control females at weeks 5 and 6 ($P_{ND} < 0.05$, $P_{HS} < 0.001$, Figures 4.3.2 a, b and c), whereas IF females consumed more high-salt food than IF males at 11 and 12 weeks of age ($P < 0.05$, Figures 4.3.2 b and d). Data also revealed the significant effect of age on food intake, which reduced relative to body mass gradually with progression in age ($P < 0.0001$, Figures 4.3.2 a, b, c and d).
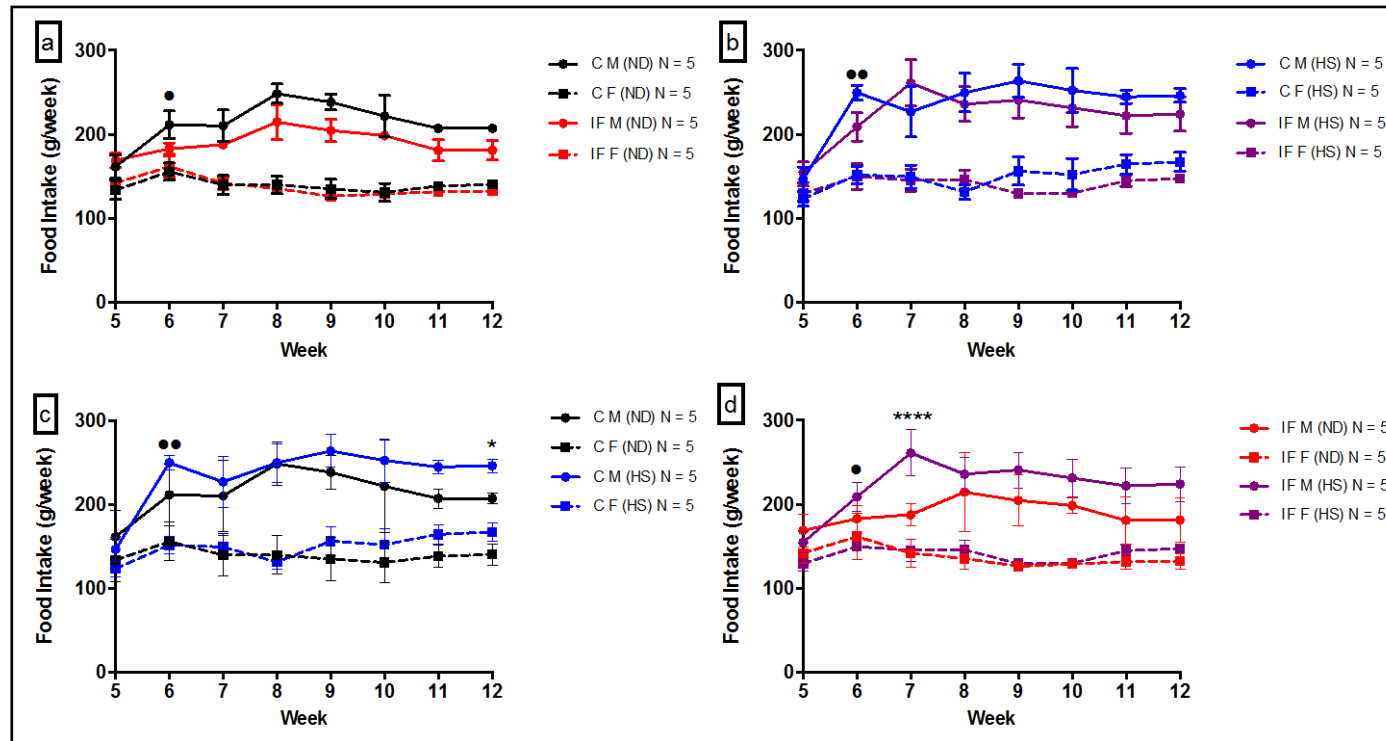
Figure 4.3.1 Food intake of control and IF offspring fed either a standard chow diet or a high-salt diet from postnatal week 5 until week 12, separated according to offspring sex. **a.** Standard chow diet (ND) intake in control (black) and IF (red) rats was comparable, with a significant increase in food consumption for control males at week 6. **b.** High-salt diet (HS) intake in control (blue) and IF (purple) rats showed similar feeding behaviour. A high-salt diet failed to increase food consumption in either control rats (**c**) or IF rats (**d**). There was a significant increase in food intake by HS IF males compared to ND IF males at postnatal week 7. Females undergoing any dietary manipulation behaved similarly, with almost constant food intake for the duration of the experiment and significantly lower intake than male littermates from week 6 onward. Data are presented as mean ± SEM. • P < 0.05, •• P < 0.01 week 6 versus week 5 in male offspring, * P < 0.05, **** P < 0.0001 HS males vs ND males. Statistical analysis was carried out using a repeated-measures two-way ANOVA followed by Tukey's post hoc test.
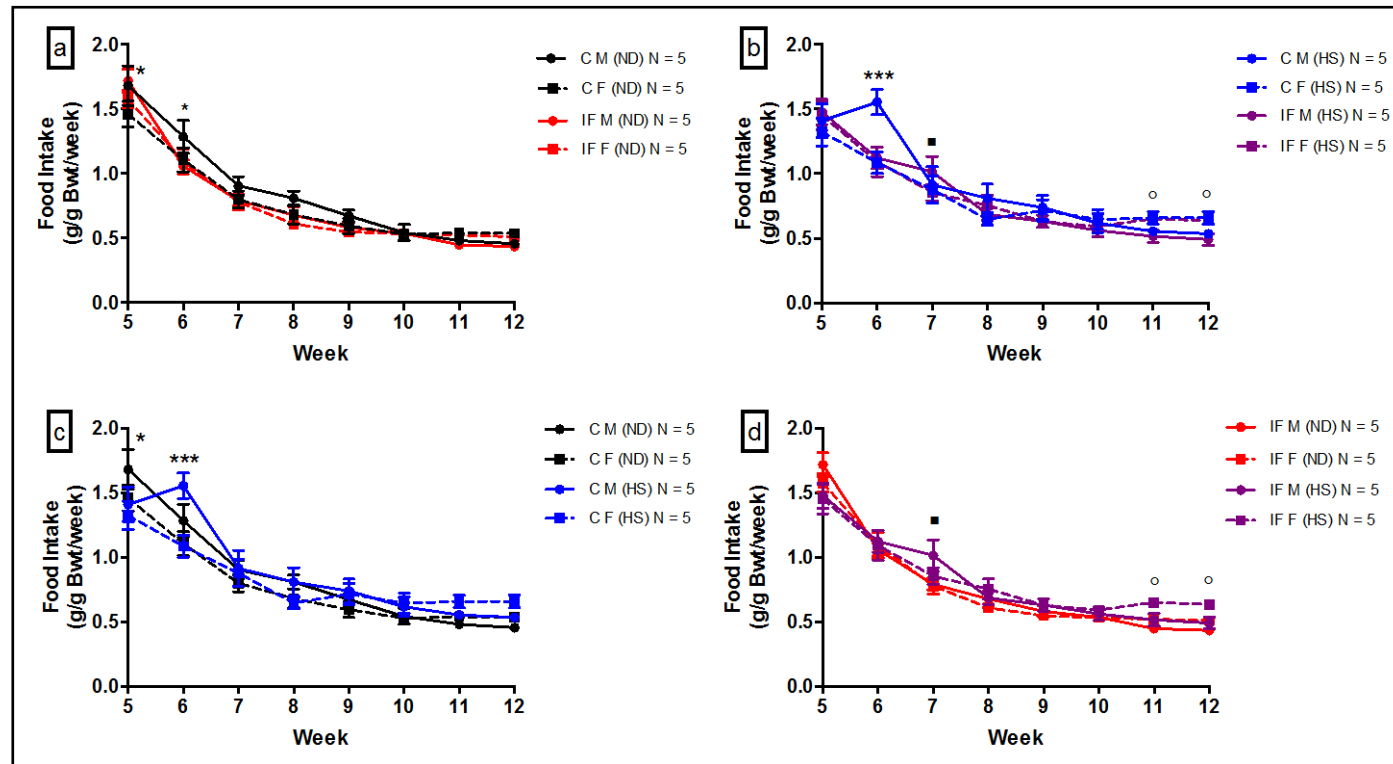
Figure 4.3.2 Food intake (normalised to bodyweight) of control and IF offspring fed either a standard chow diet or a high-salt diet from postnatal week 5 until week 12, separated according to offspring sex. Food intake gradually decreased with progression in age ($P_{Week} < 0.0001$). **a.** Standard chow diet (ND) intake in control (black) and IF (red) rats was comparable, with a significant increase in food consumption for control males at weeks 5 and 6 (* $P < 0.05$). **b.** High-salt diet (HS) intake in control (blue) and IF (purple) rats showed similar feeding behaviour. However, control males ingested more food at week 6 than IF males and control females (*** $P < 0.001$). **b and d.** There was a significant increase in food intake by HS IF males compared to HS IF females and ND IF males at postnatal week 7 (■ $P < 0.05$). IF females consumed a significantly higher high-salt diet than IF male littermates from week 11 onward (° $P < 0.05$). Data are presented as mean ± SEM. Statistical analysis was carried out using a repeated-measures two-way ANOVA followed by Tukey's post hoc test.

4.3.2 Water intake in response to a high-salt diet in control and IF offspring

The weekly water intake of all dietary treatment groups over a period of eight weeks is presented in Figure 4.3.3. Water intake was stable during the experimental period for rats maintained on a standard chow diet, with similar water intake for females of both dietary groups and males of the IF group (Figure 4.3.3 a). However, male control offspring showed a significant increase in the first week post-weaning (week 6 compared to week 5), following the same pattern as in food intake (Section 4.3.1). Interestingly, male control offspring had significantly higher water consumption than male IF offspring for almost the entire period of the experiment except at week 7 and 8 (P < 0.01 and P < 0.001 respectively, Figure 4.3.3 a). A high-salt diet, on other hand, induced a marked increase in water consumption for both sexes of control and IF groups relative to the matched sexes on a standard chow diet (P < 0.0001, Figures 4.3.3 c and d). Water intake also had a close association with food intake as salt-loaded IF male offspring showed increased water consumption for two consecutive weeks, while females showed steady water intake for the entire period (Figures 4.3.3 b and d).

Remarkably, when water intake was expressed relative to bodyweight, the differences between the sexes in both dietary groups were no longer apparent (Figures 4.3.4 a, b, c and d). Similarly to food intake, relative water intake declined with progression in age (P < 0.0001, Figures 4.3.4 a, b, c and d). Both control and IF offspring showed polydipsia resulting from a higher salt intake than a standard chow diet (P < 0.001, Figures 4.3.4 c and d). Control males drank more water than IF males that were fed standard chow at week 6 (P < 0.001, Figure 4.3.4 a). Salt-loaded control males consumed more water than matched females at week 5 and 6 (P < 0.05 and P < 0.001 respectively, Figure 4.3.4 c) and more than both sexes in the IF group of at week 6 (P < 0.001, Figures 4.3.4 b and c). Salt-loaded IF males drank more water than matched IF females at week 6 (P < 0.001, Figure 4.3.4 d).
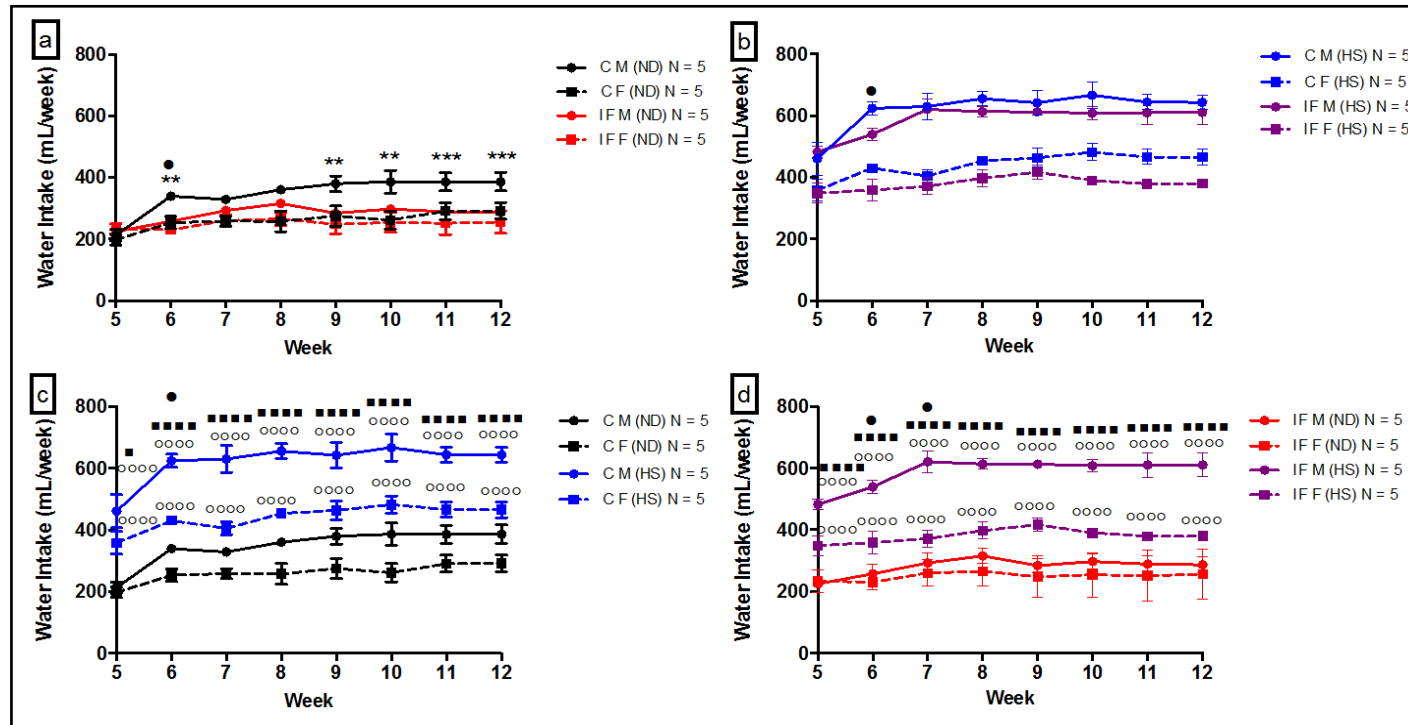
Figure 4.3.3 Water intake of control and IF offspring fed either a standard chow diet or a high-salt diet from postnatal week 5 until week 12, separated according to offspring sex. **a.** In standard ND groups, water consumption was similar between control (black) and IF (red) rats except for control males, which exhibited significantly higher water intake compared to IF males in most weeks. **b.** Control (blue) and IF (purple) rats intaking a high-salt diet showed similar drinking behaviour, with males showing significantly higher water consumption than females. **c** and **d**. Salt-loaded offspring of both sexes showed a significant increase in water intake for both control and IF groups compared to standard chow diet counterparts. Data are presented as mean ± SEM. • P < 0.05 week 6 versus week 5 and week 7 versus week 6 in male offspring, ** P < 0.01, *** P < 0.001 C males versus IF males, °°°° P < 0.0001 HS rats versus ND rats, ▪ P < 0.05, ▪▪▪▪ P < 0.0001 males versus females on HS diet. Statistical analysis was carried out using a repeated-measures two-way ANOVA followed by Tukey's post hoc test.
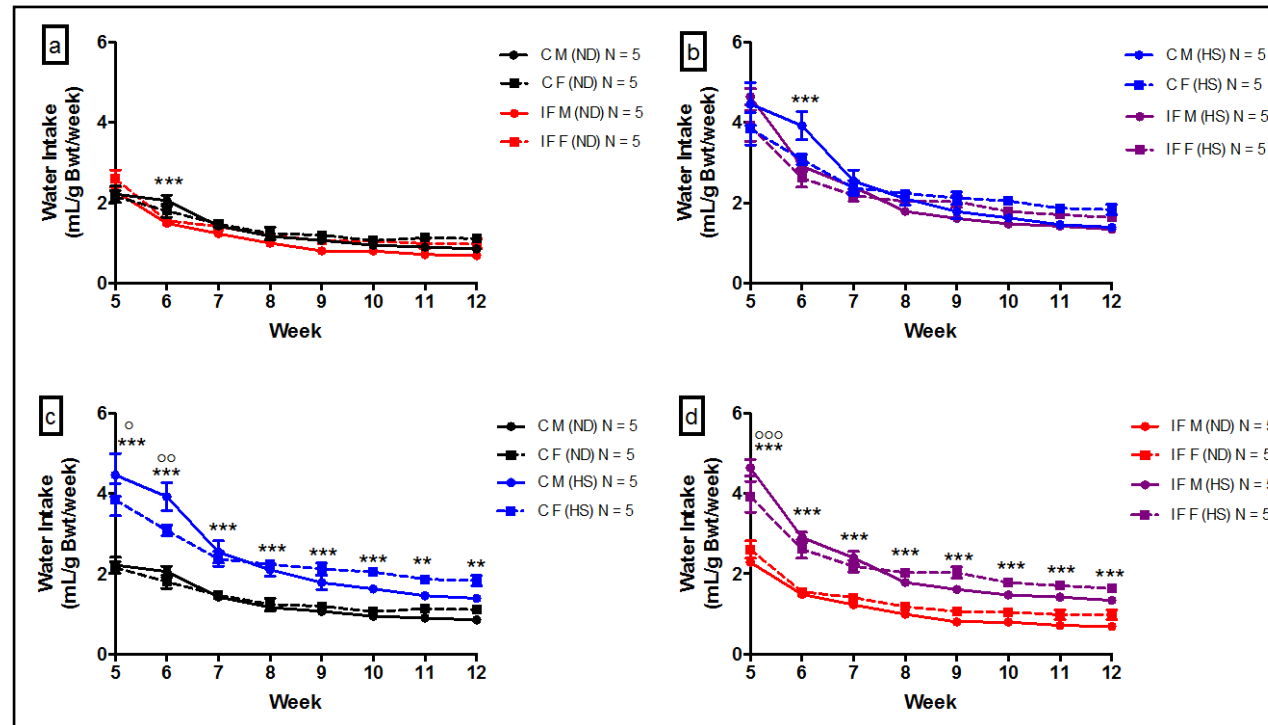
Figure 4.3.4 Water intake (normalised to bodyweight) of control and IF offspring fed either a standard chow diet or a high-salt diet from postnatal week 5 until week 12, separated according to offspring sex. **a.** In standard ND groups, water consumption was similar between control (black) and IF (red) rats except for control males, which exhibited significantly higher water intake than IF males at week 6 (*** $P < 0.001$). **b.** Control (blue) and IF (purple) rats with a high-salt diet intake showed similar drinking behaviour. However, control males showed significantly higher water consumption than females and IF males at week 6 (*** $P < 0.001$). **c** and **d**. Salt-loaded offspring of both sexes showed a significant increase in water intake in both control and IF groups compared to their standard chow diet counterparts (** $P < 0.01$, *** $P < 0.001$). **c.** Salt-loaded control males drank more water than matched females at week 5 (° $P < 0.05$) and 6 (°° $P < 0.01$) whereas **d.** salt-loaded IF males drank more water than matched females at week 5 only (°°° $P < 0.001$). Data are presented as mean ± SEM. Statistical analysis was carried out using a repeated-measures two-way ANOVA followed by Tukey's post hoc test.

4.3.3 Bodyweight response to a high-salt diet in control and IF offspring

Changes in bodyweight in rats fed with standard chow or a high-salt diet were monitored weekly until rats reached 12 weeks of age. As already shown in Section 3.3.3, male offspring were heavier than females in both control and IF groups regardless of diet ($P < 0.0001$, Figures 4.3.5 a, b and c). In the control group, bodyweight did not differ among rats fed standard chow or a high-salt diet (Figure 4.3.5 a). In the IF group, from week 8 onward, salt-loaded male offspring showed a significant increase in bodyweight compared to offspring fed a standard chow diet, whereas female offspring were significantly lighter ($P < 0.0001$, Figure 4.3.5 b). In control and IF rats fed high-salt diet, IF males were heavier at postnatal weeks 8 and 9, but at postnatal weeks 11 and 12, differences no longer existed between groups ($P < 0.01$, Figure 4.3.5 c). In contrast, female offspring in the IF group were of comparable weight to controls until 10 weeks of age, after which they became significantly lighter ($P < 0.01$, Figure 4.3.5 c).

4.3.4 Systolic blood pressure (SBP) in response to a high-salt diet

Systolic blood pressure rose in response to salt loading in both control and IF offspring in a time-dependent manner (Figure 4.3.6 a). From week 5 to week 10, the SBP of salt-loaded control male and female rats increased by 11.5 ± 2.2 and 11.0 ± 2.0 mmHg, respectively, and the SBP of salt-loaded IF male and female rats increased by 16.5 ± 2.1 and 12.0 ± 2.0 mmHg, respectively ($P < 0.01$, Figure 4.3.6 a). The SBP of IF rats of both sexes fed a high-salt diet were significantly greater in comparison to counterparts receiving a standard rat chow diet at postnatal weeks 7 and 10 ($P < 0.01$, Figures 4.3.8 b and c). Female salt-loaded control rats demonstrated a significant increase in SBP at week 7 and 10 ($P < 0.05$, Figures 4.3.7 b and c), whereas males experienced an increase at week 10 only ($P < 0.01$, Figure 4.3.7 c) compared to matched-standard chow. However, no differences in SBP emerged between the salt-loaded control and IF rats or between sexes of each group at any time (Figures 4.3.6 b, c and d).
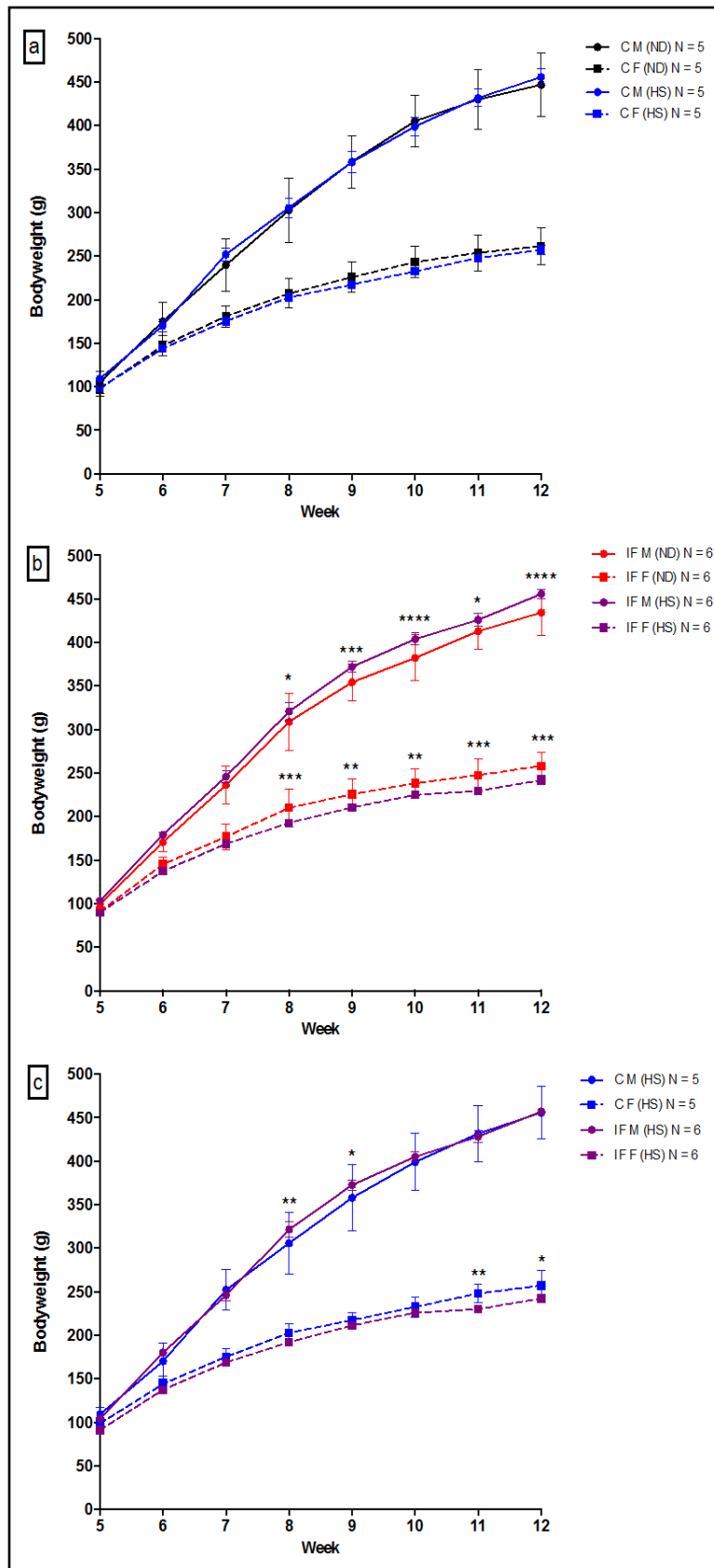
Figure 4.3.5 The effect of a high-salt diet on postnatal growth in control and IF offspring over postnatal weeks 5 to 12, separated according to sex. Offspring were weighed weekly. **a.** HS control offspring (blue) were of similar weight to ND controls (black) over postnatal weeks 5 to 12, with no differences observed. **b.** In IF offspring, HS males were significantly heavier than ND males from week 8 onward. In contrast, HS females were lighter than ND females from week 8 onward. **c.** HS IF male offspring were heavier than HS control males at weeks 8 and 9, but at weeks 11 and 12, the weights of IF male offspring were comparable to those of control offspring. IF female offspring, on the other hand, were of similar weight to controls over weeks 5 to 10, after which they became significantly lighter than controls. Data are presented as mean ± SEM. $P_{Day} < 0.0001$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ HS versus ND. Statistical analysis was carried out using a repeated-measures two-way ANOVA followed by Tukey's post hoc test.

Offspring heart rate (bpm) was unaffected by dietary salt in control and IF groups over the same period, with a lack of sex-specific effects (P > 0.05, Table 4.3.1). In comparison to the heart rate results of control and IF rats fed a standard rat chow diet in Chapter 3, Section 3.3.7, there were no differences between dietary groups or between sexes.

Table 4.3.1 Heart rate in control and IF offspring fed a high-salt diet measured using tail-cuff plethysmography

Experimental group		Control (HS) N = 5		IF (HS) N = 6	
		Male	Female	Male	Female
Heart rate (bpm)	Week 5	437 ± 4	435 ± 3	443 ± 6	433 ± 4
	Week 7	437 ± 2	433 ± 3	441 ± 2	432 ± 6
	Week 10	436 ± 1	432 ± 4	441 ± 2	436 ± 5

Values are mean ± SEM with P > 0.05 (two-way ANOVA followed by Tukey's post hoc test).
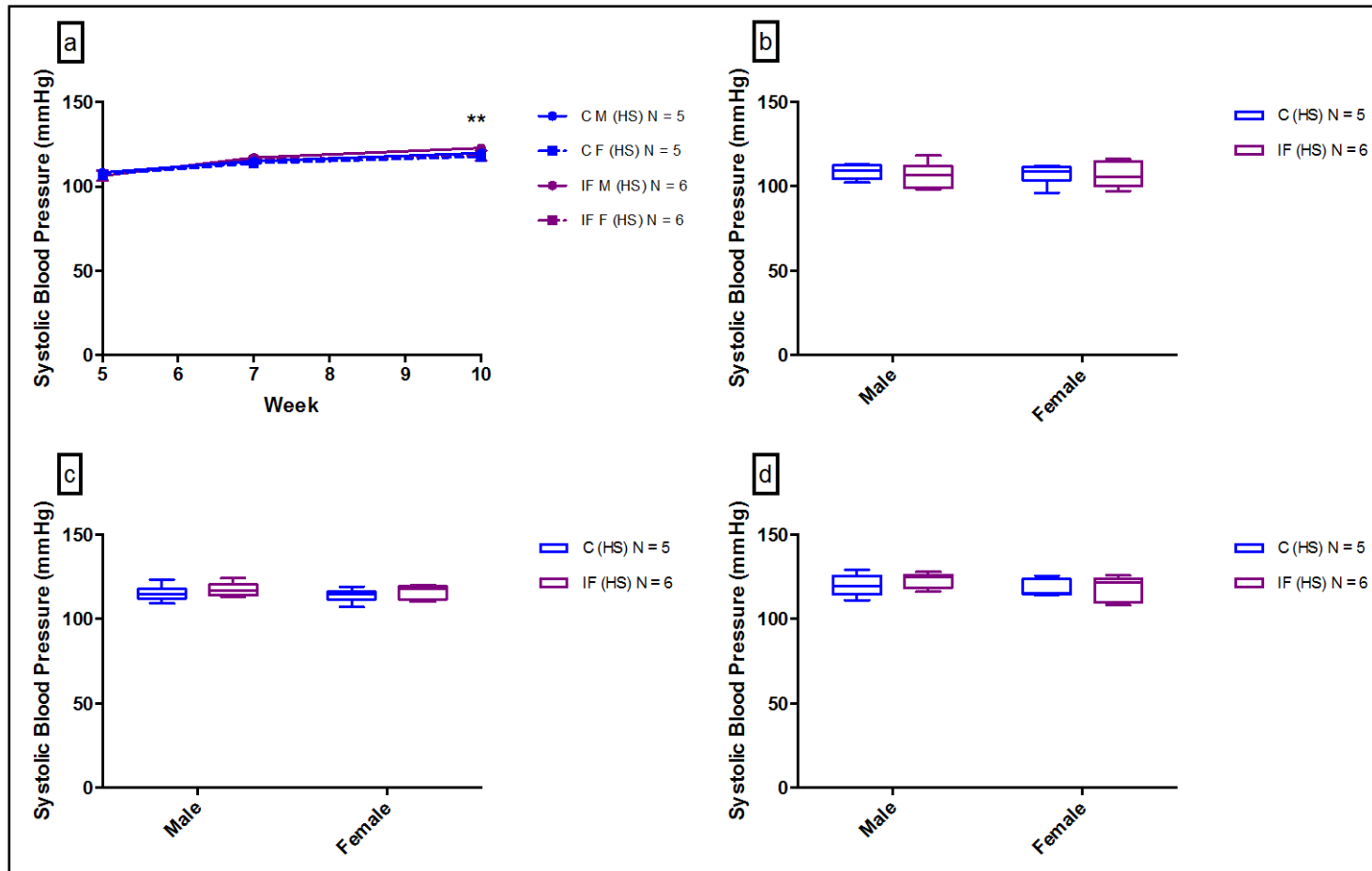
Figure 4.3.6 Comparison of systolic blood pressure in control (blue) and IF (purple) offspring in response to a high-salt diet. Blood pressure was measured **a.** over a period of five weeks, at postnatal **b.** week 5, **c.** week 7, and **d.** week 10. **a.** Systolic blood pressure increased over a period of five weeks. However, SBP was similar between dietary groups and sexes. Data are analysed as **a.** mean ± SEM, ** $P < 0.01$ between weeks 5 and 10. **b. - d.** Data expressed as box and whisker plots, $P > 0.05$. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
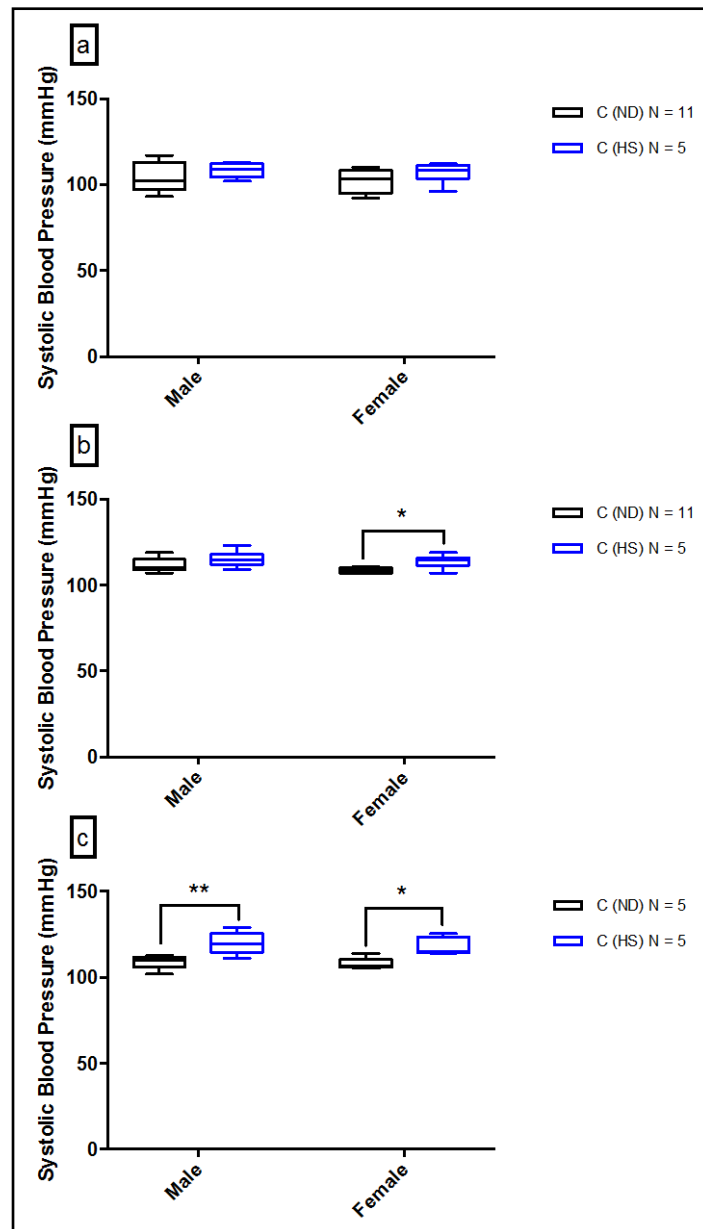
Figure 4.3.7 Comparison of systolic blood pressure in ND control (black) and HS control (blue) offspring. Blood pressure was measured at postnatal **a.** week 5, **b.** week 7, and **c.** week 10. Systolic blood pressure was similar between dietary groups and sexes at week 5. At weeks 7 and 10, control female HS rats showed a significant increase in SBP compared to ND counterparts, whereas control male HS rats showed a significant increase in SBP only at week 10 compared to ND counterparts. Data are analysed as mean/litter and expressed as box and whisker plots. * $P < 0.05$ between HS and ND females, ** $P < 0.01$ between HS and ND males. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
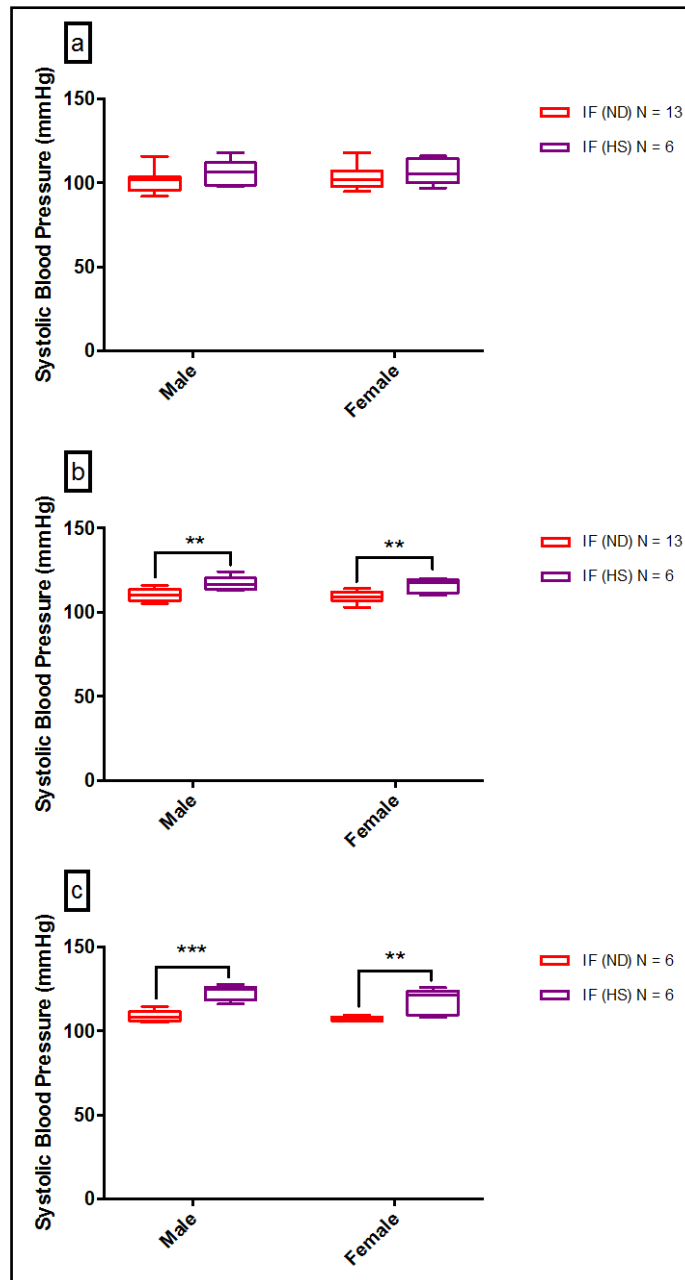
Figure 4.3.8 Comparison of systolic blood pressure of ND IF (red) and HS IF (purple) offspring. Blood pressure was measured at postnatal **a.** week 5, **b.** week 7, and **c.** week 10. Systolic blood pressure was similar between dietary groups and sexes at week 5. At weeks 7 and 10, the SBP of HS IF rats of both sexes was significantly higher compared to that of their counterparts. Data are analysed as mean/litter and expressed as box and whisker plots. ** $P < 0.01$ between HS and ND groups, *** $P < 0.001$ between HS and ND males at week 10. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

4.3.5 Salt preference and aversion in IF rats

Early developmental nutrition can determine salt preference and intake in rat offspring (Alwasel et al., 2012). In a test providing a choice between two bottles, one containing water and one normal saline (0.9% NaCl), fluid intake was measured for five consecutive days in rats at seven weeks of age exposed *in utero* to intermittent fasting or a control diet. Total fluid intake, including water and saline, did not differ between control and IF offspring ($P > 0.05$, Figure 4.3.9). However, females of both dietary groups drank significantly more relative to bodyweight than males in all series of saline concentrations (Figures 4.3.9 a, b, c and d). Total fluid intake by offspring of both dietary groups and sexes was maximal when offered the 0.9% saline solution (Figure 4.3.9 a); but as the concentration of saline increased, total fluid intake dropped gradually (Figures 4.3.9 b, c and d).

In the salt preference test, differences were observed between sexes. Per 100 g bodyweight, female rats of both dietary groups showed a strong preference for saline, ingesting significantly more salt fluid than water throughout the test ($P < 0.01$, Figure 4.3.9 a). In contrast, male rats in control and IF groups ingested similar amounts of saline and water ($P > 0.05$, Figure 4.3.9 a). This pattern of salt preference was also maintained with a 1.2% saline solution. With a series of solutions containing increasing amounts of salt (1.5%, 1.8% and 2.1%), males of both dietary groups switched their preference to water over salt solutions ($P < 0.01$, Figures 4.3.9 c, d and e). Control females, on the other hand, displayed no preference for either water or saline solutions ($P > 0.05$, Figures 4.3.9 c, d and e). IF females behaved differently, showing a preference for 1.5% saline over water ($P < 0.05$, Figure 4.3.9 c). This preference disappeared with a 1.8% saline solution, for which no preference was observed between water or saline intakes ($P > 0.05$, Figure 4.3.9 d). With a 2.1% saline solution, IF females ingested significantly more water than saline ($P < 0.05$, Figure 4.3.9 e).

To determine the threshold of aversion to salt, rats were presented with a choice of two bottles, one containing water and one containing a solution with an increasing percentage of saline over time, from 0.9% up to 2.1%, in 0.3% increments (Figure 4.3.10). Males of both dietary groups had an aversion to the saline solution at a concentration of 1.8% ($P < 0.05$, Figures 4.3.10 a and b), whereas females of the control dietary group showed a strong aversion to the saline solution at a concentration of 1.5% ($P < 0.01$, Figure 4.3.10 c). However, IF females demonstrated a higher threshold of salt aversion, evident at a concentration of 1.8% saline ($P < 0.01$, Figure 4.3.10 d).
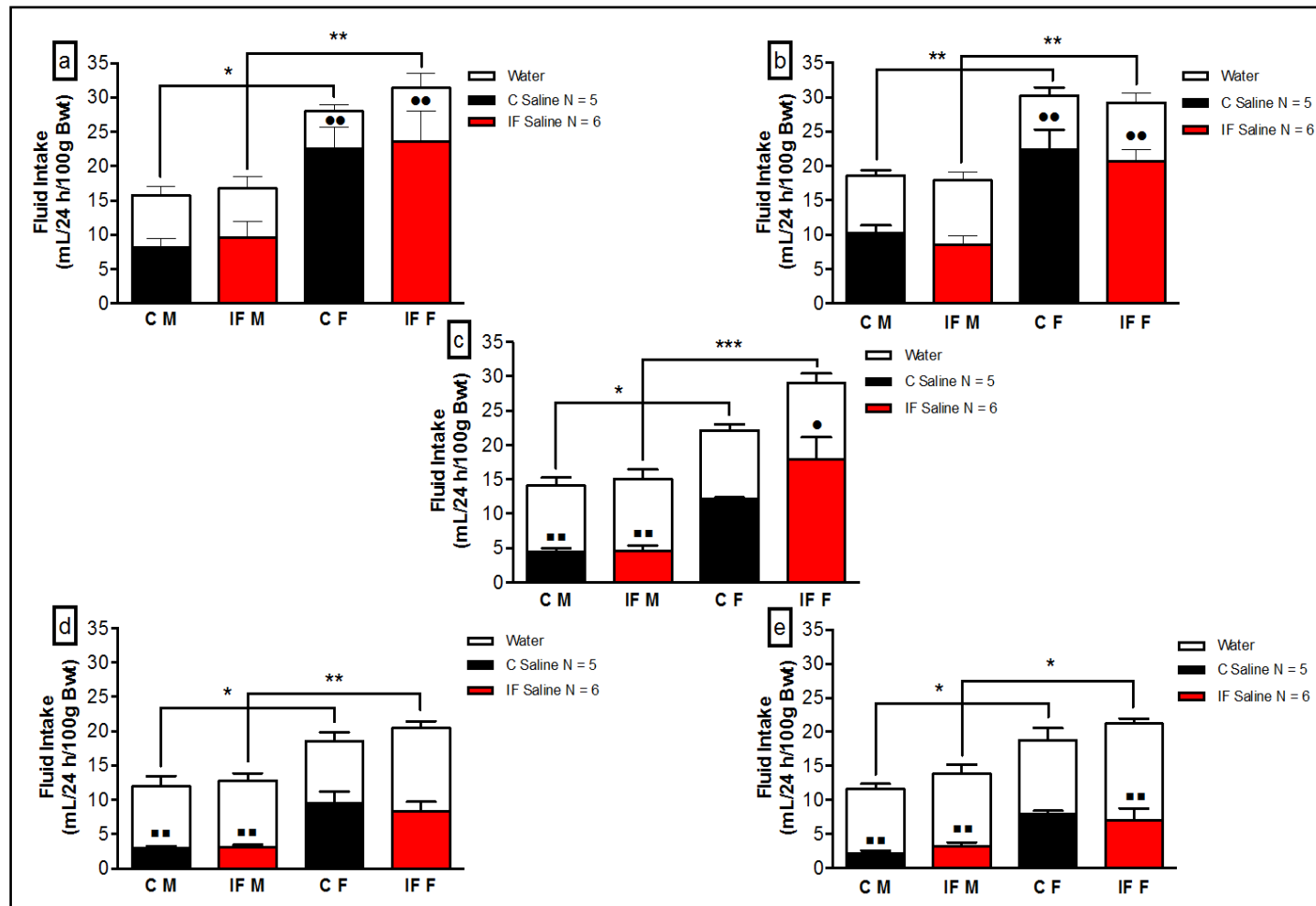
Figure 4.3.9 Salt preference and fluid intake in control (black) and IF (red) offspring at postnatal week 7. Rats were offered a choice of water or saline at concentrations of **a.** 0.9%, **b.** 1.2%, **c.** 1.5%, **d.** 1.8%, and **e.** 2.1% as drinking fluid. At different saline concentrations, total fluid intake was significantly higher in females compared to males of both dietary groups. The preference for salt significantly increased in females of both dietary groups presented with 0.9% and 1.2% saline solutions compared to water, while males of both dietary groups showed a similar preference for both fluids. From 1.5% to 2.1% saline, males of both dietary groups ingested significantly more water than saline, whereas control females lacked a preference for either saline or water. IF females showed a preference for 1.5% saline compared to water, with a similar preference for 1.8% saline and water and a higher water intake compared to 2.1% saline. Data are presented as mean + SEM. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test. * indicates differences between sexes, • indicates a significant increase in saline intake, and ▪ indicates a significant increase in water intake. * and • $P < 0.05$, ** and ▪▪ $P < 0.01$, *** $P < 0.001$. The figure uses the following abbreviations: C M, male control rats; C F, female control rats; IF M, male IF rats; IF F, female IF rats. The open bar represents water intake, whereas the coloured bar represents saline intake.
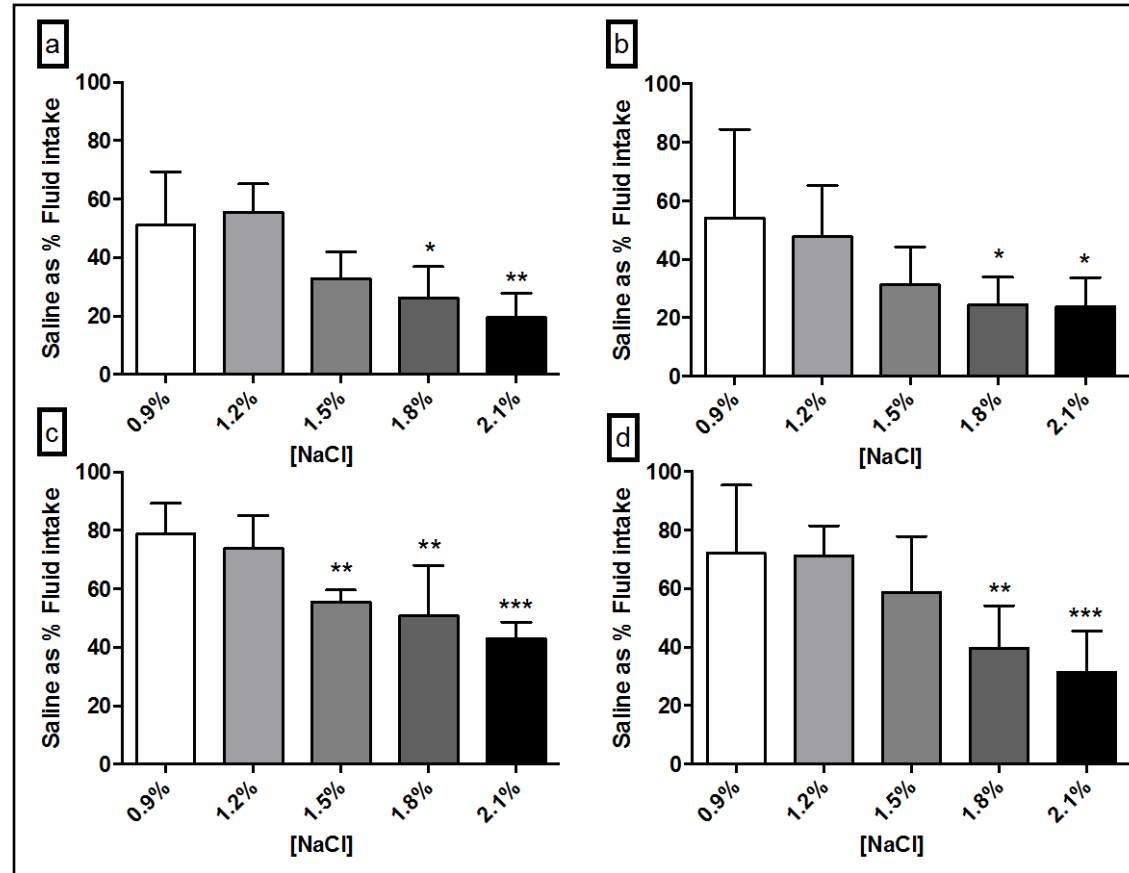
Figure 4.3.10 Salt aversion in control and IF offspring offered a choice between two bottles, one with water and one with a gradual increase of saline concentrations. Intake was measured in **a.** male control rats, **b.** male IF rats, **c.** female control rats, and **d.** female IF rats. Males of both dietary groups and IF females showed salt aversion at a 1.8% saline concentration. Females of the control group had a lower aversion threshold, at a 1.5% saline concentration. Data are presented as mean + SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to 0.9% saline. Statistical analysis was carried out using a one-way ANOVA followed by Dunnett's test.

4.3.6 Effect of prenatal exposure to intermittent fasting on extracellular fluid volume (ECFV) in rats on standard chow diet

Extracellular fluid volume was estimated in rats at 12 weeks of age exposed *in utero* to either intermittent fasting or a control diet using the *in vivo* measurement of inulin space. ECFV was similar between the two dietary groups and sexes (P > 0.05, Figure 4.3.11).
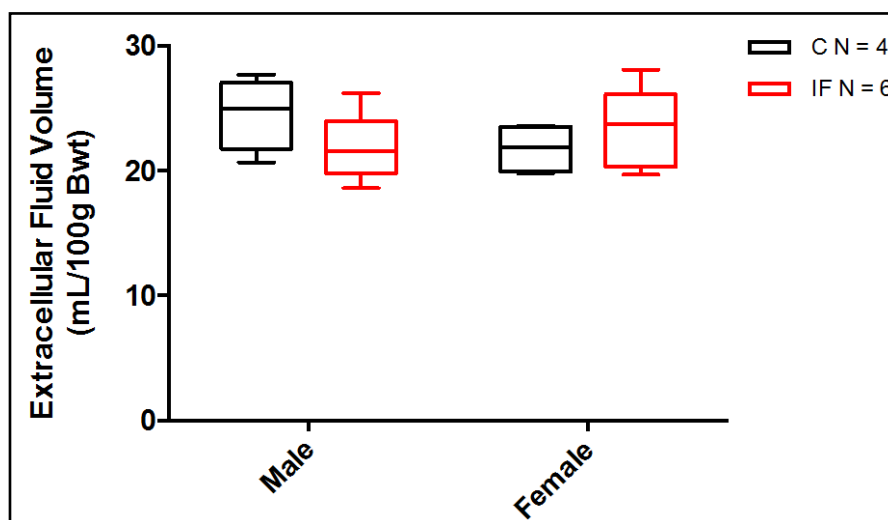
Figure 4.3.11 Effect of maternal intermittent fasting on the extracellular fluid volume of the offspring at 12 weeks of age. There were no differences between control (black) and IF (red) groups or between sexes of each group. Data are expressed as box and whisker plots. P > 0.05. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

4.3.7 Renal function in conscious rats fed high-salt diet: metabolism cages

Urine creatinine concentration decreased significantly in both male and female salt-loaded IF offspring compared to the salt-loaded control group (P < 0.01, Figure 4.3.12). While urine albumin concentration did not differ between salt-loaded control and IF groups, IF males had a significantly higher albumin concentration compared to their female littermates (P < 0.05, Figure 4.3.13). Excretion of albumin in the urine (corrected for urinary creatinine concentration) showed a similar trend, being significantly greater in salt-loaded IF males than females, with a comparable concentration between both dietary groups (P < 0.05, Figure 4.3.14). Urine NGAL concentration, an early marker for kidney injury, was measured in salt-loaded offspring at postnatal week 12. IF male offspring fed a high-salt diet showed a significant increase in urine NGAL concentration compared to salt-loaded control males (P < 0.05, Figure 4.3.15). IF female offspring, on the other hand, had higher urine NGAL concentration than control females, however, this increase failed to reach a statistically significant level (P = 0.066, Figure 4.3.15).
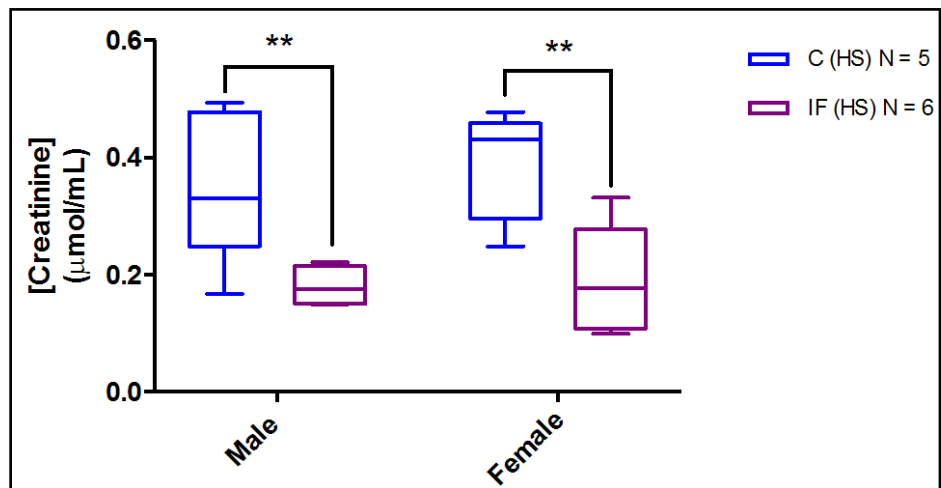
Figure 4.3.12 Effect of a high-salt diet on urine creatinine concentration in control (blue) and IF (purple) offspring at 12 weeks of age. There was a significant decrease in urine creatinine concentration in salt-loaded IF offspring compared to controls. Data are expressed as box and whisker plots. ** $P < 0.01$ IF versus control offspring. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
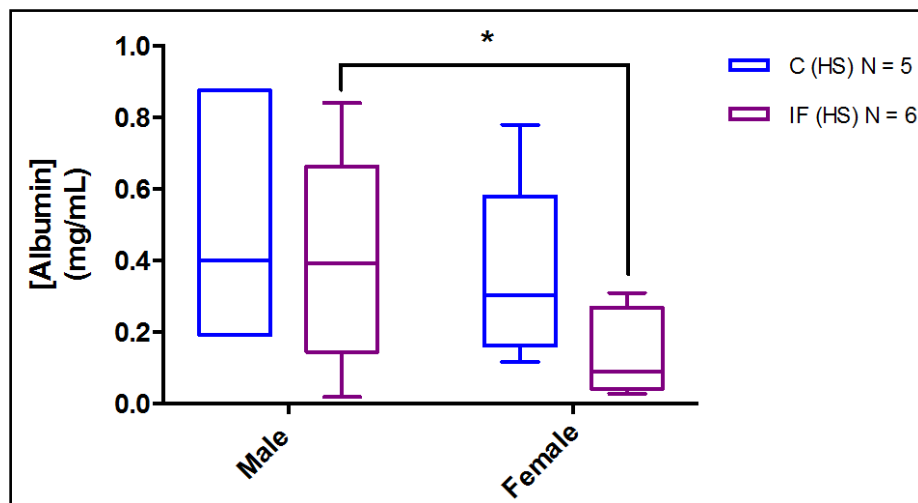
Figure 4.3.13 Effect of a high-salt diet on urine albumin concentration in control (blue) and IF (purple) offspring at 12 weeks of age. There were no differences between dietary groups; however, IF females had a lower urine albumin concentration compared to male littermates. Data are expressed as box and whisker plots. * $P < 0.05$ IF males versus IF females. Statistical analysis was carried out using a Kruskal-Wallis followed by Dunn's multiple comparison test.
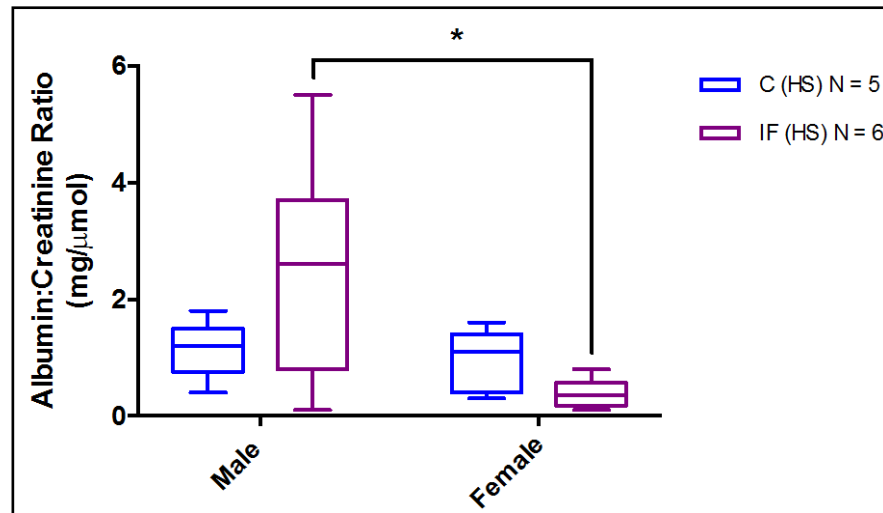
Figure 4.3.14 Effect of a high-salt diet on albumin:creatinine concentration ratio in control (blue) and IF (purple) offspring at 12 weeks of age. There were no differences between dietary groups; however, IF males had a higher albumin:creatinine concentration ratio compared to female littermates. Data are expressed as box and whisker plots. * $P < 0.05$ IF males versus IF females. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
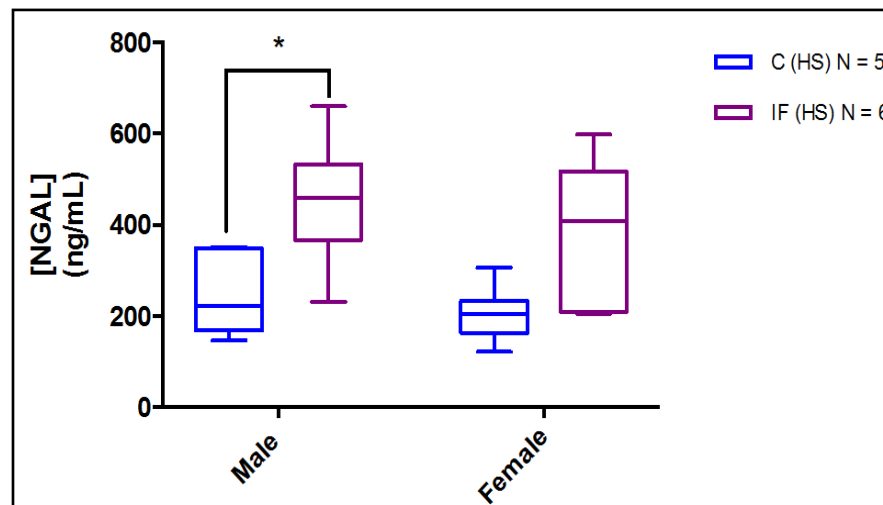
Figure 4.3.15 Effect of a high-salt diet on urine NGAL concentration in control (blue) and IF (purple) offspring at 12 weeks of age. IF male offspring showed a significantly higher urine NGAL concentration than did control males (* $P < 0.05$). IF female offspring showed an increase in urine NGAL concentration that failed to reach a statistically significant level ($P = 0.066$). Data are expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

**4.3.8 Bodyweight and terminal organ weights at 14 weeks of age in offspring fed a high-salt diet**

At 14 weeks of age, offspring from salt-loaded control and IF groups were weighed prior to renal clearance studies. As expected males were heavier than females for both dietary groups ($P < 0.0001$), but for clarity the statistical difference between sexes is not shown in Figure 4.3.16. No significant difference was noted between the dietary groups with regard to bodyweight ($P > 0.05$, Figure 4.3.16 a). In comparison to rats of the same age fed a standard chow diet (see Chapter 3), the bodyweight of females was similarly independent of dietary exposure before birth or after weaning. However, the bodyweight of salt-loaded male offspring in both control and IF groups was significantly lower compared with rats fed standard chow ($P < 0.05$, Figures 4.3.16 b and c).

Terminal organ weights expressed relative to bodyweight from salt-loaded offspring are shown in Figure 4.3.17. There were no significant differences in heart, lung, kidney or liver weights among control and IF groups ($P > 0.05$, Figures 4.3.17 a, b, c and d). However, there was a sex-specific difference in that salt-loaded females of both IF and control groups had significantly heavier heart weights compared to their male littermates ($P_{IF} < 0.05$, $P_{Control} < 0.0001$, Figure 4.3.17 a). Organ weights from salt-loaded offspring were also compared with those of offspring fed a standard chow diet (Figures 4.3.18 and 4.3.19). Relative kidney weight was significantly affected by salt intake irrespective of sex, as kidney weights were significantly increased in salt-loaded offspring compared to offspring fed a standard chow diet (Figures 4.3.18 c and 4.3.19 c). Relative liver weight was also heavier in control females fed a high-salt diet (Figure 4.3.18 d).
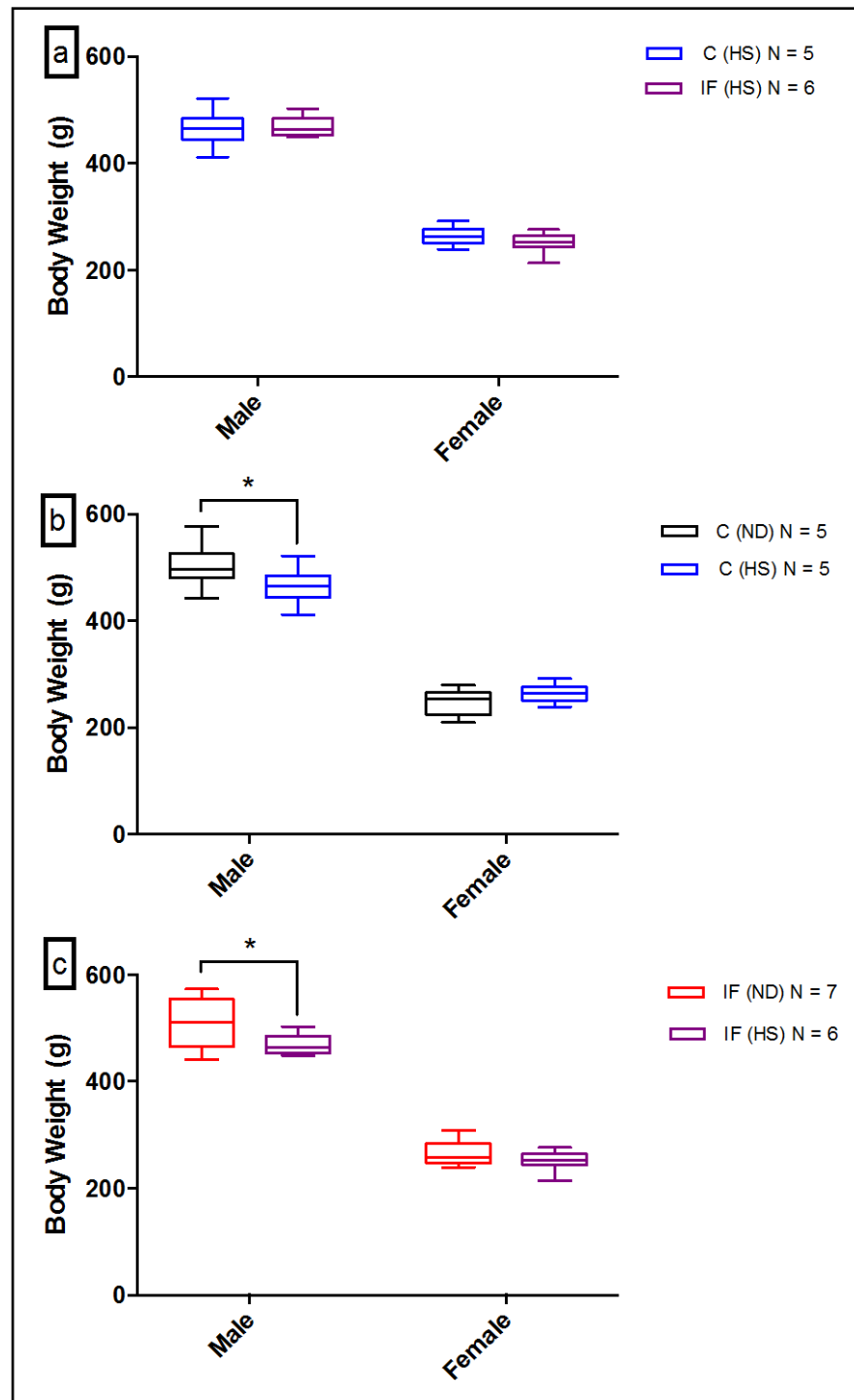
Figure 4.3.16 The effect of a high-salt diet on offspring bodyweight at 14 weeks of age. **a.** Salt-loaded offspring bodyweight was similar between IF and control groups. **b.** and **c.** HS males in both control (blue) and IF (purple) groups were lighter than ND control (black) and IF (red) counterparts (* $P < 0.05$). Data were analysed as mean/litter and expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
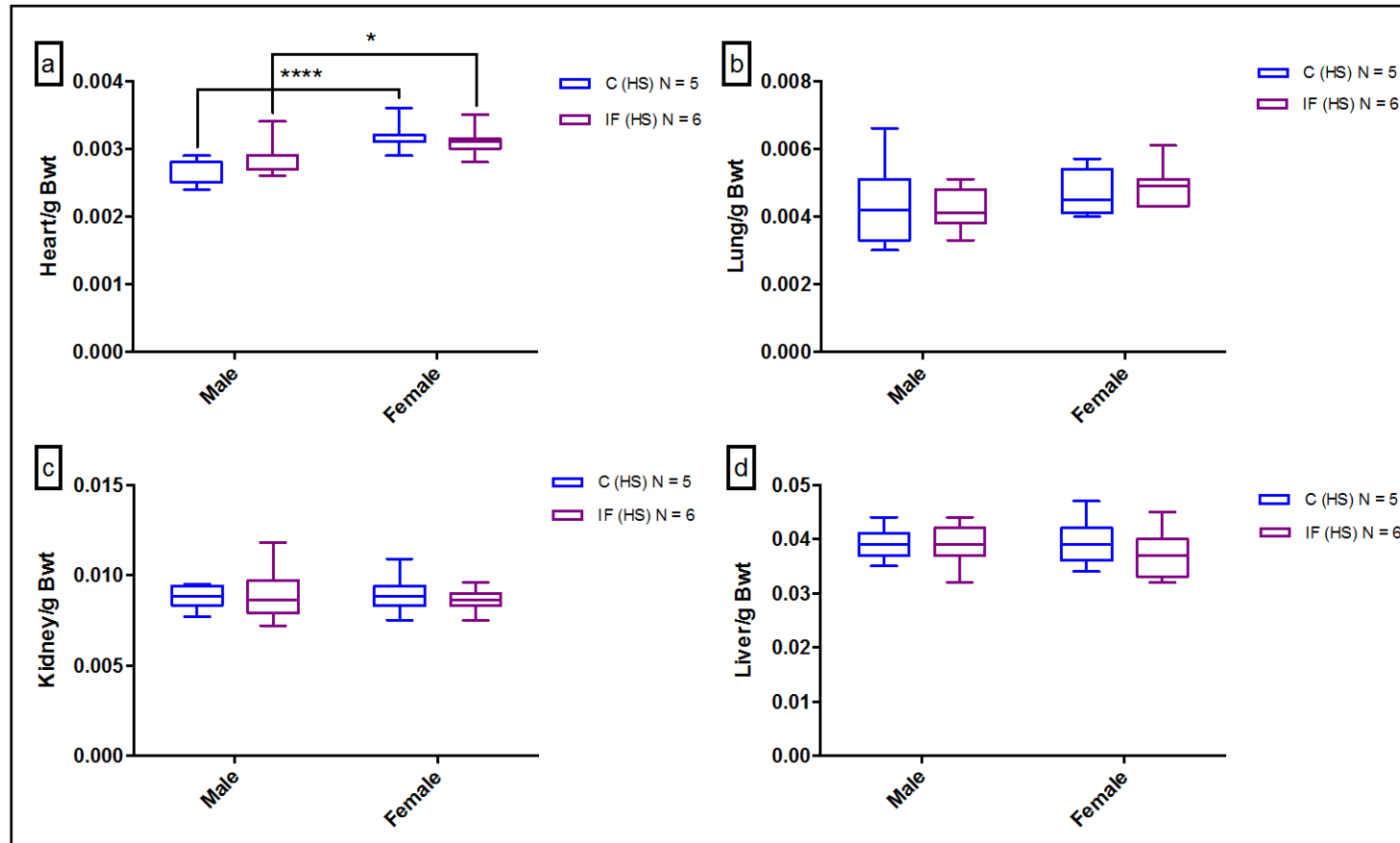
Figure 4.3.17 Terminal organ weights relative to body weight (Bwt) of HS offspring at 14 weeks of age. Organs weighed were the **a.** heart, **b.** lungs, **c.** kidneys and **d.** liver. For both sexes, relative organ weights were similar between salt-loaded IF (purple) and control (blue) groups. Females in both groups had significantly heavier relative heart weights compared to male littermates. Data were analysed as mean/litter and expressed as box and whisker plots. * P < 0.05 IF females versus IF males, **** P < 0.0001 control females versus control males. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
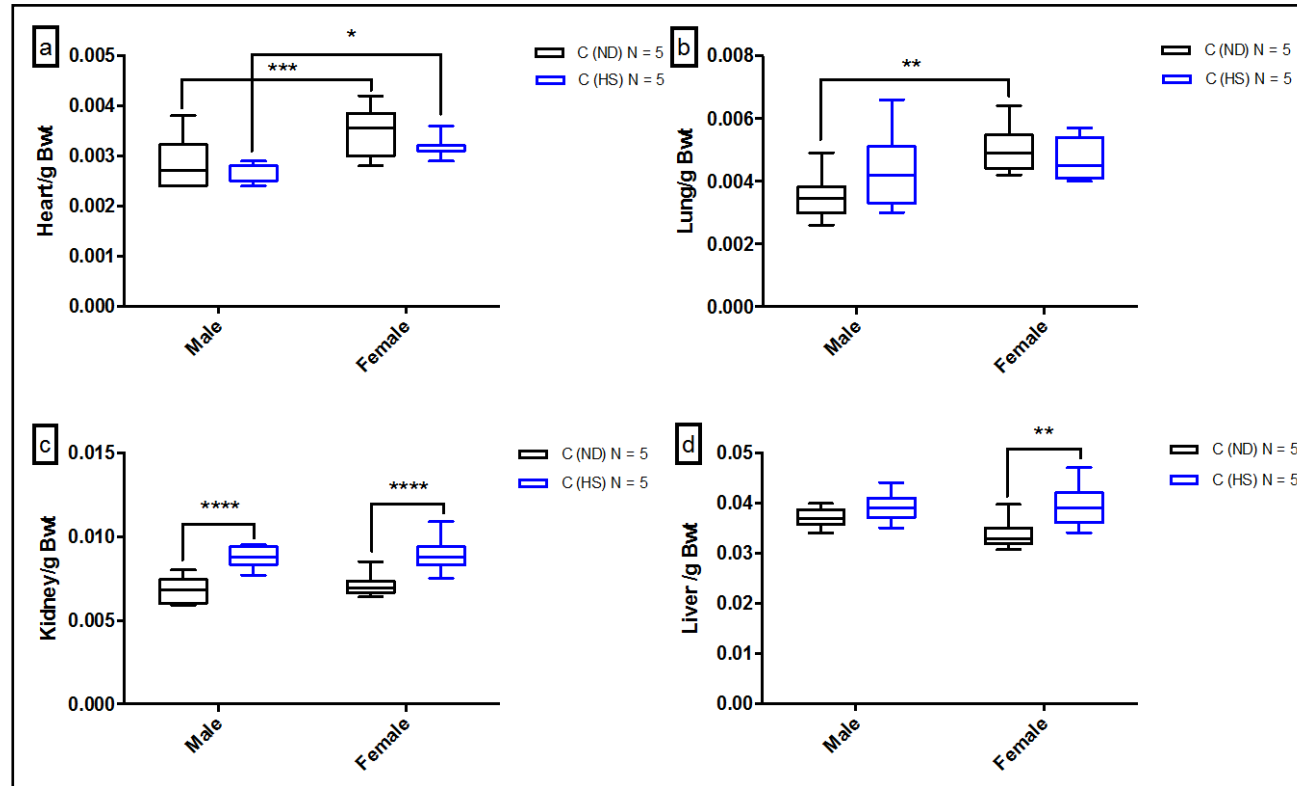
Figure 4.3.18 Terminal organ weights relative to bodyweight (Bwt) of HS control offspring (blue) and ND control offspring (black) at 14 weeks of age. Organs weighed were the **a.** heart, **b.** lungs, **c.** kidneys and **d.** liver. Relative kidney weights were significantly heavier in HS offspring compared to ND offspring (**** $P < 0.0001$). HS females had significantly heavier relative liver weights compared to ND females (** $P < 0.01$). Females in both dietary groups had heavier relative heart weights compared to male couterparts (* $P_{HS} < 0.05$, ** $P_{ND} < 0.01$). ND females had significantly heavier relative lung weight compared to ND males (** $P < 0.01$). For both sexes, relative heart and lung weights did not differ between dietary groups ($P > 0.05$). Data were analysed as mean/litter and expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
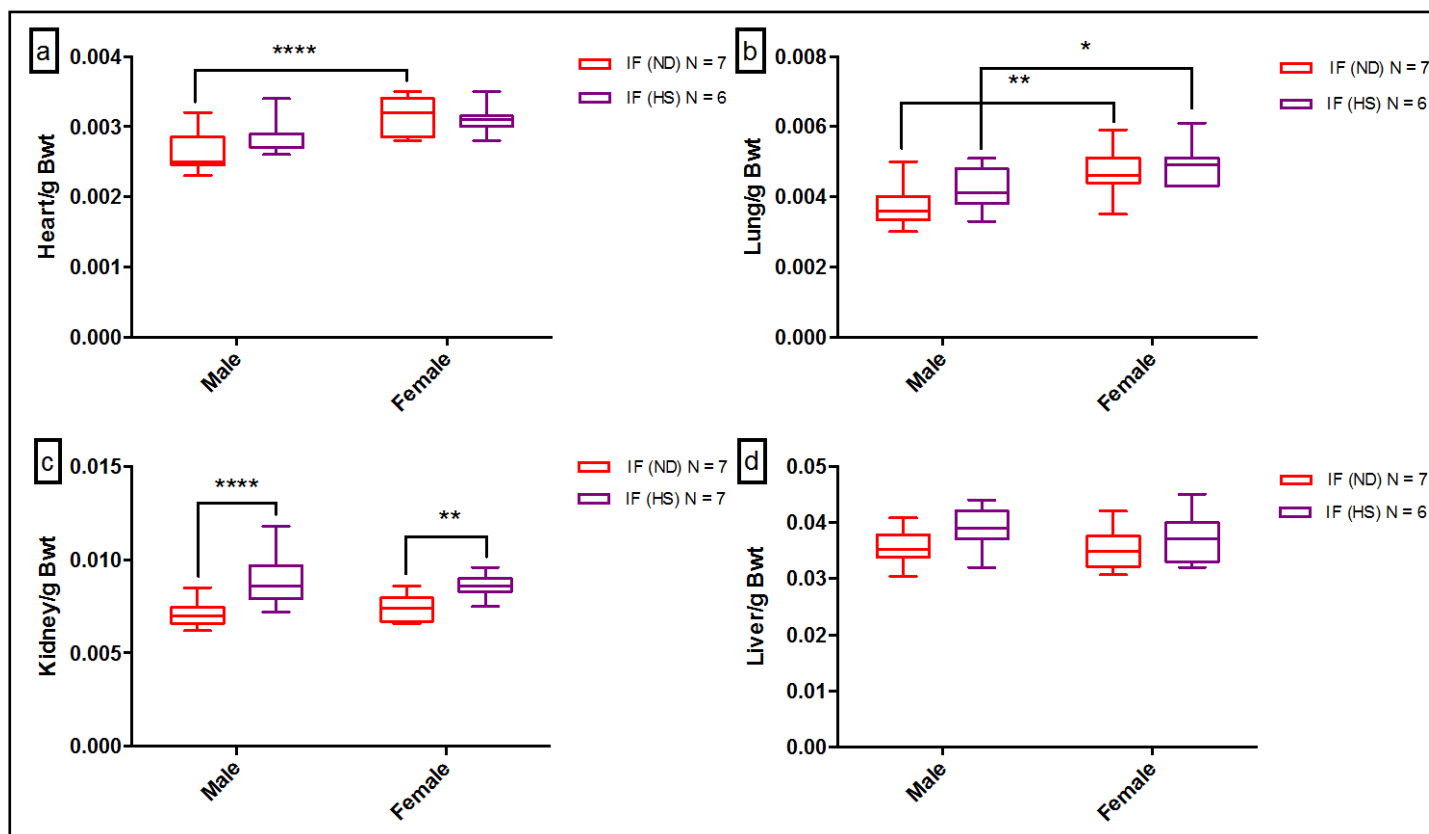
Figure 4.3.19 Terminal organ weights relative to bodyweight (Bwt) of HS IF offspring (purple) and ND IF offspring (red). Organs weighed were the **a.** heart, **b.** lungs, **c.** kidneys and **d.** liver. For both sexes, relative organ weights (heart, lung and liver) were similar between dietary groups (P > 0.05). Females in both groups had significantly heavier relative lung weights compared to male littermates. Relative kidney weights were significantly heavier in HS offspring compared to ND offspring (** P_{Male} < 0.01, **** P_{Female} < 0.0001). ND females had significantly heavier relative heart weights compared to ND males (**** P < 0.0001). Data were analysed as mean/litter and expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

4.3.9 Blood pressure in anaesthetised rats fed high-salt diet

During the renal clearance experiment, mean arterial pressure (MAP) was measured in anaesthetised rats. MAP remained relatively constant in both control and IF salt-loaded groups across the 3 h post-equilibration period ($P_{time} > 0.05$, Figure 4.3.20 a). The high-salt diet did not significantly affect MAP in any dietary group or between sexes of each group ($P > 0.05$, Figure 4.3.20 b).

4.3.10 Effect of high-salt diet on renal function in anaesthetised rats

Renal clearance studies were performed to determine the effect of a high-salt diet on kidney function in offspring at 14 weeks of age. Urine and plasma samples were collected during post-equilibration periods, after which all renal parameters were standardised to 100 g bodyweight.

4.3.10.1 Plasma electrolytes

Plasma concentrations of sodium, potassium, chloride and protein were not significantly different between salt-loaded IF and control groups or between sexes ($P > 0.05$, Table 4.3.2). Similarly, osmolality was similar between IF and control groups as well as between sexes ($P > 0.05$, Table 4.3.2).

The haematocrit of arterial blood samples did not differ between salt-loaded control and IF offspring ($P > 0.05$, Figure 4.3.21 a). However, in comparison to the haematocrit measured in offspring fed a standard chow diet (Chapter 3), dietary salt intake had an effect in that both sexes of the control group and females of the IF group had a significantly higher haematocrits than did their standard diet counterparts ($P_{Control} < 0.0001$, $P_{IFF} < 0.05$, Figures 4.3.21 b and c).
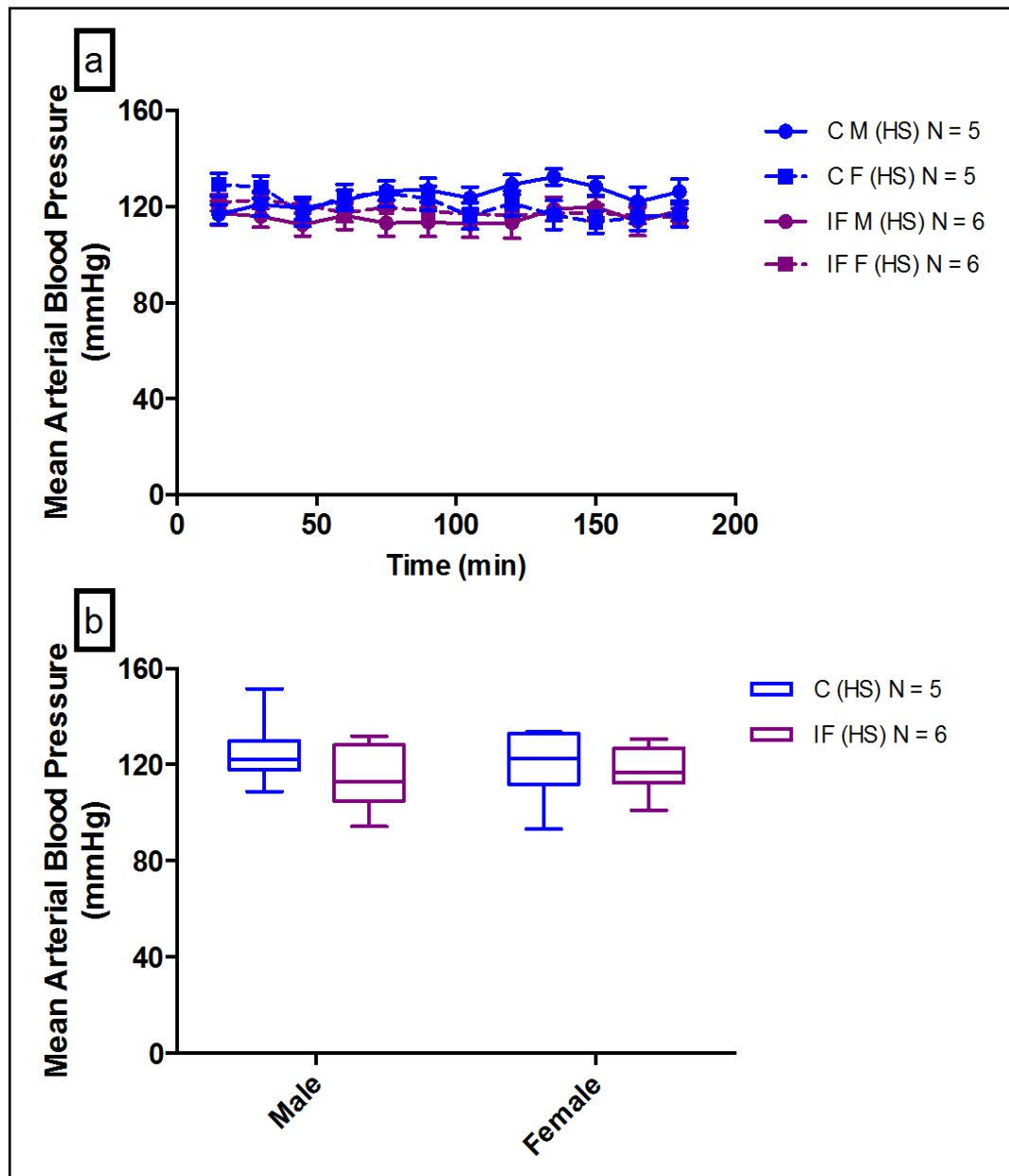
Figure 4.3.20 Mean arterial blood pressure (MAP) in 14-week-old anaesthetised offspring fed a high-salt diet (HS). **a.** Measurements taken over 15 min periods during the post-equilibration phase of the renal experiments. **b.** Overall mean of the 3 h experimental period. **a.** MAP was steady over 3 h experimental period. Data are presented as mean ± SEM. P > 0.05 IF vs control group (repeated-measures two-way ANOVA followed by Tukey's post hoc test). **b.** MAP was similar among dietary groups and sexes. The data are expressed as box and whisker plots. P > 0.05 IF versus control group. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

Table 4.3.2 Plasma electrolytes, osmolality and protein concentration measured in anaesthetised offspring fed a high-salt diet at 14 weeks of age

	Control (HS) N = 5		IF (HS) N = 6	
	Male	Female	Male	Female
Na$^+$ (mmol/L)	146 ± 4	147 ± 3	142 ± 6	146 ± 2
K$^+$ (mmol/L)	3.4 ± 0.2	2.9 ± 0.2	3.5 ± 0.2	3.2 ± 0.1
Cl$^-$ (mmol/L)	107 ± 1	106 ± 2	106 ± 1	105 ± 1
Osmolality (mOsm/kg H$_2$O)	328 ± 16	335 ± 13	334 ± 15	327 ± 11
Protein (g/100 mL)	4.34 ± 0.20	4.42 ± 0.24	3.82 ± 0.18	3.84 ± 0.19

Values are mean ± SEM with $P > 0.05$ IF versus control. Kruskal-Wallis test followed by Dunn's multiple comparisons test were used as the data were not normally distributed.
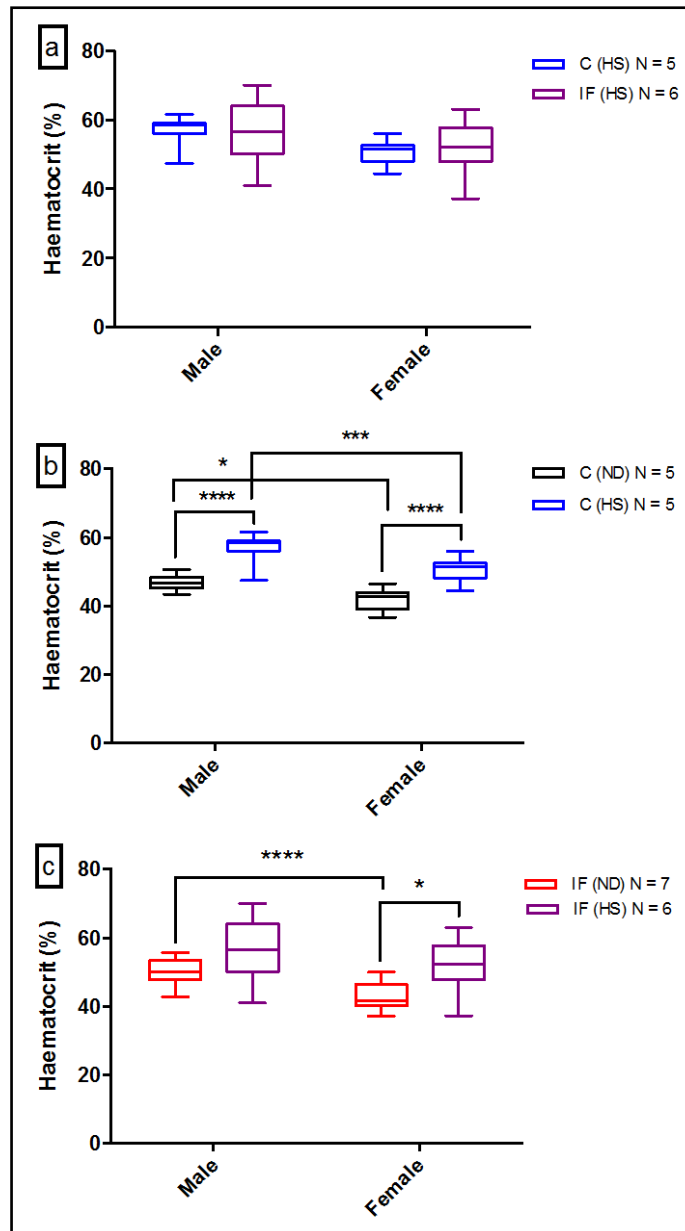
Figure 4.3.21 Haematocrit (%) measured in 14-week-old anaesthetised offspring fed a high-salt diet (HS) or standard chow diet (ND). **a.** Haematocrit was similar between the two dietary groups and sexes fed a high-salt diet (P > 0.05). **b.** Salt-loaded control offspring (blue) had a significantly higher haematocrit compared to their matched-chow diet counterparts (Black, **** P < 0.0001) with males in both dietary groups having a significantly higher haematocrit compared to their female littermates (* P_{ND} < 0.05, *** P_{HS} < 0.001). **c.** Salt-loaded IF females (purple) showed higher haematocrit compared to females on a standard chow diet (red, * P < 0.05) with IF males on standard chow having a significantly higher haematocrit compared to their female littermates (*** P < 0.0001). Data were analysed as mean/litter and are expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

4.3.10.2 Renal haemodynamics

Effective renal blood flow (ERBF) and glomerular filtration rate (GFR) were stable throughout the experiment (P_{time} > 0.05, data are not shown). The high-salt diet had a sex-specific impact on renal haemodynamics among IF offspring in that GFR tended to be lower in IF male offspring; however, this reduction failed to reach a statistically significant level (P = 0.07, Figure 4.3.22 b). Filtration fraction (FF) revealed a significant effect of salt intake in that IF male offspring had a marked decrease in FF (P < 0.05, Figure 4.3.22 c). ERBF, on other hand, was similar between the two dietary groups and sexes (P > 0.05, Figure 4.3.22 a). A comparison of these data to those presented in Chapter 3 reveals that high-salt intake significantly increased FF in the control group (P_{Male} < 0.001, P_{Female} < 0.01, Figure 4.3.23 c). GFR was also significantly greater in salt-loaded males of the control group (P < 0.05, Figure 4.3.23 a). While females showed an increase in GFR, this failed to reach a statistically significant level (P = 0.06, Figure 4.3.23 a). In contrast, a high-salt diet failed to induce any increase in renal parameters in IF offspring compared to their matched-chow diet (P > 0.05, Figures 4.3.23 b and d).

4.3.10.3 Arteriole resistance

Afferent and efferent arteriole resistances were also determined in this study. Arteriole resistance was not affected by a high-salt diet, and afferent and efferent arteriole resistances did not differ between IF and control groups or between sexes (P > 0.05, Figures 4.3.24 a and b).

4.3.10.4 Urine electrolytes

The data in Table 4.3.3 present the effects of a high-salt diet on urine electrolyte concentrations (sodium, potassium and chloride) and on urine anion gap. All urine electrolyte concentrations were comparable between salt-loaded IF and control groups (P > 0.05, Table 4.3.3). In terms of Na^+ urine concentration, sex divergence was evident in that females of both control and IF groups had significantly higher Na^+ concentrations than did their male littermates (P < 0.05, Table 4.3.3). With regards to K^+ urine concentration, only IF female offspring showed a significantly lower K^+ concentration than IF males (P < 0.05, Table 4.3.3). Urine anion gap, calculated as $[Na^+]+[K^+]-[Cl^-]$, did not differ between IF and control groups or between sexes (P > 0.05, Table 4.3.3). In comparison to the data presented in Chapter 3, a high-salt intake significantly increased Na^+ urine concentration by almost two-fold in both control and IF groups irrespective of sex (P < 0.001, Figures 4.3.25 a and b).
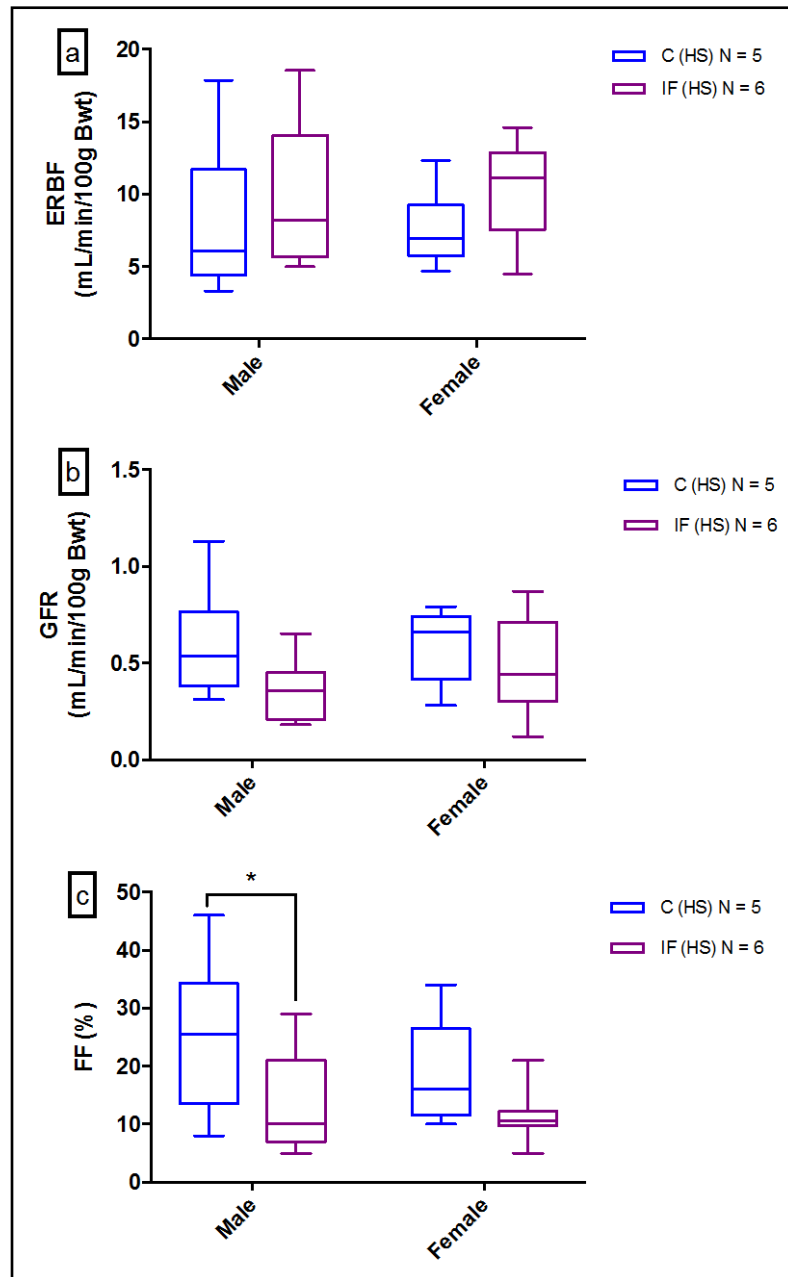
Figure 4.3.22 Renal haemodynamics in 14-week-old anaesthetised offspring fed a high-salt diet (HS). **a.** Effective renal blood flow (ERBF) **b.** glomerular filtration rate (GFR) and **c.** filtration fraction (FF) determined in anaesthetised HS offspring at 14 weeks of age. ERBF did not differ between control and IF offspring ($P > 0.05$). IF males showed a trend towards a lower GFR compared to control males ($P = 0.07$). FF was significantly reduced in IF male on HS diet compared to control counterparts (* $P < 0.05$). Data are expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
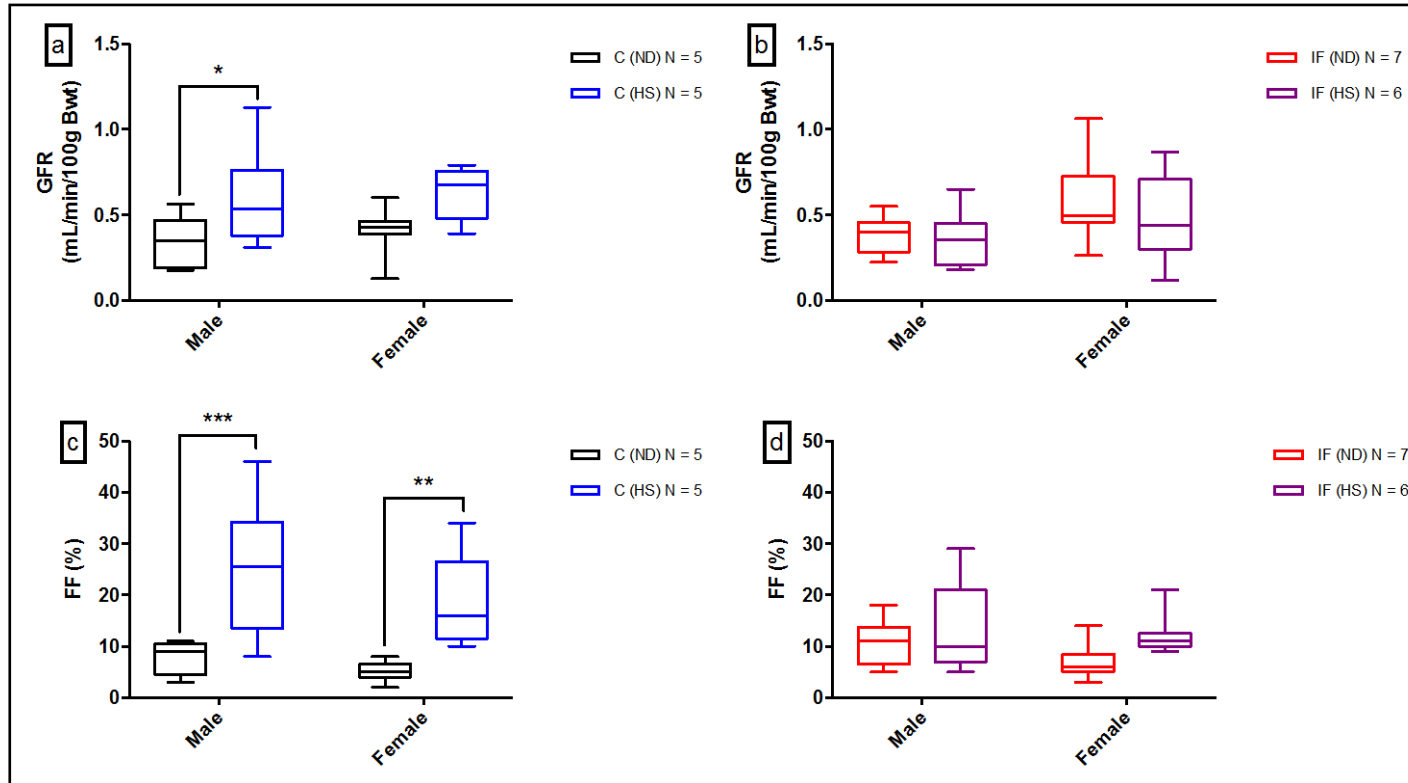
Figure 4.3.23 Renal haemodynamics in 14-week-old anaesthetised offspring fed a high-salt diet (HS) or standard chow (ND). **a. and b.** Glomerular filtration rate (GFR) and **c. and d.** filtration fraction (FF) determined in anaesthetised HS and ND offspring at 14 weeks of age. GFR and FF did not differ between HS and ND IF offspring (P > 0.05). A high-salt diet significantly increased FF in both sexes of the control group (*** $P_{Male} < 0.001$, ** $P_{Female} < 0.01$). GFR was significantly increased in HS males (* P < 0.05) but failed to reach a significant difference in females of the control group (P = 0.06). Data are expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
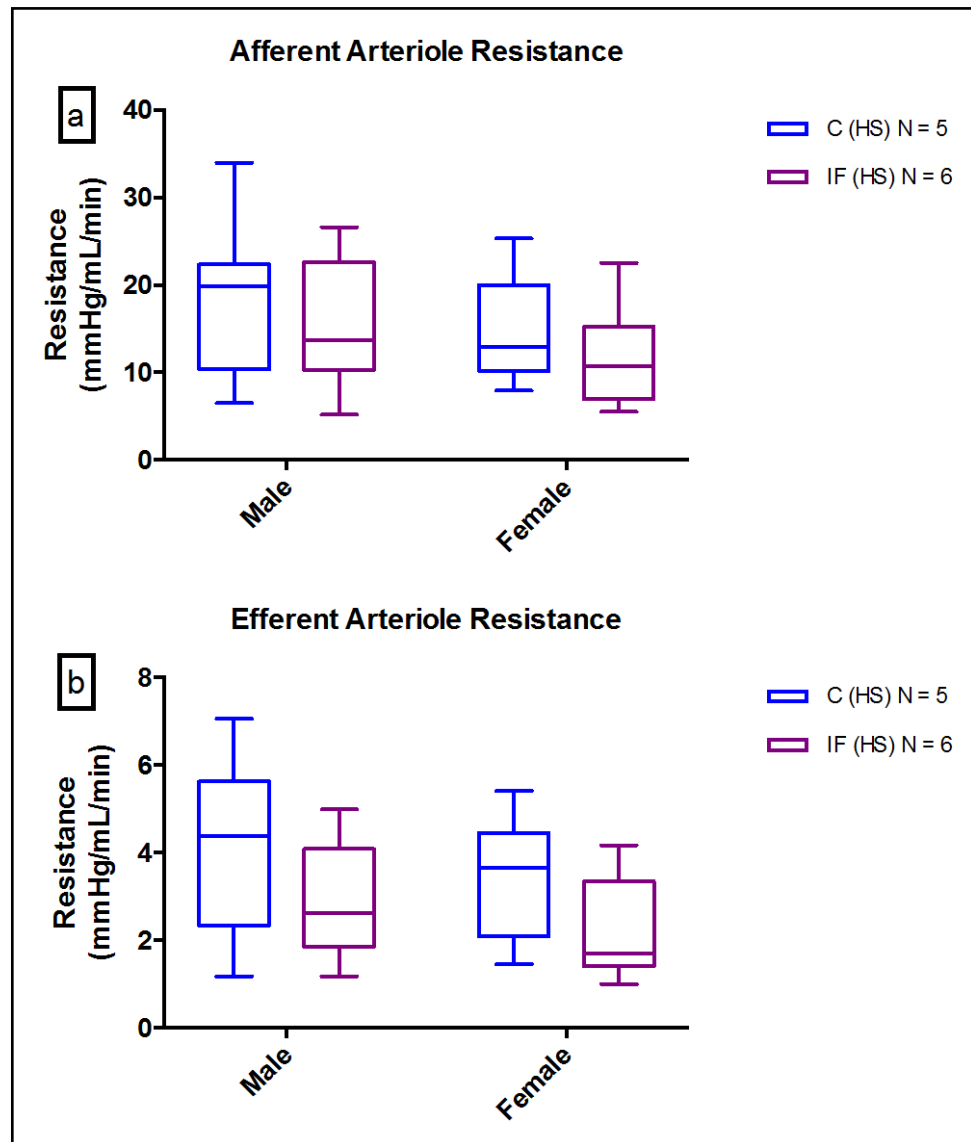
Figure 4.3.24 Afferent and efferent arteriole resistance in 14-week-old anaesthetised offspring fed a high-salt diet (HS). The effect of a high-salt diet on **a.** afferent and **b.** efferent arteriole resistance of offspring at 14 weeks of age. Afferent and efferent arteriole resistances were similar between dietary groups and sexes of each group. Data are expressed as box and whisker plots. $P > 0.05$ IF vs control group and sexes. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

Table 4.3.3 Urine electrolyte concentration, urine anion gap and osmolality measured in 14-week-old anaesthetised offspring fed a high-salt (HS) diet

	Control (HS) N = 5		IF (HS) N = 6	
	Male	Female	Male	Female
Na^+ (mmol/L)	178 ± 13	221 ± 6*	162 ± 11	203 ± 8*
K^+ (mmol/L)	75 ± 11	46 ± 5	87 ± 12	47 ± 2*
Cl^- (mmol/L)	178 ± 6	181 ± 6	154 ± 4	185 ± 9
Urine anion gap (mmol/L)	75 ± 7	85 ± 6	79 ± 9	69 ± 5
Osmolality (mOsm/kg H_2O)	737 ± 87	509 ± 27	831 ± 113	630 ± 73

Values are mean ± SEM with $P > 0.05$ IF versus control, * $P < 0.05$ female versus male littermates. (For K^+ and osmolality which were not normally distributed, Kruskal-Wallis test followed by Dunn's multiple comparisons test were used; for Na^+, Cl^- and urine anion gap which were normally distributed, two-way ANOVA followed by Tukey's post hoc test were used).
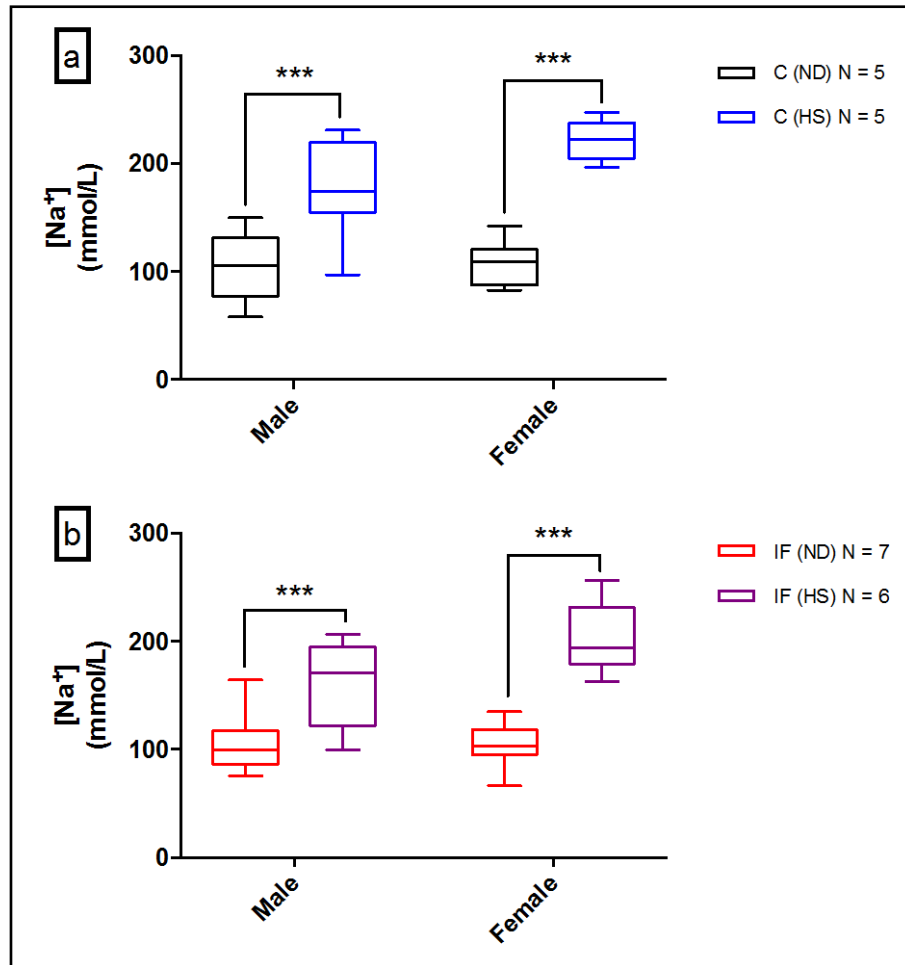
Figure 4.3.25 The effect of a high-salt diet on Na$^+$ urine concentration in 14-week-old anaesthetised offspring fed a high-salt diet (HS) or standard chow (ND). **a.** control and **b.** IF offspring at 14 weeks of age. Na$^+$ urine concentration was significantly increased in both IF and control groups fed a high-salt diet (*** P < 0.001). Data are expressed as box and whisker plots. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

4.3.10.5 Urine flow rate, osmolar excretion and free water clearance

Figure 4.3.26 a shows that urine flow rate remained fairly steady in both sexes of IF and control groups throughout the post-equilibration period of the experiment (P_{time} > 0.05). A high-salt diet did not affect overall urine flow rate in either the IF or the control group. However, sex divergence was observed in that females had a higher urine flow rate than males in both groups ($P_{Control}$ < 0.01, P_{IF} < 0.0001, Figure 4.3.26 b). This is consistent with the fixed infusion rate and lighter bodyweight of female offspring (Figure 4.3.12 a).

A high-salt diet had a sex-specific impact on the osmolar excretion of IF offspring, as osmolar excretion was significantly decreased only in IF male offspring compared to control males (P <

0.01, Figure 4.3.27 a). Females in both dietary groups had significantly higher osmolar excretion than their male littermates (P < 0.0001, Figure 4.3.27 a). In terms of free water clearance (C_{H2O}), the values were negative for IF and control groups, showing that the excreted urine was hyperosmotic to plasma. C_{H2O} was similar between IF and control groups and between sexes (P > 0.05, Figure 4.3.27 b).

4.3.10.6 Renal handling of electrolytes

To assess the effect of a high-salt diet on kidney handling of electrolytes, electrolyte excretion and fractional excretion of Na^+, Cl^- and K^+ were measured. A high-salt diet had no significant effect on renal handling of electrolytes. There were no differences in Na^+ (Figure 4.3.28 a), Cl^- (Figure 4.3.29 a), and K^+ (Figure 4.3.30 a) excretion rates between IF and control groups. However, IF male offspring showed a reduction in both Na^+ and Cl^- excretion that failed to reach the level of statistical significance (P = 0.06). The fractional excretion (FE) of these electrolytes was also comparable between IF and control groups (P > 0.05, Figures 4.3.28 b, 4.3.29 b and 4.3.30 b).

A sex difference emerged in that females in control and IF groups had significantly higher electrolyte excretion rates than their male counterparts (Figures 4.3.28 a, 4.3.29 a and 4.3.30 a). Likewise, fractional excretions of Na^+ and Cl^- was higher only in females of the IF group compared to their male counterparts (P_{Na} < 0.05, Figure 4.3.24 b; P_{Cl} < 0.001, Figure 4.3.25 b).
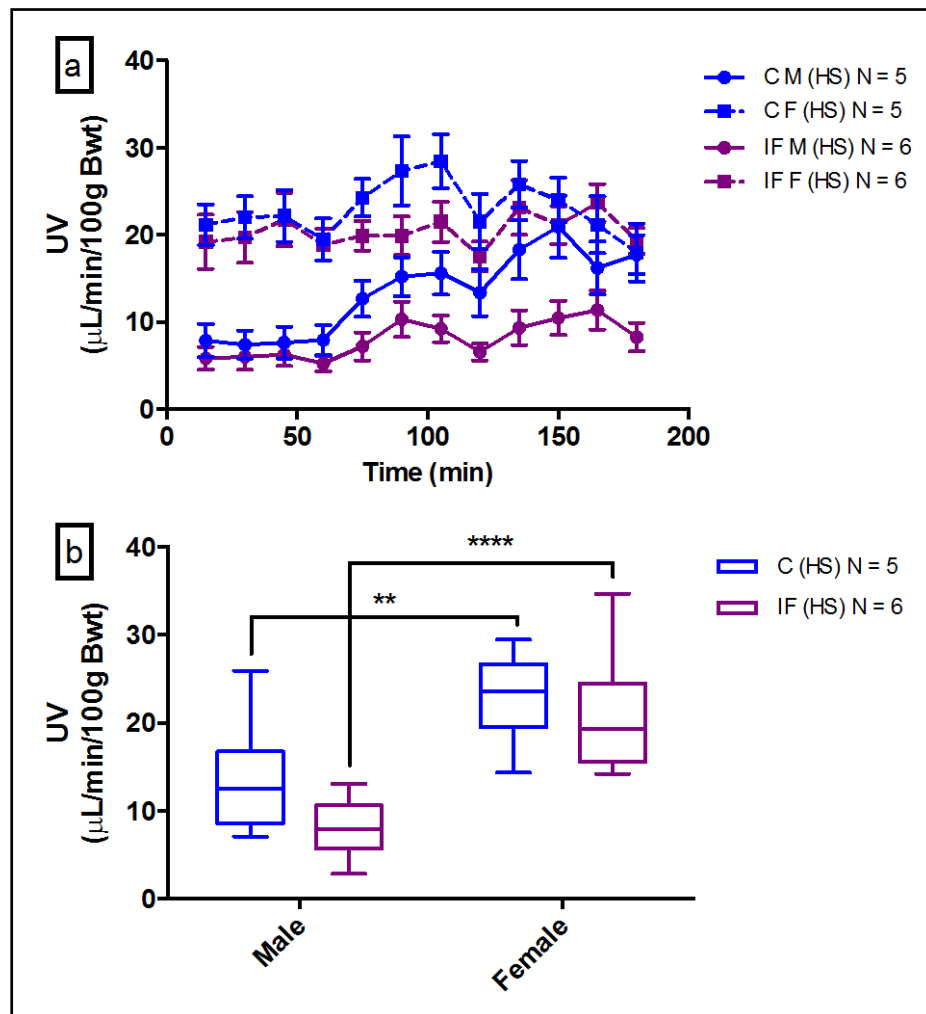
Figure 4.3.26 Urine flow rate (UV) in 14-week-old anaesthetised offspring fed a high-salt (HS) diet. **a.** Measurements taken over 15 min periods during post-equilibration phase of the renal experiments showed that UV was stable over the 3 h experimental period. Data are presented as mean ± SEM. P > 0.05 IF vs control group (repeated-measures two-way ANOVA followed by Tukey's post hoc test). **b.** Overall mean of 3 h experimental period. UV was similar between dietary groups, but females had higher UV than males in both groups. Data are expressed as box and whisker plots. P > 0.05 IF versus control group, ** $P_{Control} < 0.01$, **** $P_{IF} < 0.0001$ for male versus female comparisons. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
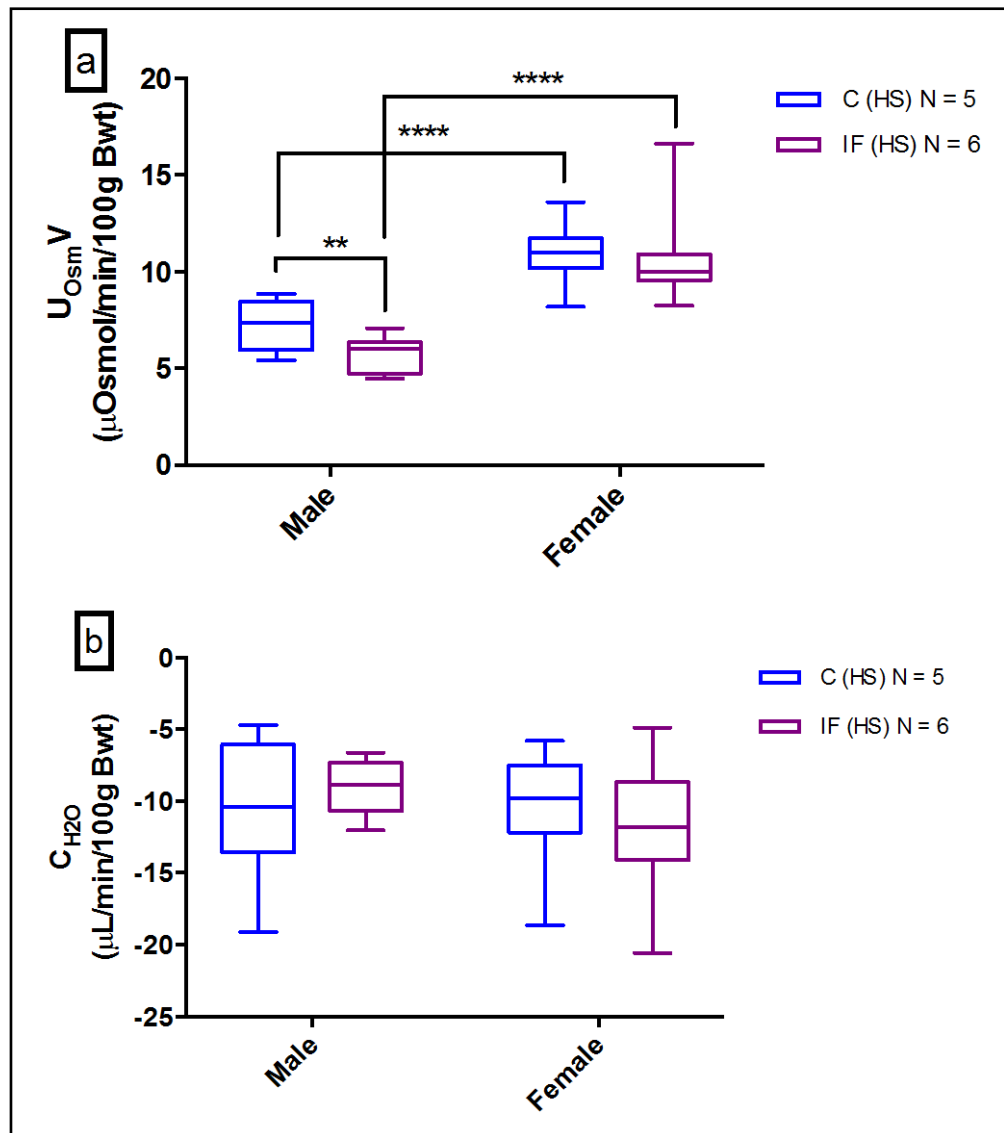
Figure 4.3.27 Osmolar excretion and free water clearance in 14 week-old anaesthetised offspring fed a high-salt (HS) diet. The effect of a high-salt diet on **a.** osmolar excretion and **b.** free water clearance of control and IF offspring at 14 weeks of age. Osmolar excretion was significantly lower in IF males compared to control males (** $P < 0.01$) with females having higher osmolar excretion than the males in both groups (**** $P < 0.0001$). Free water clearance (C_{H2O}) did not differ between the dietary groups or sexes. $P > 0.05$ dietary groups and sexes. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.
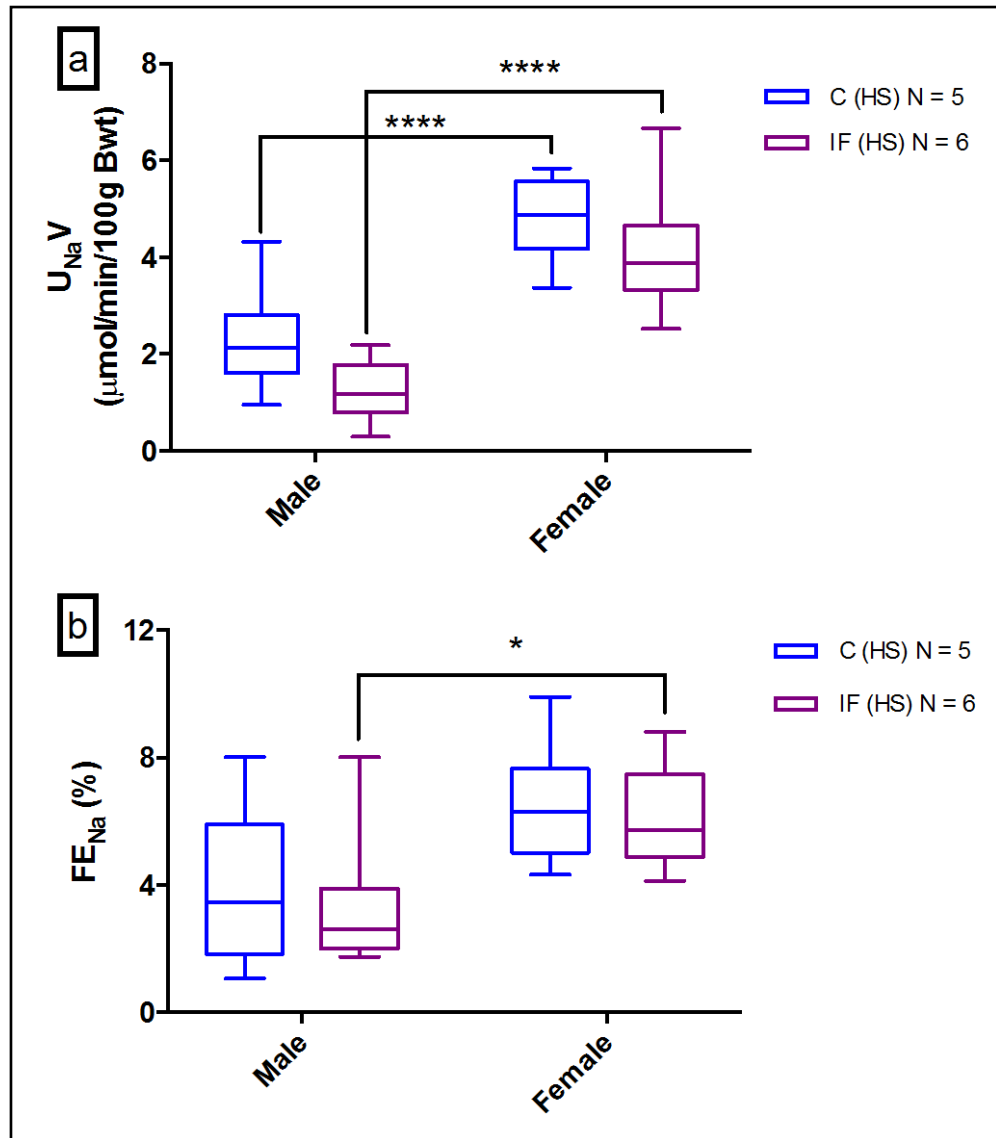
Figure 4.3.28 Sodium excretion and fractional excretion of sodium in 14 week-old anaesthetised offspring fed a high-salt diet (HS). The effect of a high-salt diet on **a.** sodium excretion and **b.** fractional excretion of sodium of control and IF offspring at 14 weeks of age. Sodium excretion and fractional excretion were similar between dietary groups (P > 0.05). Females in both dietary groups had higher sodium excretion than their males (**** P < 0.0001), yet only IF females showed higher FE_{Na} than males (* P < 0.05). Data are expressed as box and whisker plots. For sodium excretion, two-way ANOVA followed by Tukey's post hoc test was used and Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to analyse fractional excretion of sodium.
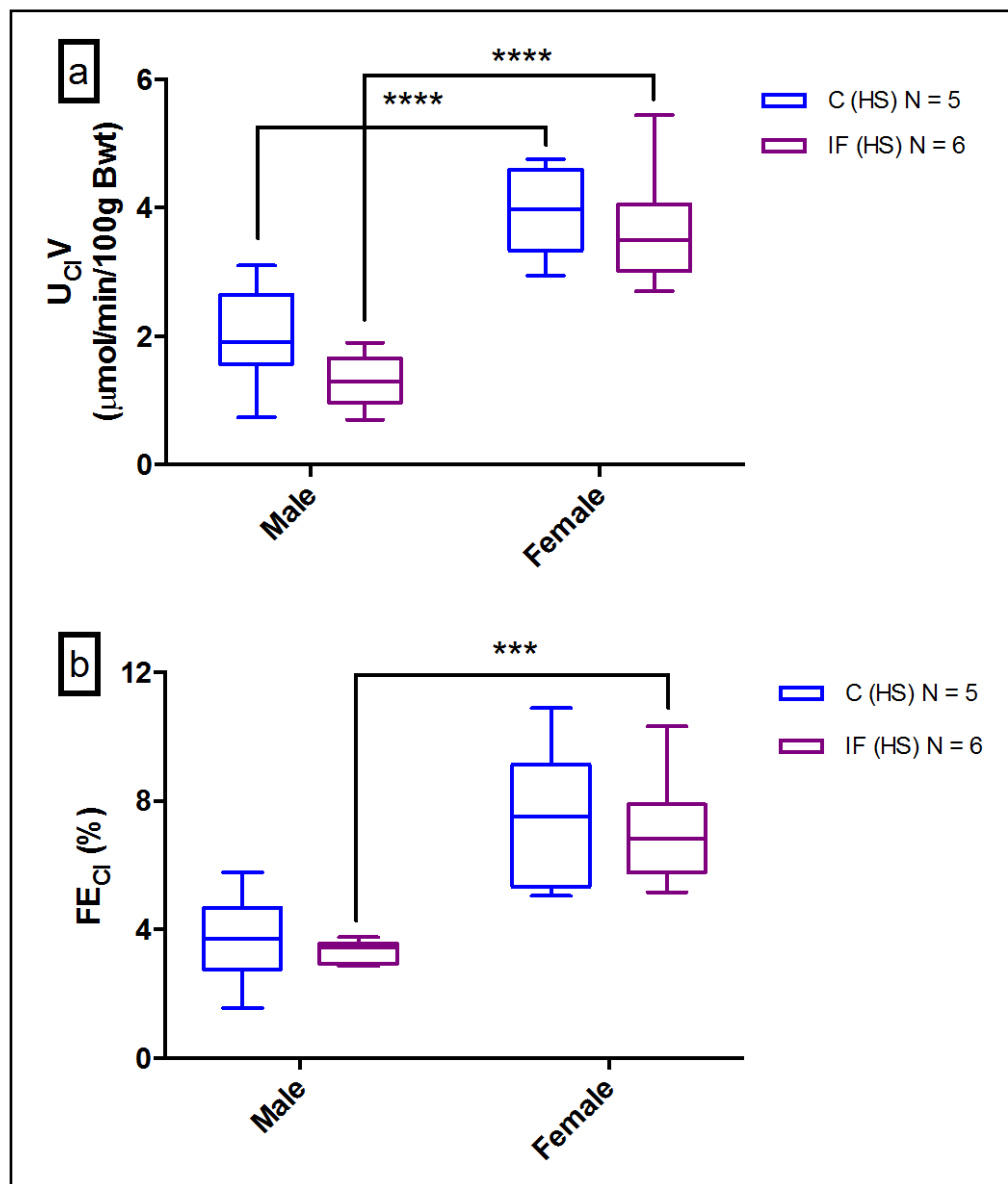
Figure 4.3.29 Chloride excretion and fractional excretion of chloride in 14 week-old anaesthetised offspring fed a high-salt diet (HS). The effect of a high-salt diet on **a.** chloride excretion and **b.** fractional excretion of chloride of control and IF offspring at 14 weeks of age. Choride excretion and fractional excretion were similar between dietary groups (P > 0.05). Females in both dietary groups had higher chloride excretion than their males (**** P < 0.0001), yet only IF females showed higher FE_{Cl} than males (*** P < 0.001). Data are expressed as box and whisker plots. For chloride excretion, two-way ANOVA followed by Tukey's post hoc test was used and Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to analyse fractional excretion of chloride.
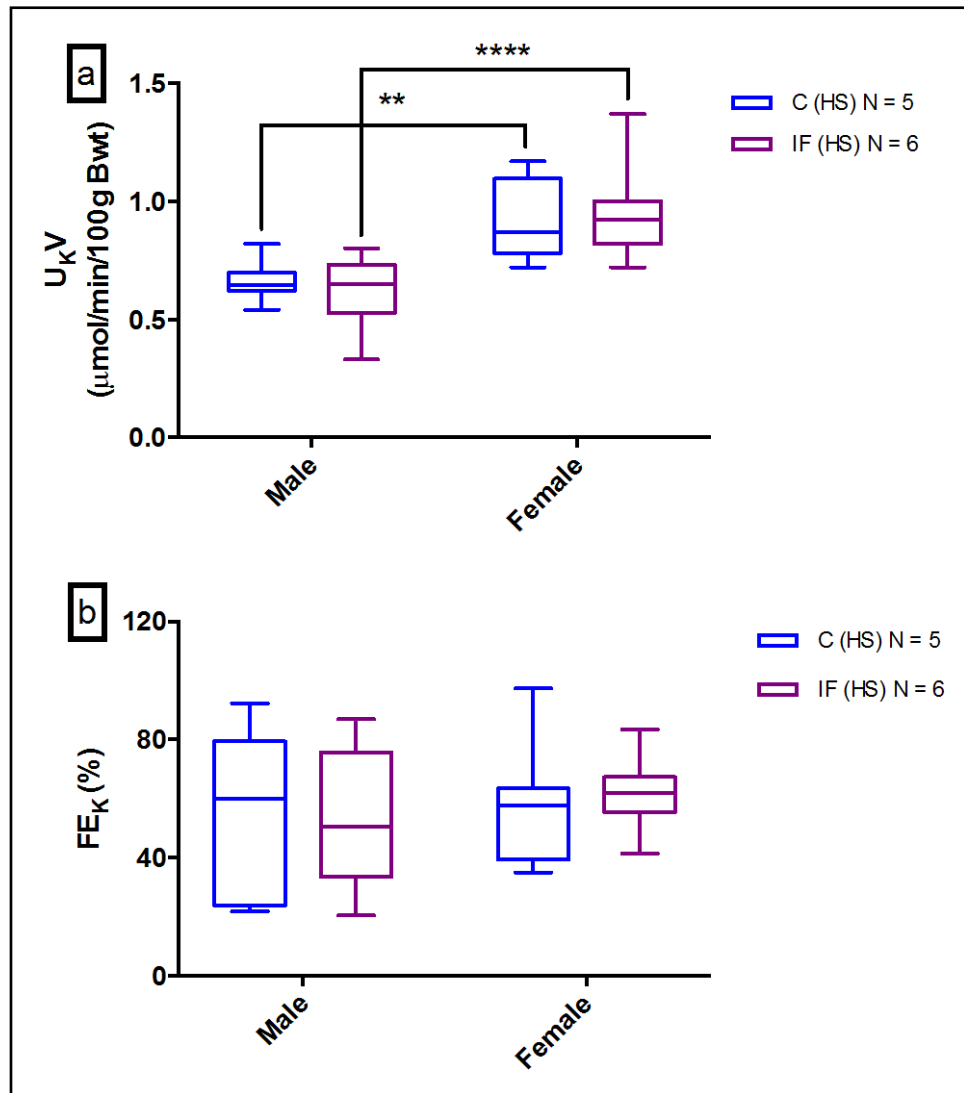
Figure 4.3.30 Potassium excretion and fractional excretion of potassium in 14 week-old anaesthetised offspring fed a high-salt diet (HS). The effect of a high-salt diet on **a.** potassium excretion and **b.** fractional excretion of potassium of control and IF offspring at 14 weeks of age. Potassium excretion and fractional excretion were similar between the dietary groups. Females in both dietary groups had higher potassium excretion than their male counterparts but with comparable FE_K between sexes. Data are expressed as box and whisker plots. P > 0.05 IF versus control group, ** $P_{Control} < 0.01$, **** $P_{IF} < 0.0001$ females versus male counterparts. Statistical analysis was carried out using a two-way ANOVA followed by Tukey's post hoc test.

4.4 DISCUSSION

Mismatches between prenatal and postnatal environments and growth patterns during early life have been linked to the development of cardiovascular and metabolic disease later in life (Fall et al., 1995; Curhan et al., 1996; Eriksson et al., 2000b; Gillman, 2005). Prenatal growth with or without low birth weight followed by accelerated catch-up growth is associated with subsequent development of obesity and increased other disease risk. The previous chapter provided insights into the impact of maternal intermittent fasting *in utero* on pre- and post-growth trajectories of IF offspring and neonatal organ weights, particularly with respect to the kidney. However, maternal dietary insult failed to programme hypertension and metabolic disorder or to alter renal function in adult offspring. This chapter therefore examined (1) the effect of prenatal intermittent fasting on post-weaning offspring feeding behaviour, water intake and pattern of body growth; (2) whether prenatal intermittent fasting can programme salt preference in adult offspring; (3) how maternal intermittent fasting over gestation influences postnatal consumption of a high-salt diet and affects renal function and blood pressure in adult offspring; and (4) if sex differences were evident with respect to the measured outcomes.

The results in this chapter clearly showed that prenatal exposure to maternal intermittent fasting did elicit changes in feeding behaviour in terms of a standard chow diet and a high-salt diet. At postnatal weeks 5 and 6, males of the IF group consumed less food (normalised to bodyweight) when provided with standard chow and a high-salt diet than did males of the control group. At 7 weeks of age, males of the IF group showed hyperphagia for a high-salt diet when compared to the IF females which normalised afterwards, whereas females of the IF group consumed more high-salt food than IF males at 11 and 12 weeks of age. Both control and IF offspring showed polydipsia resulting from a high-salt intake compared to a standard chow diet. High-salt intake and polydipsia had differing effects on body growth in the IF group; salt-loaded males were heavier while females were lighter from week 8 onward than their counterparts on standard chow diet. As expected, a high-salt intake raised blood pressure in both dietary groups at weeks 7 and 10, with no differences emerging between control and IF groups. Heart rate, on the other hand, was not affected by salt loading.

Salt preference tests confirmed the innate instinct for rats to drink more 0.9% saline than water, with similar fluid intake between IF and control groups. Females, however, had a greater salt preference and fluid intake than males in both control and IF groups. With an increase in the salt content of the saline solution, IF females showed a higher aversion threshold than did control females, whereas control and IF males displayed similar aversion responses. A high-salt diet induced hypertrophy in control and IF offspring kidneys. As a consequence, the renal function of IF male offspring was susceptible to high-salt insult. IF males had significantly lower FF and osmolar excretion, with a tendency towards lower GFR, Na^+ and Cl^- excretions that failed to reach the level of statistical significance. Urine creatinine concentration, on the other

hand, was significantly lower in salt-loaded male and female IF offspring. NGAL concentration, an early marker of kidney injury, was significantly elevated in salt-loaded IF male offspring. Together these data suggest that salt loading induced mild kidney injury in IF rats.

4.4.1 The effect of altered maternal dietary intake and a postnatal high-salt diet on feeding behaviours and patterns of body growth in control and IF offspring

The current study demonstrates that maternal intermittent fasting over pregnancy had an effect on appetite and feeding behaviour. Post-weaning intake of either a standard chow diet or a high-salt diet was equivalent between IF and control groups, and a constant amount of food was ingested throughout the post-weaning period. Male offspring consumed more food than females. However, when food intake was normalised to bodyweight, sex differences were no longer evident. The only difference observed was that IF male offspring had significantly lower intakes of both the standard chow diet and the high-salt diet compared to males in the control group in the first two weeks following weaning. After this drop in food intake, males of the experimental group showed signs of hyperphagia by consuming more high-salt diet at week 7 postnatally compared to their females of the IF group, which disappeared afterwards, whereas IF females started to consume more of the high-salt diet than IF males at 11 weeks of age and onward. This suggests that intermittent fasting may programme salt appetite in a sex-specific manner that manifests in young adulthood.

In agreement with the data outlined in this Chapter, previous studies have shown changes in appetite and feeding behaviours as a consequence of nutritional disturbance *in utero*. Vickers et al. (2000) have reported that severely calorific-restricted offspring of both sexes that are growth retarded at birth grow up to be hyperphagic when eating standard chow or when eating a hypercalorific diet of a 30% fat content. Although the mechanisms involved are not clear, the authors have suggested that insulin and leptin resistances in calorific-restricted offspring lead to neuroendocrine regulation impairment and thus stimulate appetite.

Bellinger et al. (2004) have shown that when fed a standard chow diet at 12 weeks of age, control females eat more than males. However, female offspring exposed to LP throughout pregnancy have a reduced food intake compared to control females, whereas LP males consume a similar amount of food to that of control males. Alwasel et al. (2012) have observed the opposite, with LP offspring at 4 weeks of age exhibiting hyperphagia in order to increase their salt intake to compensate for renal sodium loss. This could be a partial explanation for the similarities seen in females of both the control and the IF groups in terms of food intake, and the lower food consumption by male of the IF group compared to the males of the control group during the first two weeks post-weaning, as IF offspring fed a standard chow diet in this study did not show any alteration in renal handling of electrolytes (see Section 3.3.11.6). However, the difference in outcomes between the studies of Bellinger et al. (2004) and Alwasel et al.

(2012) can be attributed to the age of the offspring at which food intake was recorded, something which was also observed in this study: males and females of the IF group showed different patterns of feeding behaviour at different ages. Although it is unclear whether age can influence prenatal programming of appetite, a long-term study by Orozco-Sólis et al. (2009) has shown that hyperphagia does not persist beyond postnatal day 60 (~8 weeks) in LP offspring.

A prenatal undernutrition environment, aside from altering postnatal food intake, also induces a selective appetite for specific diet contents. When rats were offered a self-select diet of high fat, high carbohydrates and high protein at 12 weeks of age, LP offspring had an increased preference for fat, with females who were hypophagic when fed a standard chow diet becoming hyperphagic (Bellinger et al., 2004). Interestingly, this preference for fatty food among LP rats at 12 weeks of age is absent at 30 weeks of age (Bellinger et al., 2004), suggesting that fat preference is age-dependent.

In the current study, control and IF offspring showed similar preferences for a high-salt diet. However, IF males showed less preference for a salt diet during the first two weeks post-weaning compared to the males of the control group which disappeared afterwards, whereas IF females exhibited a sudden change in salt appetite at 11 and 12 weeks of age, consuming more of the high-salt diet than did IF males. This suggests that programming of feeding behaviour can be sex- and age-specific. Furthermore, Bellinger and Langley-Evans (2005), who generated groups of offspring from dams subjected to a LP diet at early, mid and late gestational stages so as to explore different effects on appetite among those offspring, found that females of all LP-exposed groups had less of an appetite for fat than did LP males and controls.

Using the same prenatal protein restriction protocol, Bellinger et al. (2006) measured food intake in offspring maintained on a standard chow diet at 18 months of age. Males exposed to LP at mid-pregnancy showed a greater food intake, whereas females for which LP was imposed at late or throughout pregnancy displayed hypophagia. Therefore, the timing of the nutrient insult *in utero* is a crucial element in determining feeding behaviour. Moreover, in two studies assessing food preference in response to task-food rewards in rats exposed to a 50% food restriction *in utero*, Alves et al. (2015) reported that food-restricted males showed a higher preference for sweet food than did controls at 90 days of age. Dalle Molle et al. (2015) have likewise demonstrated that food-restricted adult offspring have a higher preference than controls for palatable food that is rich in fat and sugar.

Also noteworthy is that in the current study, food intake relative to bodyweight declined with advancing age, in agreement with previous studies (Bellinger et al., 2004; Coupé et al., 2009). Food and water intake are linked closely (Ellacott et al., 2010), so it would be anticipated that as food consumption (normalised to bodyweight) decreased with age, this would be paralleled

by a similar trend in water intake. In rats fed a standard chow diet, water intake was similar between control and IF groups except at week 6, during which control males consumed more water than IF males and control females. There was a substantial increase in water intake by control and IF offspring when a high-salt diet was introduced. This polydipsia was significantly lower in males of the IF group compared with males of the control group at week 6 in parallel to lower high-salt intake of IF males during this week. These results are consistent with those of Stricker et al. (2003), who showed that an 8% high-salt diet stimulated thirst rapidly, after which the volume of water intake was proportional to food intake. Similarly, Sanders et al. (2005) showed that when offspring of dams that underwent bilateral uterine ligation during pregnancy were given high-salt (2% NaCl) drinking fluid at 12 weeks of age, their water intake increased significantly to a level similar to that of control offspring.

These results align with reports that visceral Na^+ osmoreceptors detect osmolytes in the gastrointestinal tract and stimulate thirst prior to any significant increase in systemic plasma osmolality (Morita et al., 1997; Striker et al., 2002). Alwasel et al. (2012) have also observed polydipsia in LP offspring at four weeks of age as a result of increased food intake. Zhang et al. (2011) have observed that water restriction in pregnant dams over the last three days of gestation results in greater thirst in male offspring due to an increase in Ang II type 1 (AT_1) and 2 (AT_2) receptors along with the expression of intracerebroventricular (ICV) angiotensin-II and angiotensinogen in the forebrain. The latter study highlights that combining food and water restriction in the intermittent fasting model could programme thirst in IF offspring.

With regard to the effect of a high-salt diet on growth rates, IF males showed accelerated growth between postnatal weeks 8 and 9, after which their growth rates slowed to match those of control males. IF females had growth rates similar to those of control females until postnatal week 10, then there was an unexpected slowing of growth which predominated. Moreover, IF offspring fed a high-salt diet showed different growth trajectories compared to IF offspring fed a standard chow diet. Salt-loaded IF males exhibited accelerated growth from week 8, with a subsequent sudden shift in body growth after week 12 to end up with lighter bodyweights than standard chow-fed IF males at week 14. The opposite scenario was seen with salt-loaded IF females, which started with a lighter weight from week 8 to week 12 and ended up with a similar weight to IF females on a standard chow diet at week 14. This suggests that IF males on a high-salt diet retained more sodium and water compared to salt-loaded IF females at that age. This was reflected by the magnitude of blood pressure which increased from week 5 until week 10 postnatally: IF males showed an increase of 16.5 mmHg compared to IF females which demonstrated an increase of 12.0 mmHg.

Manning and Vehaskari (2005) have reported that when low- and high-salt diets were introduced to LP offspring three weeks postnatally, female HS offspring were significantly lighter than control ND offspring between 8 and 10 weeks of age and continued to be so until

16 weeks of age. Stewart et al. (2009) applied a similar salt-modified diet to LP offspring at three weeks of age. While food intake was significantly reduced in both control and LP rats by almost 35% compared to rats fed a low-salt diet, both control and LP offspring on a high-salt diet gained a significant amount of weight as a result of sodium and water retention. The reason for the different responses of HS offspring between the two studies is not clear.

It is therefore evident that multiple complex factors such as postnatal diet, sex and age interact with prenatal insults to influence metabolic function and predispose offspring to disease. These findings are in agreement with various epidemiological studies demonstrating that FGR and being born small for gestational age but experiencing accelerated growth and obesity in youth can predict the development of metabolic and cardio-renal diseases later in life (Eriksson et al., 2001a, b; Langley-Evans, 2006).

Animal experiments have further corroborated the link between prenatal undernutrition and postnatal hypercalorific nutrition and its effect on bodyweight. Vickers et al. (2003) have demonstrated that food restriction *in utero* decreases offspring locomotor activity and energy expenditure levels, leading to obesity. Additionally, when this prenatal insult is followed by a hypercaloric (30% fat) diet after weaning, locomotor activity decreases further. Bellinger et al. (2006) have imposed protein restriction either throughout gestation or at different stages of pregnancy and have similarly noted a reduction in physical activity in all LP females and in males exposed to LP in early gestational stages at 9 and 18 months of age. The study concluded that prenatal protein restriction as a first insult does not elicit obesity without postnatal dietary challenge as a second insult. However, in contrast to the findings of the current study, Hoppe et al. (2007) found that rats exposed to LP *in utero* followed by a post-weaning high-salt diet did not experience altered body growth at PD 30 or 135.

Differences between offspring exposed to a prenatal intermittent fasting regimen and other dietary manipulation(s) may arise from different maternal food intake patterns. Rats are nocturnal animals, and as such, when they were deprived of food overnight, it would be predicted that this pattern of repeated food deprivation during their active phase would result in an altered feeding time pattern and a disturbed sleep-wake cycle, leading to circadian rhythm dysregulation. Hence, Ramadan fasting is placed in a circadian regulation context since food and fluid ingestion are shifted from daylight hours to post-sunset hours in humans and sleep-wake cycle patterns, energy balance, and muscle and psychomotor performance are all impaired. Accordingly, circadian rhythms are altered in Ramadan fasting, but the mechanisms underlying this alteration remain unclear (Reilly and Waterhouse, 2007).

All daily behavioural and physiological processes are cyclical in nature and are controlled and synchronised by an endogenous timing system called the circadian clock, the master pacemaker of which is located in the hypothalamic suprachiasmatic nucleus (SCN) (Schibler

and Sassone-Corsi, 2002; Feillet et al., 2008). The SCN controls peripheral clocks located in tissues such as the liver (Peirson et al., 2006), the intestine (Sládek et al., 2007), and the placenta (Wharfe et al., 2011), through neuronal pathways and signals which are not yet fully understood.

The circadian clock is involved in the regulation of distinct metabolic processes such as lipid and glucose synthesis (Yang et al., 2006). Additionally, nutrients and metabolic signals can promote the synchronisation of circadian machinery. In a study by Damiola et al. (2000), rats and mice were subjected to a restricted diet in which food was available for only four hours during the day, during normal sleeping hours. While those animals displayed circadian shifts in peripheral tissue genes such as those in the liver and kidneys, the SCN retained its synchrony with the external light-dark cycle. In another study by Orozco-Solís et al. (2011), rats exposed to LP during pregnancy and lactation showed circadian alterations in several genes regulating appetite and energy metabolism at PD 17 and after weaning. These changes were associated with hyperphagia and indicate that the circadian clock undergoes metabolic programming. Accordingly, it is necessary in future to determine the implications of maternal intermittent fasting during pregnancy on the circadian clock and how the regulation of maternal circadian rhythm might influence disease onset later on in offspring.

4.4.2 Impact of prenatal intermittent fasting on salt preference and ECFV in IF offspring: sex-specific effects

The previous chapter revealed that exposure to intermittent fasting *in utero* does not evoke hypertension in IF offspring. Extracellular fluid volume, which depends on plasma sodium content, is involved in the regulation of blood pressure (De Wardener et al., 2004). Since food intake of the standard chow diet was similar between IF and control groups from week 7 onward and renal handling of sodium was unaltered in IF offspring (see Section 3.3.11.6), the current study has examined ECFV and salt preference and aversion in IF offspring fed a standard chow diet.

Inulin space analysis is the most common method used to determine ECFV (Addanki et al., 1967). The data in this study showed that prenatal intermittent fasting in dams did not impact ECFV, which was comparable between IF and control groups, being approximately 24% of total bodyweight. These results are in agreement with previous studies (Barratt and Walser, 1969; Alwasel et al., 2012) and may underscore the similarities in blood pressure between IF and control groups (see Section 3.3.7). In terms of salt preference and aversion, females had a higher salt preference and total fluid intake than their counterpart males, with no differences between IF and control groups. Nevertheless, when the salt content of the saline solution was increased from 0.9% to 2.1% in 0.3% increments, IF females had a higher threshold of aversion at 1.8% saline concentration compared to control females. This is consistent with

feeding behaviour, as IF females consumed more of the high-salt diet at postnatal weeks 11 and 12. Hence, intermittent fasting during pregnancy appears to induce an enhanced preference for salt in female offspring but not in males.

As mentioned earlier, thirst and salt appetites first become evident in newborn rats two and three days after birth, respectively (Ellis et al., 1984; Leshem et al., 1994). Twelve days after birth, rats first responded to a sodium deficit, and at 24 days of age, rats are able to regulate NaCl and water intake to balance body fluid tonicity (Leshem et al., 1993). Increased salt preference has been reported in various prenatal programming models. For instance, four-week-old rats exposed *in utero* to LP exhibited a marked increase in 0.9% saline ingestion and fluid intake as well as an expansion of ECFV and an increase in total body water content. The increased preference for sodium in those LP rats was driven by renal sodium wasting (Alwasel et al., 2012).

A study by Nicolaidis et al. (1990) mimicked human vomiting episodes during pregnancy by treating pregnant Wistar rats with polyethylene glycol to induce dehydration through reduced extracellular fluid volume. This led to an increased saline preference among treated offspring compared with untreated controls. In the context of Ramadan fasting, Rabinerson et al. (2000) and Joosoph et al. (2004) have reported an increased risk of hyperemesis gravidarum (morning sickness) in fasting pregnant women especially during the first month of pregnancy, and recent studies have demonstrated a reduction in the amniotic fluid indices of pregnant women who fast during summer time (Seckin et al., 2014; Sakar et al., 2015). Thus, if IF offspring were to be subjected to food and water deprivation, as is the case during Ramadan fasting in humans, their salt preference programming may well be more pronounced. Moreover, another *in utero* stimulus also increases the salt appetite of offspring: Dahl salt-sensitive rats exposed to a low-salt diet during pregnancy also showed an increased salt preference in both sexes (Hara et al., 2014). Argüelles et al. (2000) have observed that even offspring whose mothers underwent a partial aortic ligation had an enhanced salt preference for a low-concentration saline solution (0.6%) compared to controls.

One of the mechanisms underlying salt appetite and thirst development involves an increase in the activity of the renin-angiotensin system. In a study by Möhring et al. (1975a, b), male rats underwent renal artery constriction, and this resulted in an elevation of blood pressure and an induction of sodium retention. When these hypertensive rats were offered a choice between water and 0.9% saline as drinking fluids, they drank more saline than controls even when their body sodium content was already higher (Möhring et al., 1975b). Plasma Ang II concentration also increased by 50%, leading the authors to suggest that renal artery constriction activated the renin-angiotensin system, which in turn induced salt appetite.

Butler et al. (2002) found that administration of losartan, which blocks Ang II (AT_1) receptors, in pregnant dams from day 2 until day 19 of gestation resulted in adult female offspring ingesting significantly more hypertonic saline and water intake than controls. The LP rat model exhibits upregulation in the expression of renal AT_{1a} (Sahajpal and Ashton, 2003) and AT_2 (McMullen and Langley-Evans, 2005; Alwasel et al., 2010b) and brain AT_{1a} (Pladys et al., 2004) receptors. The upregulation of brain AT_{1a} can play a role in the development of salt appetite in LP offspring, as such an increase enhances sensitivity to Ang II, which in turn is a powerful stimulus for thirst and salt appetite (Fitzsimons, 1998). Fitts et al. (2007) have found that a small increase in the circulating concentration of Ang II induced by intravenous infusion at 50 ng/kg bodyweight/min overnight is able to enhance salt appetite in rats.

In addition to the RAS, changes in brain structure, mainly in the hypothalamus, have implications regarding feeding behaviour. Offspring exposed to protein restriction throughout gestation and lactation showed decreased volume of the whole brain and of the hypothalamic paraventricular nucleus. This was associated with an increase in neural density but a decrease in neurons staining for neuropeptide Y, cholecystokinin and galanin, which are the key peptides involved in appetite control (Plagemann et al., 2000). In the current intermittent fasting model, IF female offspring showed a reduction in relative brain weight at PD 1. This was associated with a cognitive deficit in IF females (Dr Nick Ashton, personal communication). It is therefore tempting to speculate that changes in brain structural development may also predispose IF females to increased salt appetite.

4.4.3 Impact of high-salt diet on blood pressure and renal function in IF offspring

Chapter 3 revealed that offspring exposed *in utero* to intermittent fasting did not show any impairment in renal function at 14 weeks of age. While studies of protein restriction during pregnancy have found a 13% to 28% reduction in nephron numbers in LP offspring, the total GFR among those offspring was unchanged due to hyperfiltration of the remaining glomeruli (Langley-Evans et al., 1999; Manning and Vehaskari, 2001). Woods et al. (2001a) have additionally found that with a 25% nephron deficit in LP offspring, the total GFR per gram kidney weight drops by only 11%, indicating a 20% increase in the GFR of remaining individual nephrons. IF offspring therefore may have adapted to preserve kidney function in postnatal life. Based on these findings, dietary salt loading was undertaken in order to challenge the kidneys and identify potential deficiencies in renal excretory capacity.

A 4% high-salt diet was sufficient to increase blood pressure in both control and IF offspring at 7 and 10 weeks of age with no significant differences emerging between the two dietary groups or sexes at the point of measurement. However, the magnitude of increase in blood pressure at postnatal week 10 compared to week 5 was greater in IF male offspring (16.5 mmHg), reaching significance in comparison with control females (11 mmHg). Furthermore, if the values of each

individual offspring are taken rather than the mean per litter, males of the IF group showed a significantly greater increase in blood pressure at week 10 compared to week 5, than IF females and control offspring of both sexes.

One of the mechanisms underlying the development of hypertension is sodium retention and expansion of ECFV (Manning and Vehaskari, 2001; Meneton et al., 2005). Salt loading stimulates sodium excretion and vasodilation as an adaptation to an increase in plasma volume. When this adaptation is inadequate, ECFV increases, including blood volume, which increases cardiac output and blood pressure (de Wardener et al., 2004). Hypertensive rats exposed to prenatal protein restriction showed an increase in ECFV, total body fluid and total body sodium content at four weeks of age, as a result of greater salt intake rather than sodium retention (Alwasel et al., 2012). In contrast, offspring exposed to dexamethasone during pregnancy became hypertensive at postnatal day 60, displaying higher tissue sodium content in the liver and femoral skeletal muscles as a result of a reduction in sodium excretion (Celsi et al., 1998). Nevertheless, sodium retention does not always result in an expansion of ECFV and therefore hypertension. Studies have shown that skeletal muscles and skin can accumulate sodium at higher levels than plasma (Kopp et al., 2013); therefore they are considered important extra-renal salt handling tissues (Foss et al., 2017). A study by Titze et al. (2005) showed that skeletal muscles have an osmotically neutral Na^+/K^+ exchange where sodium accumulates at the expense of potassium loss. However, skin is osmotically inactive causing only sodium retention without leading to water retention or hypertension. Since IF male offspring tended to show a reduction in sodium excretion without further increase in the blood pressure, the extra-sodium could be deposited in the skin.

In order to study how postnatal salt intake interacts with prenatal insults, Stewart et al. (2009) offered diets of low salt (0.03% LS), standard salt (0.3% SS), and high-salt (3% HS) to LP offspring at weaning and assessed blood pressure at six weeks of age. The study found that LP *in utero* induced hypertension in offspring maintained on an SS diet and that hypertension was amplified by ~20 mmHg in LP rats fed an HS diet, with no impact of the same diet on control rats. A LS diet, on the other hand, had normalised blood pressure among LP offspring. Thus, hypertension is salt-sensitive in the LP model. Alwasel and Ashton (2012) reported similar salt sensitivity in the LP rat, which exhibited a rightward shift in the pressure natriuresis curve. Stewart et al. (2009) also assessed SS animals at 51 weeks of age following early exposure to either an HS or an LS diet. Brief early exposure to an LS diet prevented the later development of hypertension, whereas nephrosclerosis was observed in animals briefly exposed to an HS diet. LP offspring exposed to an LS diet lost their salt sensitivity when challenged with an HS diet between 43 and 49 weeks of age, suggesting the existence of a developmental window during which hypertension may be reprogrammed (Stewart et al., 2009).

Contrary to this, a study by Langley-Evans and Jackson (1996) showed that prenatal exposure to protein restriction caused hypertension in LP rats at six weeks of age, with LP blood pressure being 20 mmHg higher than that of controls. However, while administration of 1.5% saline as a drinking solution at postnatal week 7 for seven days raised the blood pressure of control offspring by 31 to 41 mmHg, the same administration had no effect on hypertensive LP rats. Also in response to salt challenge, urinary volume and Na^+ excretion rose to similar levels in control and LP rats. Therefore, the mechanism underlying the resistance of LP offspring to develop hypertension following salt loading is unknown. Both studies used methods similar to those used in the current study to measure blood pressure, yet the magnitude of the blood pressure increase in response to salt intake in those studies was almost two-fold higher than that observed here.

With regards to renal function, salt-loaded controls presented a significant increase in renal haemodynamics and in electrolyte handling over controls fed a standard chow diet, which is consistent with previous studies in which animals displayed either an increase or no change in GFR in response to salt loading (Sanders et al., 2005; Dickinson et al., 2007; Ruta et al., 2010). However, a postnatal high-salt diet created a potential susceptibility to renal dysfunction among adult males exposed *in utero* to intermittent fasting. IF males showed a significant reduction in FF, osmolar excretion and urine creatinine concentrations by approximately 46% along with higher NGAL concentrations of up to 80% compared to control offspring. Also, IF males displayed a wide range of albumin:creatinine concentration ratios that were significantly greater than that of IF females. GFR and Na^+ and Cl^- excretions were reduced in IF male offspring by almost 35%, although this reduction did not achieve statistical significance. The reduction in electrolyte excretion could be a consequence of a decline in GFR or an alteration in the mechanism of tubular sodium reabsorption, as water clearance was unaffected. Despite sodium retention in IF male offspring, plasma sodium concentration was similar to that of control males. In salt-loaded IF females, on the other hand, GFR tended to be increased compared to IF females fed standard chow, with no differences emerging between IF females and controls. This implies that IF offspring are not adapting to the salt/fluid load appropriately. Interestingly, both control and IF offspring exhibited higher haematocrit and kidney hypertrophy in response to salt intake, as previously reported (Hoppe et al., 2007; Ofem et al., 2009). Renal sodium retention is usually accompanied by water retention, thus leading to ECVF expansion. The haematocrit in that case should be reduced, but the increase in haematocrit in this study implies movement of fluids from the extracellular compartment to the intracellular compartment which may cause the hypertrophy seen in the kidney.

The urine anion gap was also calculated as an indicator of the kidney's function and ability to acidify urine appropriately. The normal range should be at a value approaching zero or slightly positive. In this study the urine anion gap in both sexes of the control and the IF offspring fed a high-salt diet was almost four-fold higher than offspring on a standard chow diet (Section

3.3.11.4). This suggests the presence of renal tubular acidosis, with the kidneys unable to excrete ammonium ions, providing further evidence of renal dysfunction following a high-salt challenge.

De Wardener et al. (2004) have demonstrated that a small increase (1 to 3 mmol/L) in plasma sodium plays a role in the mechanisms by which a high-salt diet raises blood pressure. In the current study, plasma sodium concentration ranged from 2 to 4 mmol/L higher in salt-loaded offspring compared to offspring fed a standard chow diet, yet this increase was not statistically significant. Celsi et al. (1998) noted a similar pattern in dexamethasone-treated offspring, which had altered renal function without any changes in plasma sodium concentration; instead, tissue sodium content was elevated. This may be the case in the current study, as well. Pressure natriuresis is a central mechanism for the regulation of sodium-fluid balance (Figure 4.4.1, Hall et al., 2012). A high-salt diet causes an increase in the body sodium content which in turn causes an expansion of ECFV that shifts the pressure-natriuresis relationship to the right, thus leading to an increase in blood pressure which is required in order to match sodium excretion to sodium intake (Hall et al., 2012).

The observed reduction in renal parameters among IF offspring is consistent with reports from two previous programming models. Sanders et al. (2005) induced a nephron deficit in rat offspring via bilateral ligation of uterine arteries during pregnancy (IUS). Under basal conditions, GFR, renal blood flow, and FF were significantly increased without any change in blood pressure in adult IUS offspring. At 12 weeks of age, a high-salt solution (2% NaCl) was introduced, and renal haemodynamic function was evaluated at 16 weeks of age; IUS offspring became hypertensive and displayed reduced renal haemodynamic parameters and elevated urinary albumin concentration. However, salt intake did not affect renal parameters in control rats.

In a model in which nephron number was reduced by 25% in mice born with two kidneys (HET2K) and by 65% in mice born with one kidney (HET1K), both groups of mice showed normal renal function and blood pressure up to one year of age (Ruta et al., 2010). With (5%) high-salt loading, MAP increased by 5 and 9.1 mmHg in HET2K and HET1K respectively, corresponding to the relative reduction in nephron number. Both groups displayed lower creatinine clearance, osmolar excretion, sodium excretion and fractional excretion, but there was no sign of albuminuria and no change in haematocrit (Ruta et al., 2010). Plasma sodium concentration (144 mmol/L) did not differ between groups and was similar to that found in the present study.
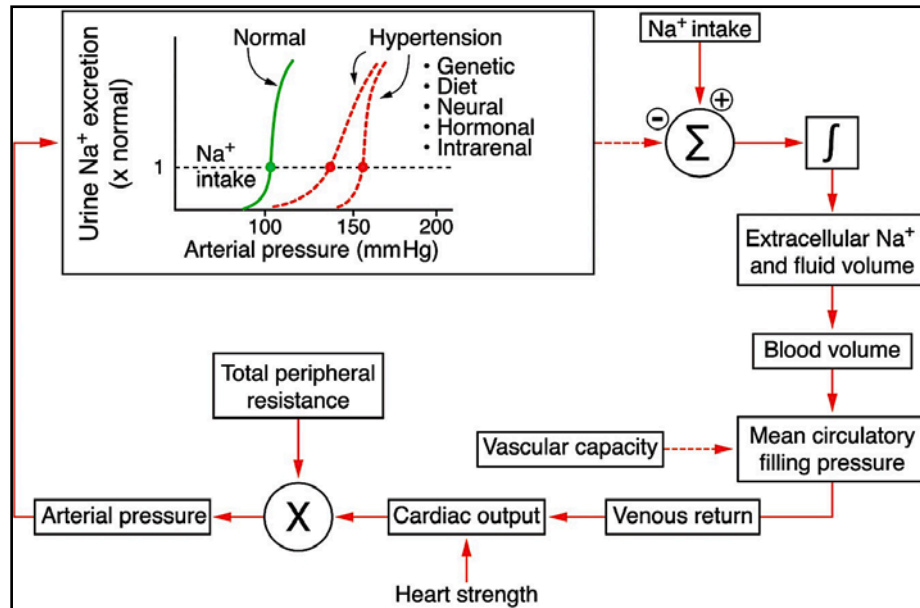
Figure 4.4.1 The cycle for regulation of arterial pressure through the renal-body fluid feedback mechanism. Taken from Foss et al. (2017).

Thomson et al. (2004) have proposed the tubulo-centric principle of GFR control to describe the salt paradox of early diabetes. According to this principle, an increase in salt intake leads to ECF expansion, which induces a primary decrease in tubular reabsorption upstream from the macula densa. The subsequent increase in sodium concentration in the macula densa activates tubuloglomerular feedback and overwhelms primary vasodilation, causing a decline in GFR. Findings in the current study would be consistent with this principle. As mentioned earlier, salt loading does not always lead to expansion of ECFV, since salt can accumulate in osmotically inactive tissues such as the skin (Titze et al., 2005).

Protein-restriction models have shown similar alterations in sodium handling. Manning et al. (2002) were the first to propose that sodium retention in protein-restricted offspring is linked to an increase in mRNA and protein expression of two key renal sodium transporters, bumetanide-sensitive Na-K-2Cl co-transporter (BSC1) and thiazide-sensitive Na-Cl co-transporter (TSC), at the thick ascending limb and the distal convoluted tubule, respectively, before the onset of hypertension at four weeks of age. BSC1 and TSC limit the rate of sodium reabsorption in nephron segments and play critical roles in regulating Na balance and extracellular volume. Therefore, the upregulation of these two transporters causes increased sodium and water retention, leading to ECFV expansion and hence an increase in blood pressure (Manning et al., 2002). However, Manning et al. (2002) based their conclusion on an increase in the expression of the transporters without any assessment of renal sodium handling *in vivo*. Alwasel et al. (2009; 2012) disagreed with the latter study and showed that 4-week old offspring exposed to a LP diet *in utero* excreted more sodium rather than reabsorbed more

sodium. The protein-restricted offspring displayed increased mRNA and protein expression of BSC1, but such an increase in the expression of this transporter was not matched to functional data *in vivo* using specific drugs to target transporter activity (Alwasel et al., 2009; 2012).

Dagan et al. (2007) evaluated the effect of prenatal dexamethasone treatment on offspring sodium transporters in the proximal tubule using *in vitro* microperfusion. Dexamethasone-treated offspring showed a 50% increase in proximal tubule volume absorption compared to controls, and this was accompanied by a 50% increase in Na^+/H^+ exchanger activity as well as an abundance of Na^+/H^+ exchanger isoform 3 protein (NHE3) in the segment responsible for sodium reabsorption. These results support the hypothesis that alteration in sodium handling at any segment of the renal tubules can mediate hypertension in prenatal programming models. The findings of Ledoussal et al. (2001) support the notion that NHE3 plays a crucial role in Na^+ handling and hypertension development. NHE3-null mice placed on a low-salt diet showed a reduction in GFR as well as renal salt wasting and hypotension. Boer et al. (2005) found that spontaneously hypertensive rats (SHR) demonstrated a significant increase in proximal sodium reabsorption, leading to a decrease in fractional sodium excretion. However, neither creatinine clearance nor fractional post-proximal sodium excretion were altered. An increase in sodium retention is coupled with an increase in NHE3 at both mRNA and protein levels in SHR compared to control rats (Sonalker et al., 2004). Furthermore, as discussed earlier, even dexamethasone-treated offspring showed sodium retention associated with a 40% reduction in GFR and fractional excretion of sodium at 60 days of age (Celsi et al., 1998). This reduction in GFR was related to a reduction in nephron number, which was 60% lower than that of controls, and sodium retention was confirmed by a significant increase in tissue sodium content in dexamethasone-treated offspring over controls.

Taking the data together, the current study would be consistent with the hypothesis that IF offspring have a reduced nephron endowment at birth, as evident through their lower kidney weight. The reduction in kidney weight did not persist at PD 12; however, it is unclear if this recovery in kidney weight reflects recovery in nephron number. Elwan (PhD Thesis, 2016) found that in a model of intermittent food and water restriction *in utero*, nephron number was reduced by 30% at birth and remained lower until adulthood. However, these results were from male offspring only, as the author did not consider sex differences. In the current study, young adult IF offspring maintained on a standard chow diet appeared to undergo compensatory adaptation and were able to sustain normal blood pressure and renal function up to 14 weeks of age (see Section 3.4.6.1). Several studies in LP offspring have reported normal renal function despite low nephron number (Langley-Evans et al., 1999; Manning and Vehaskari, 2001). Hyperfiltration in the remaining nephrons, in response to nephron loss, may affect the progression of renal disease (Cullen-McEwen et al., 2003) and may contribute to glomerulosclerosis and hypertension, creating a vicious cycle of worsening renal damage and increasing blood pressure (Brenner, 1983; Brenner and Chertow, 1994).

Maritz et al. (2009) and Nenov et al. (2000) have argued that a congenital nephron deficit as a first hit is not sufficient to produce overt renal disease or hypertension. However, nephron deficit increases susceptibility to a second hit as a factor toward manifesting renal disease. Following this argument, IF offspring in the current study were challenged with a high-salt diet for 10 weeks post-weaning. A marked sex difference in renal function was observed whereby IF male offspring were more sensitive and vulnerable to salt loading as interpreted by a reduction in renal haemodynamics and renal Na^+ excretion. This sodium retention could be related to upregulation in sodium transporters, a hypothesis which is supported by the findings of Brenner and Chertow (1994), who proposed that reduced final nephron numbers cause sodium hypofiltration and an expansion of ECFV, leading to the development of hypertension.

An interesting question arises from these observations: why did the sodium retention and renal haemodynamic reduction phenomena apparent in IF male offspring not exacerbate their rise in blood pressure over matched control males? It could be that alterations in kidney function and sodium retention precede the onset of more pronounced hypertension. More research is required to provide evidence for this hypothesis, including nephron counting, measurement of sodium transporter expression at mRNA and protein levels, and evaluation of the functionality of each renal tubule segment and total body sodium content.

Urine albumin and albumin:creatinine concentration ratio were measured in this study as predictors of renal dysfunction. The values were in agreement with the findings of Nwagwu et al. (2000) in LP rats, and no differences were observed between IF and control groups. However, salt-loaded IF male offspring showed a wide range of albumin:creatinine concentration ratio values that were significantly higher in comparison to salt-loaded IF females. This is consistent with renal clearance results and would suggest that IF male offspring undergo an accelerated deterioration in kidney morphology, particularly in the selective permeability of the glomerular basement membrane, which is responsible for preventing albuminuria in a normal kidney. The filtration membrane barrier consists of fenestrated endothelial cells, glomerular basement membrane (GBM) and the slit membrane (Tojo and Kinugasa, 2012). The fenestrated endothelial cells have a surface layer (ESL) that consists of adsorbed plasma components and glycocalyx that forms the first barrier for albumin filtration (Satchell, 2013). The second barrier is the GBM which consists of a meshwork of extracellular matrix proteins that acts as a size and a charge barrier for proteins. The slit membrane that spans the gap between podocyte processes acts as a final fine filter (Tojo and Kinugasa, 2012). Albuminuria can occur due to an impairment of any of the components of the filtration barrier. Dysfunction of ECL particularly in glycocalyx (Satchell, 2013), defects in GBM components such as laminins and type IV collagen (Suh and Miner, 2013), damage of slit membranes and detachment of podocytes (Figure 4.4.2) as well as impairments of proximal tubules to reabsorb albumin (Tojo and Kinugasa, 2012) all contribute to albuminuria

occurrence.
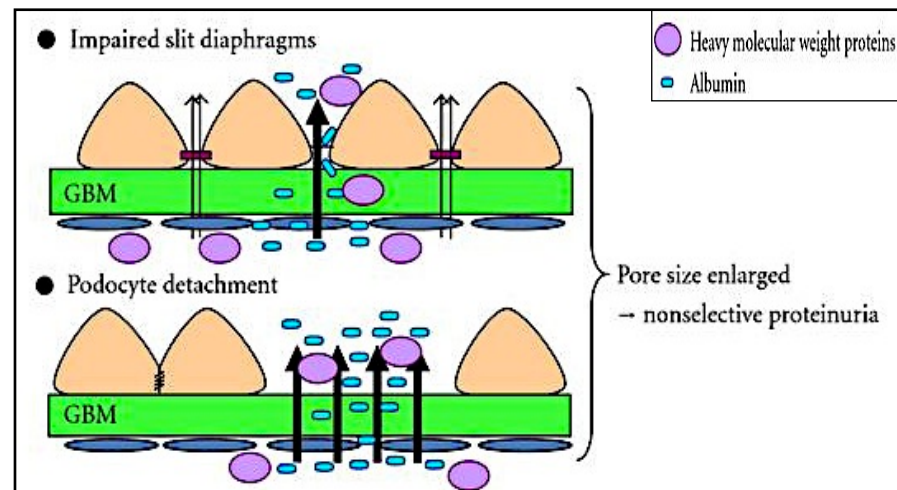
Figure 4.4.2 Diagram illustrates damage to the slit membrane (diaphragm) and the detachment of podocytes leads to protein and albumin filtration. GBM, glomerular basement membrane. Taken from Tojo and Kinugasa (2012).

NGAL, a marker for early renal injury, was also measured. When kidney damage occurs, NGAL is produced systematically. It is also produced locally by the injured tubules. Another, third way for NGAL to be produced is through inflamed vasculature or activated neutrophils/macrophages, often seen in chronic kidney disease (Schmidt-Ott et al., 2006). NGAL was observed to rise up to twentyfold in serum and a hundredfold in urine, respectively, 48 h before acute kidney injury (AKI) which was identified by creatinine measurements (Mårtensson and Bellomo, 2014). Therefore, it can be used as a biomarker to predict AKI. The release of NGAL by kidney injury can also act as a local defensive mechanism, as NGAL mediates iron transport and therefore reduces the oxidative stress that can be caused by excess extracellular iron (Mårtensson and Bellomo, 2014). NGAL is positively correlated with severity of kidney injury, which highlights the relevance of NGAL for prognosis of chronic kidney disease (Patel et al., 2015). Values recorded in IF rat urine were almost ten-fold higher than that which has been reported previously under normal conditions and normal diet but similar to the values indicated in acute renal injury, indicating that a high-salt diet induces renal injury (Han et al., 2012). A high-salt diet can stimulate inflammation through altering immune cell function either by direct activation of T lymphocytes (T cells) or indirect activation of antigen presenting cells (APC) such as macrophages and monocytes (Figure 4.4.3, Foss et al., 2017). Therefore, the rise in NGAL concentration seen in this study is a consequence of high-salt intake, especially as the kidneys were hypertrophic. Interestingly, though, IF males showed a two-fold higher NGAL concentration compared to controls, denoting more subtle cellular kidney injury. This study therefore suggests that prenatal intermittent fasting may significantly increase kidney sensitivity to the effects of a postnatal high-salt diet and that IF males are more susceptible to this sensitivity than are IF females.
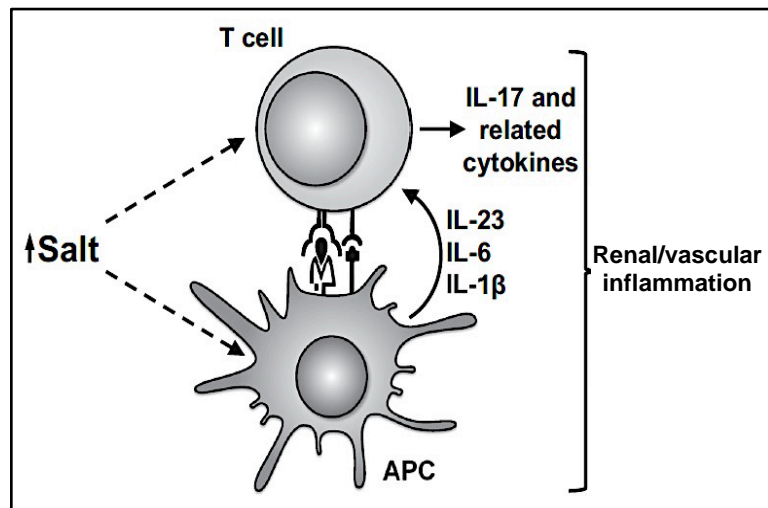
Figure 4.4.3 Salt diet can activate immune cells such as T cells or antigen presenting cells (APC) and stimulate inflammation by release of several cytokines. IL, interleukin. Modified from Foss et al. (2017).

Other factors that may contribute to renal deterioration and hypertension development are oxidative stress and the renin-angiotensin system. In conditions of maternal dietary protein deprivation, Stewart et al. (2005) found that intrarenal oxidative stress was increased in LP offspring at four weeks of age before the onset of hypertension. Treatment of LP offspring in the pre-hypertensive stage with a reactive oxygen species scavenger such as a superoxide dismutase mimetic prevented hypertension development as well as reduced renal oxidative stress. However, renal oxidative stress was exacerbated with a high-salt diet, leading to a further rise in blood pressure and nephrosclerosis, which was reversed through the application of a low-salt diet.

Alwasel et al. (2010b) additionally found that offspring exposed *in utero* to protein restriction exhibited alterations in the renin angiotensin system, as protein expression of Ang II AT$_1$ and AT$_2$ receptors was reduced over the period of nephrogenesis, and this may have contributed to the nephron deficit observed in LP offspring. By the weaning period, renal Ang II AT$_1$ (Vehaskari et al., 2001) and AT$_2$ (Sahajpal and Ashton, 2003) and plasma angiotensin converting enzyme activity (Langley-Evans and Jackson, 1995) were upregulated. The renin-angiotensin system coexists in the proximal tubule (Seikaly et al., 1990; Braam et al., 1993) and plays an important role in regulating proximal tubular transport (Cogan, 1990; Cogan et al., 1991; Quan and Baum; 1996). Therefore, it is important to determine whether the renin-angiotensin system affects renal tubule transport in the IF model.

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4.5 SUMMARY

The data presented in this Chapter showed that females had greater salt preference than males in both control and IF groups. Maternal intermittent fasting *in utero* thus programmes salt preference in a sex-specific manner, as IF female offspring displayed a higher salt aversion threshold than did control female offspring. ECFV, on the other hand, was comparable between dietary groups and sexes. Both sexes of IF offspring exhibited different pattern of feeding behaviour on both standard chow diet and high-salt diet. A postnatal high-salt diet caused polydipsia in both IF and control groups but altered growth trajectories in IF offspring only, among which IF males were heavier and IF females lighter than IF offspring fed a standard chow diet. In response to high-salt intake, blood pressure rose in both control and IF offspring.

In the control group, as a consequence of salt loading and higher blood pressure, renal haemodynamics and renal electrolyte handling increased significantly. Interestingly, the study clearly indicates that prenatal intermittent fasting as a first insult alone does not result in renal dysfunction and hypertension in IF offspring, but it increases the vulnerability of the offspring to a second insult such as a high-salt diet. Salt-loaded IF males showed deterioration in renal function, with reduced renal electrolyte handling and haemodynamics as well as a significant increase in a marker that detects early renal injury, NGAL. Therefore, a repeated pattern of intermittent fasting leading to maternal undernutrition, can have effects on the developing kidney, such that when challenged by a high-salt intake during the postnatal period, sex-specific renal dysfunction is observed in the offspring.

CHAPTER 5

GENERAL DISCUSSION

5.1 DISCUSSION

Prenatal nutritional perturbation is a widespread condition that not only occurs during famines and diseases, but also in both underdeveloped and developed countries. It is considered as an essential health problem as it can have an adverse impact on fetal growth, birth outcomes and long-term health consequences (Ravelli et al., 1999; Brodsky and Christou, 2004; Li et al., 2010). Under intrauterine sub-optimal nutritional conditions, the fetus undergoes morphological and metabolic adaptation which helps it to sustain growth *in utero*; however, this adaptation increases the risk of cardio-renal and metabolic disease later in life (Langley-Evans, 2009), often in a sex specific manner (Sundrani et al., 2017).

The research described in this thesis modelled the intermittent fasting pattern undertaken by pregnant Muslim women during the practice of Ramadan fasting, and investigates the impact on the mother's and the offspring's health with specific reference to cardio-renal function. Ramadan fasting is one of the five pillars of Islam. It is a religious obligation which healthy adult Muslims around the world subscribe to on a fixed annual basis and involves abstaining from food and drink from dawn to sunset for a duration of 30 days. Pregnant women are allowed to break their fast and compensate for any fasting days that they miss after delivery. However, custom means that many pregnant Muslim women, estimated as 70% to 90% of the global population, amounting to approximately 230 million Muslim women of reproductive age (Daley et al., 2017), do participate in the religious observance of Ramadan. Thus many Muslim women may fast with their families despite their pregnancy, and despite the exception that they are granted. Therefore they undergo a pattern of maternal intermittent fasting (IF) with possible health effects on the mother and child. The nature of intermittent fasting is different from ongoing calorific-restriction, which has been widely studied. Currently, there is a lack of research regarding the full influence of fasting during pregnancy. Nevertheless, detrimental effects on maternal and fetal health have been documented (Malhotra et al., 1989; Dikensoy et al., 2009; Seckin et al., 2014; Sakar et al., 2015).

A greater understanding of how Ramadan fasting exposure impacts on maternal and fetal outcomes should assist Muslim women around the world to make more informed decisions about their fasting participation during Ramadan. As has been recently commented there is a need for such studies so that advice given to Muslim women can be less inconsistent and more evidence-based (Daley et al., 2017).

The placenta represents the interface between the maternal environment and the fetus, and thus plays an important role in fetal programming. Epidemiological and animal studies have reported changes in placental morphology (Coan et al., 2010; Sandovici et al., 2012) and alterations in amino acid transport in response to maternal undernutrition (Glazier et al., 1997; Jansson and Powell, 2006; 2007; Sibley, 2009), such as a reduction in system A transporter

activity in FGR (Glazier et al., 1997; Jansson et al., 2006). In Ramadan fasting, changes in placental weight and dimensions are the only parameters that have been assessed to date (Alwasel et al., 2010a; 2014), demanding further investigations into how placental function may be altered in this situation.

The current study had two main aims. The first was to develop a rat model which mimics aspects of Ramadan fasting in humans to investigate the effects of intermittent fasting during pregnancy on maternal physiology, fetal growth and metabolic profile, and metabolomics, functionality and morphology of the placenta. Since sex-specific differences have been observed in a number of developmental diseases (Sundrani et al., 2017), the sex-dependent specificity of fetal responses to maternal intermittent fasting were also examined.

The second aim was to determine the long-term impact of maternal intermittent fasting *in utero* on the offspring's health. This assessment focused on the growth trajectory and feeding behaviour of the offspring and on the impact of this dietary regimen on cardiovascular, metabolic and renal function.

A number of important findings were made in this research which are likely to apply to humans to various degrees. Maternal intermittent fasting affected maternal physiology, including reducing concentrations of plasma glucose, glucagon and amino acids reflective of altered maternal nutrient status. The maternal dietary regimen also impaired fetal growth and metabolic homeostasis, resulting in lower insulin and amino acid concentrations accompanied by a significant reduction in system A transporter activity (*in vivo*) in fetuses of both sexes. The metabolomic profile was altered in the placentas of both sexes, with more pronounced changes in IF males. Nevertheless, prenatal intermittent fasting was not associated with low birth weight or hypertension or the manifestation of glucose intolerance and insulin resistance in the offspring. It did not result in ECFV expansion or renal dysfunction in the adult offspring. However, exposure to intermittent fasting *in utero* altered growth trajectory and feeding behaviour of the offspring, exposing a higher salt aversion threshold in females compared to males. Although challenging the IF offspring with a high-salt diet caused an increase in blood pressure in both sexes, this was not different from controls. However there was a sex-dependent change in renal function in the IF males. These outcomes imply that there is a significant negative impact to be expected from intermittent fasting in humans that stretches over a month long with impacts likely to affect organs, overall growth and development, as well as a risk of programming for diseases later in life.

The importance of the study presented in this thesis is that is has advanced our understanding of how the placenta responds to maternal intermittent fasting and has revealed that some effects elicited occur in a sex-dependent manner, which could also apply to human pregnancy. Overall, this study has shown that maternal intermittent fasting has an impact on fetal and

placental growth and function, but the long term effects on cardio-renal function in the adult offspring appear to be minimal. In this respect, the IF model developed in the current study is unique among developmental programming models, and warrants further investigation in order to fully understand how the intrauterine environment affects the adult phenotype.

5.1.1 The IF model: limitations and relevance to human Ramadan practice

Since the month of Ramadan follows the lunar rather than the solar calendar, the start of the Ramadan fasting month shifts forward every year by 11 days. The length of each day, and the corresponding duration of the daily fast, can vary dependent on the season of the year in which Ramadan falls. The duration of the daily fast therefore lasts between 9 and 19 hours per day, depending on season and geographical location. For pregnant Muslim women who decide to fast, their period of intermittent fasting can fall into any stage of their pregnancy (Bragazzi, 2014). In this IF model, maternal intermittent fasting was imposed on the pregnant dams from the beginning of gestation until day 21, and involved the removal of food on a daily basis during the rats' active phase from 5 pm until 9 am the following day.

There are some important limitations to the rat model, as it did not completely resemble human Ramadan fasting. Ramadan fasting includes abstinence from drinking, whereas the rats had water available to them at all times. The dam's water intake was not restricted due to the design of the study which wanted to study the influence of food deprivation alone. In the future, the model could be further refined to better match the human practice of Ramadan fasting by looking at the combined effects of food and water deprivation to animal welfare regulations and the terms of the project license that was granted. Also, the model involved fasting for almost the entire period of the rats' pregnancy rather than just a fraction of it. During Ramadan, pregnant women fast for only one month of their pregnancy, which equates to about 2.3 - 2.5 days in a rat's pregnancy. Food deprivation was imposed on the rats' pregnancies throughout 21 days to establish the new model and investigate the maximum effect as a baseline to build upon and then refine the model to be more relevant to human fasting.

Nevertheless, a pattern of intermittent food restriction during the rats' active time was implemented, where the rats were food restricted for 16 hours per day, equivalent to the minimum hours pregnant Muslim women fast during summer time (between 16 to 19 hours per day). This rat model established a baseline from which to obtain insight into the broader impacts of intermittent fasting on pregnancy, mimicking the repeated pattern of fasting then feeding, and a step towards understanding how changes in the food intake pattern influences pregnancy outcome in rats in order to know what the impacts of intermittent fasting on humans are, and whether the food deprivation on the pregnant mother can be sufficiently compensated for during the intermission period where food is available.

5.1.2 The impact of maternal intermittent fasting on the mother, fetus and placenta

5.1.2.1 Maternal physiology

The study clearly shows that intermittent fasting affected maternal health. Dams exposed to intermittent fasting consumed 30% less food throughout pregnancy. The eight hours during which food was available to the experimental group was not enough for the rats to adequately compensate for the food withdrawal during the night. As a consequence, their daily weight gain over the course of pregnancy was significantly reduced, starting from GD 8 onward. These phenotypic features correspond with previous reports on Ramadan fasting and its effect during pregnancy in humans. It was found that by fasting for 11 h and 45 min, 91.9% of fasting pregnant women suffered a deficiency of more than 500 kcal compared to the recommended daily calorific intake of 2,500 kcal (Arab, 2004), which resulted in reduced body weight especially during the second and third trimesters (Kiziltan et al., 2005). Therefore, having half a day available to achieve the recommended calorific intake was insufficient.

The reduction in calorific intake resulting from Ramadan fasting during pregnancy is associated with several maternal metabolic changes, such as hypoglycaemia, aggravated insulin resistance, decreased amino acid concentration and increases in ketone and free fatty acid concentrations (Prentice et al., 1983; Malhotra et al., 1989; Mirghani et al., 2004; 2005). These metabolic alterations are defined as "accelerated starvation" (Metzger et al., 1982), which has more impact during the second part of pregnancy (Butte et al., 1999). In this study, the pregnant dams exposed to intermittent fasting showed metabolic changes consistent with accelerated starvation, including reduced glucose, glucagon and essential and non-essential amino acid concentrations, whereas the insulin concentration remained unchanged.

During normal pregnancy conditions, pregnant women as well as rats undergo two metabolic phases (Knopp et al., 1973; Anderson et al., 1980; Baynouna Al Ketbi et al., 2014). The first half of pregnancy is the anabolic phase, which is characterised by increased insulin sensitivity and tissue glycogen and adipose tissue fatty acid accumulations with decreased hepatic glucose production. The food intake at this stage is directed at maternal stores and is reflected by maternal weight gain (Knopp et al., 1973; Anderson et al., 1980). The second half of pregnancy is the catabolic phase featuring increased tissue insulin resistance and insulin secretion in which maternal insulin concentrations are up to 50% higher than in non-pregnant women (Herrmann et al., 2001). Fat stores are mobilised to meet the mother's physiological needs and nutrient intake is shifted to the fetus to provide it with the nutritional requirements for its rapid growth at this stage. Hence, maternal weight gain reflects fetal growth in the latter part of pregnancy (Knopp et al., 1973; Anderson et al., 1980).

The pattern of maternal intermittent fasting in the present study interrupted the normal anabolic/catabolic cycle, which led to further mobilisation and depletion of the maternal nutrient stores to sustain a normal fetal growth rate. It impacted on the dams' weight gain which was significantly lower in IF dams during pregnancy. As a consequence, a greater fall in maternal glucose concentration and aggravated insulin resistance occurred, increasing the risk of gestational diabetes mellitus (Mirghani and Hamud, 2006).

The IF rat model also gave an insight into the effect of intermittent fasting on maternal organs. In normal pregnant rats, both liver and kidney weights increase significantly with the progression of pregnancy (Davison and Lindheimer, 1980; Borlakoglu et al., 1993; Kuriyama et al., 2000; Cornock et al., 2010). The weight increases happen due to a transitory swelling of parenchymal cells of the liver (Borlakoglu et al., 1993) and an increase in water content in the kidney (Davison and Lindheimer, 1980). Intermittent fasting in the present model led to a reduction in the relative liver weight and an increase in the relative kidney weight. The alteration in liver weight may be caused by depletion of glycogen content, changes in parenchymal cells and functionality of the liver, as has been seen previously in food-restricted pregnant rats (Kuriyama et al., 2000). Renal hypertrophy, on the other hand, may be caused by increased water content and an alteration in renal function. Pregnant women who fasted for more than 17 h/d also showed an alteration in renal function, with an increase in blood urea nitrogen and potassium concentrations, haematocrit, and blood and urinary neutrophil gelatinase-associated lipocalin (NGAL), an early biomarker for renal injury (Bayoglu Tekin et al., 2016). These findings provide evidence of a greater risk of renal dysfunction in pregnant women as a consequence of Ramadan fasting.

Overall, the data suggest that the IF rat model replicates some of the effects of Ramadan fasting on pregnant women. The study provides evidence that intermittent fasting has a negative impact on the mother's health in terms of food consumption, weight gain, organ weights and metabolic status (Figure 5.1). Further work should address the impact of intermittent fasting at different stages of pregnancy and examine changes in maternal renal function and liver metabolism. Nevertheless, the results of the current study are valuable as they give an insight into the risks that Muslim women undertake by fasting at any stage during pregnancy, regarding the buildup of their nutritional stores and their ability to provide adequate nutrition for the growth of their babies as well as the correlating time frames within which these processes take place and that adequate compensation for food deprivation does not occur.

5.1.2.2 The fetal phenotype

The impact of pregnant Muslim women fasting during the month of Ramadan on fetal health has been studied previously. Fetuses exposed to Ramadan fasting for 10 h/d showed reductions in fetal breathing movements which were attributed to maternal hypoglycaemia,

highlighting the sensitivity of fetal organ functionality to a low concentration of glucose (Mirghani et al., 2004). In more recent studies where the duration of Ramadan fasting was more than 17 h/d, decreases in fetal biparietal diameter, fetal head circumference and fetal femur length (Sakar et al., 2015) along with birth weight (Savitri et al., 2014) were found - possible indicators for a degree of fetal compromise. Inadequate fluid intake and dehydration while fasting during summer also negatively impacted the amniotic fluid index (Seckin et al., 2014; Sakar et al., 2015).

Sex-specific differences in response to Ramadan fasting have also been reported. Male fetuses exposed to Ramadan fasting in mid-gestation were taller and the ratio of head circumference to bodyweight was significantly greater than in girls who had a shorter gestational period. It was seen that in boys, brain growth was protected at the cost of trunk growth (Alwasel et al., 2011). Furthermore, the birth rate of boys during or following the month of Ramadan is decreased, likely caused by death *in utero* (van Ewijk, 2011). Therefore, one of the major objectives of this research was to determine the effect of maternal intermittent fasting on fetal development in a sex-specific manner.

Maternal intermittent fasting did not affect litter size, but restricted fetal growth as fetal weights and anthropometric measurements, in particular fetal length and head circumference, were reduced in both sexes. The fetal weights for liver, brain and kidney relative to bodyweight were also reduced in proportion to the growth restriction in IF fetuses. Under nutritional insult in the womb, the metabolism of the fetus is modulated to protect its survival and the preservation of its most important organs like the brain, at the cost of the growth and development of more peripheral organs, especially relevant in the IF dams where there was a decrease in blood glucose concentration observed. To measure the impact of restricted intrauterine growth on vital organ growth in the fetus, the fetal brain/liver weight ratio was calculated as an indicator of the fetal brain being spared at the expense of abdominal organs (Mitchell, 2001). Both fetal sexes of the IF group displayed an increase in brain/liver weight ratios, indicating that the fetal brains were protected despite the reduced maternal food intake and associated low blood glucose concentration.

Although the brain weights in the IF group were spared, this does necessarily mean that structure was normal. According to Zhang et al. (2010), 50% food restriction during gestation and lactation caused male offspring to exhibit a reduction in the density of nitric oxide synthase neurons in the hippocampus, leading to impaired learning and memory function at postnatal day 70. Also, a low-protein diet during the first two weeks of pregnancy hindered astrogenesis, neuronal differentiation and programmed cell death in the cerebellum and cortex of offspring during the first 2 weeks of age (Gressens et al., 1997). Moreover, Saintonge and Côté (1987) found a positive correlation between maternal glucose index and brain cell number and

myelination. These studies imply that maternal intermittent fasting may lead to an altered the development of the fetal brain.

The present study also showed that maternal intermittent fasting increased the risk of congenital malformations such as snout face deformity, exencephaly and stillbirth among female fetuses only (Chapter 7, Appendix). Nevertheless, these malformations were rare, affecting only 12 female fetuses out of 36 litters. These results can be linked to ketone formation following prolonged intermittent fasting and maternal low blood glucose (Metzger et al., 1982). Ketones diffuse easily from the maternal circulation to the fetus, giving rise to neurological malformations (Hunter and Sadler, 1987). For instance, mice fetuses exposed to food restriction for a period of 24 to 30 h displayed vertebral, costal deformities and exencephaly (Runner and Miller, 1956). In an *in vitro* study, mice embryos at GD 9 exposed to D-β-hydroxybutyrate, a ketone body, for 24 h developed neurological malformations and growth restriction (Hunter and Sadler, 1987).

Importantly, the rat model allowed an assessment of changes in the fetal environment against the background of altered maternal metabolism. Exposure to maternal intermittent fasting impaired not only overall growth but also altered fetal metabolic status. During normal pregnancy as fetal growth accelerates with the progression of gestation, fetal insulin concentration significantly increases to promote glucose utilisation by the fetal organs leading to lower fetal plasma glucose concentration (Girard et al., 1977; Hay, 1991; Lesage et al., 2002). It is worth noting that the IF fetuses in the current study had hypoinsulinaemia, predicted to be a factor contributing to the growth restriction that was observed. However, unexpectedly, fetal plasma glucagon and glucose concentrations did not differ significantly from those of the control group. Thus, the IF fetuses were able to maintain normal glucose concentrations in spite of maternal low blood glucose concentrations. The interesting question that arose from these observations was how was fetal glucose concentration maintained in IF fetuses and what were the potential sources of fetal glucose: were placental glycogen or fetal liver glycogen involved? Therefore, both tissues were analysed for glycogen content and only the fetal liver of the IF group showed a reduction in the glycogen stores. Previous studies have shown similar depletion of fetal liver glycogen content and activation of gluconeogenesis under prolonged starvation of pregnant animals (Ballard and Oliver, 1964; Goodner and Thompson, 1967).

Fetuses exposed to maternal intermittent fasting also showed reductions in essential and non-essential amino acid concentrations which mirrored reductions in their mother's circulation. Alanine was one of the amino acids that was impacted the most, as were the BCAA which were reduced in both sexes of IF fetuses. Whilst alanine is a preferred substrate for system A amino acid transporter, it is worthy of comment that other system A amino substrates such as glycine and glutamine were unaffected, highlighting that there were selective effects on both maternal and fetal amino acid concentrations. Yet, the transplacental transfer of ^{14}C-MeAIB, a system A

substrate, was diminished in the IF dietary group. This leads to the speculation that the differential effect on fetal plasma concentration of amino acids that serve as substrates for a common transport mechanism, reflects the overlapping amino acid substrate specificity for several distinct transport mechanisms, as well the potential influence of placental metabolism. Sex-specific differences also emerged: males of the control group had higher concentrations of lysine, arginine and tyrosine compared to control females. However, females responded differently to intermittent fasting with reductions in phenylalanine, tryptophan and tyrosine concentrations, indicating an alteration in system L activity. It is therefore interesting to note that reductions in placental system A and system L activities are implicated and that this is associated with a fetal growth restricted phenotype, mirroring the observations made in human pregnancy (Mahendran et al., 1993; Glazier et al., 1997; Jansson et al., 2002b; Jansson and Powell, 2007; Sibley, 2009). Despite the reduction of maternal amino acid concentration, fetuses of the IF group were still able to maintain significantly higher plasma amino acid concentrations relative to maternal concentration, consistent with the preservation of active placental transport mechanisms. Further, the fetal to maternal amino acid concentration ratio of some amino acids showed a significant increase in the IF dietary group; this may reflect diminished maternal amino acid concentration as well as reduced fetal tissue amino acid extraction.

Collectively, maternal intermittent fasting impaired fetal growth, with impacts on organ weights, and altered fetal metabolism in a sex-specific manner (Figure 5.1), which may have detrimental metabolic consequences in the offspring's later life (Figure 5.2). This negative influence on fetal health therefore provides a strong rationale to propose that pregnant Muslim women should be discouraged from fasting during Ramadan.

5.1.2.3 The placental phenotype

Babies who had been exposed to Ramadan fasting during the second or third trimester of pregnancy had reduced placental weight at birth, but achieved normal birth weight (Alwasel et al., 2010a). This suggests that placental efficiency was increased and the placentas were able to preserve their activity adequately and sustain fetal growth despite their reduced size (Alwasel et al., 2010a).

In the present rat model, the effect of maternal intermittent fasting was assessed not only on placental weight, but also the morphology and the functionality of the placenta by investigating nutrient transport via system A using *in vivo* and *in vitro* approaches. Furthermore, placental metabolomics were also analysed. Maternal intermittent fasting did not affect placental weight or placental morphology, although placental efficiency was reduced in both sexes of the IF group. Interestingly, sex-specific differences were found with respect to placental morphology. Female placentas had a smaller labyrinth but larger junctional zones, relative to total placental

area: the opposite was seen in males. A previous study on Sprague-Dawley rats at GD 18 had identical findings (Reynolds et al., 2015). A detailed histological study has not been conducted on human placenta exposed to Ramadan fasting. However a study by Alwasel et al., (2014) demonstrated that placentas of male babies had greater thickness than that of the females, whereas female placental surface areas differed according to different geographical regions.

To explore the mechanism behind the growth restriction of the IF fetuses, activity of the placental system A amino acid transporter was studied. Previous studies have reported alterations in system A activity in the placenta in response to nutrient deprivation (Jansson et al., 2006; Coan et al., 2010; 2011) linked to the onset of FGR in rats (Jansson et al., 2006) and the severity of FGR in humans (Glazier et al., 1997). *In vivo* maternofetal clearance of ^{14}C-MeAIB across the placentas of IF fetuses was significantly reduced in both sexes. However, Na$^+$-dependent uptake of ^{14}C-MeAIB into isolated plasma membrane vesicles *in vitro* was unaltered in the placentas of IF fetuses. There are several possible explanations for the differences between the outcomes observed in the two methods. First, the data were normalised to different divisors: placental weight for maternofetal clearance of ^{14}C-MeAIB and vesicle protein content for vesicle uptakes. Secondly, environmental regulatory components may contribute to the down-regulation in system A observed *in vivo* which are lost *in vitro*. Thirdly, transport across the basal trophoblast plasma membrane (i.e. the fetal-facing plasma membrane of syncytiotrophoblast layer III) could be the rate-limiting step in this scenario.

To gain further insight, mRNA and protein expression of placental system A transporter isoforms were measured. At the mRNA level, sex differences were apparent in response to the intermittent fasting regimen; male placentas of the IF group displayed significant increases in the gene expression of *Slc38a1*, *Slc38a2* and *Slc38a4* whereas no changes were observed in IF females. Sex-specific effects on *Slc38a* isoforms have been reported previously in which pregnant rats exposed to high fat (HF), high salt (HS), or high fat and salt (HFSD) diet showed an increase in *Slc38a2* and *Slc38a4* mRNA in male placentas, but no effects in female placentas (Reynolds et al., 2015). At the protein level, SNAT1 and SNAT2 expression were found to be unchanged in the isolated plasma membrane vesicles derived from the maternal-facing plasma membrane of syncytiotrophoblast layer II of IF placentas compared with controls. If SNAT1 and SNAT2 protein expression matches to system A activity, this would be entirely consistent which the results from the *in vitro* study, which demonstrated that system A activity, as measured by ^{14}C-MeAIB uptake into plasma membrane vesicles, was unchanged in IF vesicles compared to controls. Therefore, as SNATs proteins, the key to system A transporter activity, and *in vitro* system A activity were unaltered, this indicated that the reduction in maternofetal clearance occurs downstream of this plasma membrane. Also, it is important to keep in mind that these results reflect a single point in gestation and FGR could be manifest due to changes in system A activity prior to this gestational day. Furthermore, changes in mRNA levels are not always mirrored by changes in protein expression; the absence of a direct

relationship between mRNA and protein expression for these SNAT isoforms has been documented previously by Desforges et al. (2006) and Jansson et al. (2006).

The observation that uptake of the system A substrate ^{14}C-MeAIB into vesicles across the maternal-facing plasma membrane of syncytiotrophoblast layer II was unaffected in the IF dietary group, whilst some system A amino acid substrates in fetal plasma were altered, led to an investigation profiling placental metabolites to try and gain a more complete understanding of how placental function might be affected by maternal intermittent fasting. Two clear findings were revealed. The first was that intermittent fasting resulted in alterations in 28 placental metabolites, which were generally raised in both sexes. In particular there was a marked increase in ophthalmic acid, a marker of oxidative stress (Soga et al., 2006), in both sexes of IF placentas. This observation implies that intermittent fasting is associated with the induction of oxidative stress, which is known to contribute to FGR. Oxidative stress has been reported to increase towards the last third of Ramadan fasting and to persist for a month beyond Ramadan in men and non-pregnant women (Faris et al., 2012). The potential impact on pregnant women has not been documented, but this aspect is certainly worthy of further investigation, especially as oxidative stress in the placenta is associated with adverse pregnancy outcomes (Myatt and Cui, 2004).

The second finding was that male and female offspring exhibited different adaptations in response to intermittent fasting; male placentas showed an increased in aromatic amino acids (tryptophan, phenylalanine and tyrosine) whereas female placentas did not. These results are in agreement with the fetal amino acid data in which males of the IF group had higher aromatic amino acid concentrations compared to the females. All of these amino acids are transported by system L, which gives rise to the speculation that system L may be upregulated in the male placentas of the IF group relative to their female counterparts.

Collectively these observations suggest that maternal intermittent fasting has impacts on placental metabolism and function with some effects manifest in a sex-specific manner (Figure 5.1).

5.1.3 The short- and long-term effect of prenatal intermittent fasting on birth weight, growth trajectory and cardio-renal function of the offspring

5.1.3.1 Birth weight, organ weights and body growth trajectory

Previous studies have reported that the effects of exposure to Ramadan fasting on birth weight have yielded conflicting outcomes. Several studies have reported that exposure to Ramadan fasting during pregnancy did not affect neonatal birth weight (Cross et al., 1990; Arab and Nasrollahi, 2001; Kavehmanesh and Abolghasemi, 2004; Alwasel et al., 2010a; Barker et al.,

2010a; Ozturk et al., 2011; Seckin et al., 2014; Daley et al., 2017). On the other hand, other studies conducted in Michigan (Almond and Mazumder, 2011) and the Netherlands (Savitri et al., 2014) found that newborns exposed *in utero* to Ramadan fasting during early pregnancy had low birth weight. These contradictory findings between the studies could depend on the season (length of the day) and the trimester of the pregnancy in which Ramadan fell. In this context, Herrmann et al. (2001) found a correlation between pregnant women fasting for 13 h or longer and a risk of preterm delivery. Additionally, Prentice et al. (1983) found that newborn babies who were exposed to prenatal Ramadan fasting showed increased incidences of special care unit admissions. In contrast, most of the previous studies (Kavehmanesh and Abolghasemi, 2004; Ozturk et al., 2011; Awwad et al., 2012; Petherick et al., 2014), including a recent report by Daley et al. (2017), did not find any evidence of preterm delivery in response to Ramadan fasting.

In the current rat model, the impact of maternal intermittent fasting during pregnancy on birth weight, neonatal organ weights and offspring body growth trajectories was determined. The study found that maternal intermittent fasting affected neither the litter size nor the mean birth weight (PD 1). As the IF fetuses were lighter than controls at GD 21, this means that the fetuses in the IF dietary group must have undergone a phase of rapid growth during the last 2 days of gestation, with a 97% increase in their body mass, to achieve a similar birth weight as the control group. However, despite the equal birth weights, all IF dams had preterm deliveries by half a day. These findings accord with observations made with respect to Ramadan fasting in humans (Herrmann et al., 2001; Alwasel et al., 2010a; Seckin et al., 2014). In contrast, most other maternal protein- and calorific-restriction studies have not shown any inclination towards preterm delivery; yet neonatal birth weight remains stunted (Lucas et al., 1997; Vickers et al., 2000; Almeida and Mandarim-de-Lacerda, 2005; Desai et al., 2005a; Alwasel et al., 2010b). The fetuses subjected to intermittent fasting *in utero* may overcome the restriction of intrauterine growth at GD 21 and reach normal body weight at birth by an increase in the efficiency of the placenta to transport nutrients, coupled with further mobilisation of maternal body reserves. Therefore, it is notable that the phenotype of the IF model differs in these respects from other models of food restriction during pregnancy, and this may be related to the repeated cyclical pattern of restricted maternal food intake in the IF model. In this context, the daily feeding/fasting rhythms that are usually harmonised to the animal's activity/rest cycle and which regulate the synchronised transcriptional regulation of anabolic and catabolic processes, are going to be perturbed, and this is likely to lead to ensuing derangement of circadian signalling pathways that drive effects on transcription, the downstream production of metabolites and gut microbiota abundance that result in altered metabolic homeostasis (Longo and Panda, 2016).

Even though the neonates of the IF group had normal birth weight, there were differences in the neonatal organ weights. The neonates had a significant reduction in relative kidney weight

in both sexes and a reduction in relative brain weight in female neonates. Remarkably, the fetal brain which was protected at GD 21, was shown to be altered at birth, as indicated by a significant reduction in the fetal brain/liver weight ratio in both sexes of the IF group. Body growth was asymmetrical, with the brain affected differently to other organs. Interestingly, children who were exposed to Ramadan fasting, especially during the first trimester when organogenesis occurs, showed a higher number of sensory and mental disabilities (Almond and Mazumder, 2011). Different findings have been reported in other undernutritional studies where 'brain sparing' phenomena were prevalent (Desai et al., 1996; Saito et al., 2009; Agale et al., 2010), again emphasizing that the IF model has a different phenotype in the offspring compared to other programming models. Hence, interestingly, the IF model presents a contrasting dietary programming model to other nutrient-perturbed programming models to investigate the complexities of metabolic and epigenetic regulation that may underlie these distinctive phenotypes. A postnatal behaviour study that was carried out in our laboratory revealed a cognitive deficit in the offspring which was induced by maternal intermittent fasting throughout pregnancy or even during the first three days of gestation in a sex-specific manner (Dr Nick Ashton, personal communication). Female offspring showed impaired recognition memory starting at the juvenile stage, whereas males developed memory loss at adulthood. Since the maternal glucose index positively related to fetal brain neuronal myelination and numbers (Saintonge and Côté, 1987), low blood glucose in IF dams may negatively influence the fetal brain. These results are in contrast to what was reported by Gressens et al. (1997), who stated that the brain has the plasticity to overcome any alteration in brain architecture induced by protein-restriction during pregnancy, whereas the changes that occurred *in utero* in response to maternal intermittent fasting have a permanent impact.

Although birth weights were unaffected in IF offspring, maternal intermittent fasting altered their growth trajectory in a sex-dependent manner. Male IF pups exhibited a sudden, significant slowing of weight gain from PD 18 until week 10. It is interesting to note that the decrease in growth rate in the male IF offspring coincided with the shift towards eating solid food, which normally starts at 16 days of age in rats (Thiels et al., 1990). Indeed, there was a significant reduction in food consumption (relative to bodyweight) in IF males at postnatal weeks 5 and 6. One reason behind this could be an increase in leptin secretion or/and sensitivity which stimulates energy expenditure and lowers food consumption (Friedman and Halaas, 1998). Such an increase in leptin concentration may be associated with hypothalamic leptin resistance and thus increases in body weight gain later in adulthood. This speculation warrants further investigation, but the above notion is supported by various previous studies. Vickers et al. (2005) demonstrated that undernutrition during pregnancy programmed the rat offspring's appetite and reduced energy expenditure; conditions that were reversed by leptin administration during the first 10 days after birth. Song et al. (2007), on the other hand, showed that LP offspring at postnatal weeks 4 and 12 had higher plasma leptin concentrations associated with leptin resistance. With respect to Ramadan fasting, no similar reports have

been published which describe the effects of fasting exposure *in utero* on the growth patterns of the offspring during childhood and young adulthood. However in the Dutch famine, and in contrast to the findings of this study, male offspring became obese at 19 years of age if they were exposed to famine over the first two trimesters of pregnancy (Lumey et al., 1993; Ravelli et al., 1999; Selassie and Sinha, 2011), whereas female offspring developed obesity due to maternal undernutrition at any stage of pregnancy (Ravelli et al., 1999), suggesting that they were protected from any potential effects of leptin resistance. This divergence between the sexes implies differences exist in the regulatory mechanisms that underlie these phenomena and this is of particular interest in the context of this study, as in contrast to males, the growth curves of female IF offspring did not differ from those of control females, again highlighting sex-dependent differences in post-natal developmental trajectories.

Together, these observations demonstrate that maternal intermittent fasting has impacts on post-natal growth trajectory, with sex-dependent differences apparent (Figure 5.2). Intriguingly, although IF fetuses showed restriction of growth at GD 21, at birth (PD 1) they had achieved the same weight at their control counterparts, suggesting highly adaptive mechanisms were invoked in the later stages of pregnancy, and the impact of these phenomena on the future development of metabolic disease was further investigated.

5.1.3.2 Offspring cardio-renal function

Epidemiological and animal studies have shown that restricted weight or disproportionate organ weights at birth, as observed in this study, increase the risk of developing hypertension, coronary heart disease and diabetes in offspring adulthood (Barker et al., 1992; 1993; Langley-Evans et al., 1996a). Moreover, protein- or calorific-restriction for a short period of pregnancy or throughout gestation raised blood pressure in rat offspring from a young age (Langley-Evans et al., 1994a; Woodall et al., 1996; Kwong et al., 2000; Manning and Vehaskari, 2001; Sahajpal and Ashton, 2003; Alwasel and Ashton, 2009) and beyond, with a sustained elevation at 10 weeks (Ellis-Hutchings et al., 2010), 12 weeks (Manning and Vehaskari, 2001), 21 weeks (Langley and Jackson, 1994) which may persist for the rest of their lives (Langley-Evans et al., 1994a; Woodall et al., 1996).

The mechanisms responsible for the widely reported increases in blood pressure are not fully understood; however the kidney is likely to be involved. The kidney is an organ that is particularly vulnerable to maternal nutrient restriction, leading to impaired fetal nephrogenesis which ultimately may contribute to offspring hypertension (Sahajpal and Ashton, 2003; Alwasel et al., 2010b). For example, studies on calorific restriction (Lucas et al., 1997; Vickers et al., 2000; Brennan et al., 2008) and protein restriction in rats (Woods et al., 2001; Vehaskari et al., 2001; Courrèges et al., 2002; Sahajpal and Ashton, 2003; Alwasel et al., 2010b) have consistently shown that a poor maternal diet has a negative impact on kidney weight and

nephron number. Such an observation suggests that nephron endowment may be altered in the smaller kidneys of IF neonates. Future studies should confirm nephron number in the IF offspring using non-biased stereology techniques.

On the basis of the widely reported association between poor maternal nutrition and development of hypertension in the offspring, the main objective of the second half of the study was to determine the effects of intermittent fasting during pregnancy on offspring blood pressure, glucose tolerance, insulin resistance and renal function.

Surprisingly, maternal intermittent fasting did not induce hypertension in either sex of the IF offspring at 5, 7, 10 or 14 weeks of age. We cannot exclude the possibility that hypertension may manifest at later stage of life beyond 14 weeks of age, but on the basis of the available data presented in this study, it seems plausible that perturbation of nutrient intake during pregnancy does not always cause an increase in offspring blood pressure. Consistent with the lack of increase in blood pressure, ECFV was also unaltered in the IF offspring.

At 12 week of age, both sexes of the IF offspring failed to display any sign of glucose intolerance or insulin resistance, indicative of the onset of diabetes. However, development of diabetic traits may not appear until after this age. For example, previous studies have suggested that maternal malnutrition *in utero* altered offspring glucose and insulin tolerance only during the later stages of life. Ozanne et al. (1996) found that at 3 months of age, LP male offspring showed improved glucose tolerance and increased skeletal muscle insulin sensitivity. However, as the offspring reached 15 months of age, they developed glucose intolerance (Ozanne et al., 2003). Female LP offspring, on the other hand, were able to maintain normal glucose and insulin concentrations up to 15 months of age (Hales et al., 1996; Sugden and Holness, 2002) but by the age of 21 months, they had developed insulin resistance associated with a higher risk of type 2 diabetes (Fernandez-Twinn et al., 2005). Thus, there is the possibility that IF offspring may go on to develop insulin and glucose intolerance after 12 weeks of age. Further studies will be required to confirm or refute this notion.

Various studies of prenatal nutritional perturbations have reported long-term effects on renal function in the offspring that were associated with nephron deficit and high blood pressure (Almeida and Mandarim-de-Lacerda, 2005; Alwasel et al. 2010). However in the present study, offspring exposed *in utero* to intermittent fasting had normal renal function at 14 weeks of age, despite having smaller kidneys at birth. One explanation for the normal renal outcome is that the IF offspring were able to overcome a speculated nephron deficit and maintain normal function through an increase in filtration area as a result of hyperfiltration by the remaining individual nephrons. This speculation is based on previous studies in which offspring subjected to protein restriction *in utero* were born with 13% to 28% nephron deficits but had unchanged GFR due to hyperfiltration of the remaining glomeruli (Langley-Evans et al., 1999; Manning and

Vehaskari, 2001). In addition, Woods et al. (2001a) reported that LP offspring with a 25% reduction in nephron number only had an 11% decrease in the total GFR per gram kidney weight. This apparent mismatch was explained by a 20% increase in the GFR of remaining individual nephrons.

Overall, this preliminary assessment of the offspring suggests that in young adults exposure to intermittent fasting *in utero* has no effect on basal cardio-renal function, at least up to 14 weeks of age (Figure 5.2). Thereby this research highlights that maternal intermittent fasting induced a different phenotype to calorific- and protein-restriction models where the offspring developed hypertension at a very young age (4 weeks of age). However, these outcomes do not exclude the possibility of alterations in common genes or gene pathways, the gatekeepers, that have been proposed to be altered by any type of nutritional insult *in utero*, which may manifest in metabolic disorders at later stages of life. An identification of an impact of intermittent fasting on the gatekeepers deserves to be investigated.

5.1.3.3 Offspring cardio-renal function in response to postnatal salt loading challenge

Development of hypertension is linked with higher salt consumption (Meneton et al., 2005) which can be programmed *in utero*. In humans, offspring demonstrated enhanced salt preference when the mother suffered moderate to severe emesis during pregnancy (Crystal and Bernstein 1995; 1998; Leshem, 1998). Pregnant women who fast during Ramadan have been reported to be at greater risk of hyperemesis gravidarum (morning sickness) which became worse during the first month of pregnancy (Rabinerson et al., 2000; Joosoph et al., 2004). In rats, administration of polyethylene glycol during pregnancy, to mimic human vomiting episodes during pregnancy, induced a greater saline preference among treated offspring (Nicolaidis et al., 1990). Protein-restriction during pregnancy also proved to programme salt appetite in LP offspring at 4 weeks of age as a result of renal sodium wasting (Alwasel et al., 2012). Consequently, this study examined salt preference and salt aversion in the offspring of the IF group to determine whether the maternal environment affected salt sensitivity. Female offspring of both the control and IF groups had greater salt preferences than the respective male offspring; females of the IF dietary group also exhibited a higher threshold for salt aversion than control females. Maternal intermittent fasting, therefore, programmed salt preference in a sex-specific manner. With regards to the salt loading challenge, the females of the IF group consumed more high-salt diet than males from week 11 onward, which is consistent with the enhanced salt preference of female rats. The salt loading was associated with polydipsia and altered growth trajectory. The salt-loaded females of the IF group were lighter, whereas salt-loaded IF males were heavier, than IF offspring on a standard chow diet. This suggests that salt-loaded males of the IF group increased their body weight due to water and sodium retention. Why this did not occur in the IF females too is currently unclear.

One mechanism through which salt appetite may be programmed is an alteration in brain structure, specifically that of the hypothalamus. Offspring that were protein-restricted during gestation and lactation had reduced total brain volume and hypothalamic paraventricular nucleus volume in parallel with decreased staining of the key peptides involved in appetite control (Plagemann et al., 2000). Central renin-angiotensin activity also plays a role in the programming of salt appetite in LP offspring (Pladys et al., 2004; Fitts et al., 2007) as expression of brain Ang II receptors (AT_1) has been found to be greater in LP offspring in comparison to controls. In a separate study in our laboratory, female offspring of the IF group were shown to have a reduction in relative brain weight at PD 1 which was associated with a cognitive deficit at weaning (Dr Nick Ashton, personal communication). Alterations in the structural development of the brain may therefore predispose the females of the IF group to an increased salt appetite, and this merits further investigation.

Salt loading elevated offspring blood pressure in both control and IF groups at 7 and 10 weeks of age respectively, without any apparent significant differences between the two dietary groups. However, the increase in blood pressure from 5 to 10 weeks was of greater magnitude in IF male offspring (16.5 mmHg) than other groups, reaching a significant difference in comparison to females of the control group (11 mmHg). A previous study by Stewart et al. (2009) reported that protein restriction during gestation induced hypertension in LP offspring that was exacerbated when fed a high-salt diet after birth; on the other hand, a low-salt diet had no effect on blood pressure. Hence, hypertension is salt-sensitive in the LP model. This finding is supported by Alwasel and Ashton (2012) who reported similar salt sensitivity and a rightward shift in the pressure natriuresis curves for LP rats. One mechanism leading to the development of hypertension in response to a high-salt diet is sodium retention and expansion of the ECFV (Manning and Vehaskari, 2001; de Wardener et al., 2004; Meneton et al., 2005).

Salt loading caused renal hypertrophy in both dietary groups, either as a consequence of water retention or hypertension. In the present study, a high-salt diet and an increase in blood pressure were matched with significant increases in renal haemodynamics and electrolyte handling in the control group, which is in agreement with previous studies showing either an increase or no change in GFR in offspring challenged with salt loading (Sanders et al., 2004; Dickinson et al., 2007; Ruta et al., 2010). Pressure natriuresis plays a mechanistic role in maintaining a sodium-fluid balance (Hall et al., 2012). An increase in the body sodium content with salt loading leads to an expansion of ECFV. Therefore, a right-ward shift in the pressure-natriuresis relationship occurs, causing blood pressure to elevate to match sodium excretion to sodium intake (Hall et al., 2012).

Salt loading revealed impairments in renal functional, albeit of a modest nature, in adult male offspring that had been subjected to intermittent fasting *in utero*. Male IF offspring showed significant decreases in FF, osmolar excretion and urine creatinine concentration in comparison

with controls. GFR and Na$^+$ and Cl$^-$ excretions also tended to be lower in the IF males, but this reduction failed to reach statistical significance. The trend towards a reduction in electrolyte excretion could be attributed to a fall in GFR or changes in tubular sodium reabsorption; the lack of change in fractional excretion of Na$^+$ points to the former as being the more likely mechanism. The salt-loaded IF males also had significantly higher urine albumin:creatinine concentration ratios than their IF female counterparts, which is indicative of kidney damage, in particular the selective permeability of the glomerular basement membrane, leading to albumin leakage. The salt-loaded IF females, however, did not show a significant difference in renal function compared with the control females, and also none in comparison to the IF females fed standard chow. Therefore, these data suggest that the male IF offspring failed to adapt to salt/fluid loading adequately, whereas the females were able to accommodate the increase in salt intake. This postulate is supported by the significantly higher concentration of NGAL, a marker of renal injury, in the urine of IF male rats compared to control males.

The mechanisms through which the salt-induced renal damage occurs in IF males requires further investigation. Evidence from other programming models points towards altered renal tubular sodium transport as a primary mechanism leading to the development of hypertension. Dagan et al. (2007) found that offspring treated with dexamethasone during pregnancy displayed an increase in proximal tubule volume absorption accompanied by an up-regulation in Na$^+$/H$^+$ exchanger isoform 3 (NHE3) protein expression and activity. Manning et al. (2002) proposed that young LP offspring retained more sodium as a result of an increase in both mRNA and protein expression of two renal sodium transporters, the bumetanide-sensitive Na-K-2Cl co-transporter (BSC1) and the thiazide-sensitive Na-Cl co-transporter (TSC), which in turn would lead to the onset of hypertension. However, Manning et al. (2002) based their conclusions on a molecular assessment of renal sodium transporter expression alone, without assessing sodium handling *in vivo*. Alwasel et al. (2009; 2012), on the other hand, showed that while young LP offspring exhibited up-regulation in mRNA and protein expression of BSC1 in response to increased sodium delivery to the thick ascending limb of the renal tubule, the transporter was not functional *in vivo* and did not increase sodium retention. In fact, LP offspring showed a sodium-wasting phenotype rather than evidence of sodium retention (Alwasel et al., 2009; 2012). Hence, there is evidence that renal sodium transporter expression and activity are altered in other dietary programming models, but this has not been explored in the IF rat model as yet. Further investigation of these aspects would better characterise the molecular mechanisms underlying the renal dysfunction observed.

Overall, it remains unclear whether IF offspring have a nephron deficit, although they do have lower kidney weights at birth. Nonetheless, any putative nephron deficit was not enough to produce overt renal disease or hypertension in young adult IF offspring maintained on a standard chow diet, indicating that they have sufficient renal reserve capacity under basal conditions. However, introducing a high-salt diet for 10 weeks post-weaning, as a second stressor hit, increased their susceptibility towards manifesting renal disease, particularly in the

IF male offspring, as summarised in Figure 5.2. Hence, this facet of the study also highlighted the existence of sexual dimorphic responses in the IF offspring, and a differential susceptibility to impaired renal function. This study therefore suggests that fasting pregnant women run the high risk of predisposing their offspring to diseases later in life following a postnatal nutritional stressor.

5.1.4 SUMMARY

In summary, a novel rat model of intermittent fasting during pregnancy has been developed in order to mimic the repeated daily pattern of fasting undertaken by women during Ramadan. A number of metabolic and fetal/neonatal developmental effects associated with Ramadan fasting in pregnant women are replicated in this model (Figures 5.1 and 5.2), implying that it is a suitable tool for further investigations into the effects of Ramadan fasting on fetal development and pregnancy outcomes. It was shown that maternal intermittent fasting during pregnancy adversely affects the mother's health not only in respect of food intake and weight gain during pregnancy, but also regarding her organs and metabolic status. The fetuses *in utero* were growth restricted and exhibited altered metabolic profiles with sex-specific differences and reductions in liver glycogen contents. Placental metabolites were altered, with particularly profound changes in the IF males, but in both fetal sexes, restricted growth *in utero* was accompanied by a reduction in the *in vivo* activity of placental system A transporter that may contribute to the observed FGR. Prenatal intermittent fasting also had consequences for the offspring later in life, altering growth trajectory and feeding behaviour. It programmed salt appetite at an early adult stage only in females. The study also showed that changes in the postnatal environment, such as a high-salt challenge, identified deficiencies in the renal excretory capacity and provoked hypertension in adulthood, with sexual dimorphic responses being clearly evident.

These findings have clear connotations for pregnant Muslim women who engage in fasting during the Ramadan season. It is reasonable to hypothesise that this form of intermittent fasting has negative effects on the mother's physiology and fetal development and growth, as well as longer-term effects on the cardio-renal function of the offspring later in life. The mechanisms underlying such developmental programming require further exploration and elucidation at the molecular level. Once this information is made available to Muslim women, they will have the necessary knowledge to exempt themselves from Ramadan fasting for the health of their offspring. The global significance of empowering Muslim women in this way is far-reaching, and as recently commented on by Daley et al. (2017) could be profound, in the context that there are ~ 230 million Muslim women around the world who are of reproductive age, estimated to give birth to 713 million babies during their lifetime.

A RAT MODEL OF INTERMITTENT FASTING

Food deprived from 5 pm until 9 am from GD 1 (vaginal plug) until GD 21 (harvesting day), Term 23 days

Maternal Health

- 30% lower food intake
- ↓ weight gain
- Liver hypotrophy
- Kidney hypertrophy
- ↓ [glucose]
- ↓ [glucagon]
- Normal [insulin]
- ↓ [essential and non-essential amino acids]

Fetal Adaptation

- No effect on litter size
- FGR
- ↓ fetal length & head circumference
- ↓ organ weights
- ↑ brain/liver weight ratio
- ↓ liver glycogen contents
- Normal [glucose] & [glucagon]
- ↓ [insulin]
- ↓ [essential and non-essential amino acids]

Placental Adaptation

- No change in placental weight
- ↓ fetal:placental ratio
- Normal morphology: ↑ JZ/total area & ↓ LZ/total area in females compared with males
- Altered placental metabolomics with more profound changes in IF males
- ↓ maternofetal clearance of ^{14}C-MeAIB in both sexes
- Unaltered uptake of ^{14}C-MeAIB into placental vesicles
- Unaltered SNAT1 & SNAT2 protein expression
- ↑ *Slc38a1*, *Slc38a2* & *Slc38a4* mRNA in IF male placentas

Figure 5.1 Diagram illustrates the impact of maternal intermittent fasting during pregnancy on maternal health, fetal growth and metabolism and placental morphology, metabolomics and functionality at GD 21. Increased (↑); decreased (↓); FGR, fetal growth restriction; GD, gestational day; JZ, junctional zone; LZ, labyrinth zone; SNAT, sodium-coupled neutral amino acid transporter.
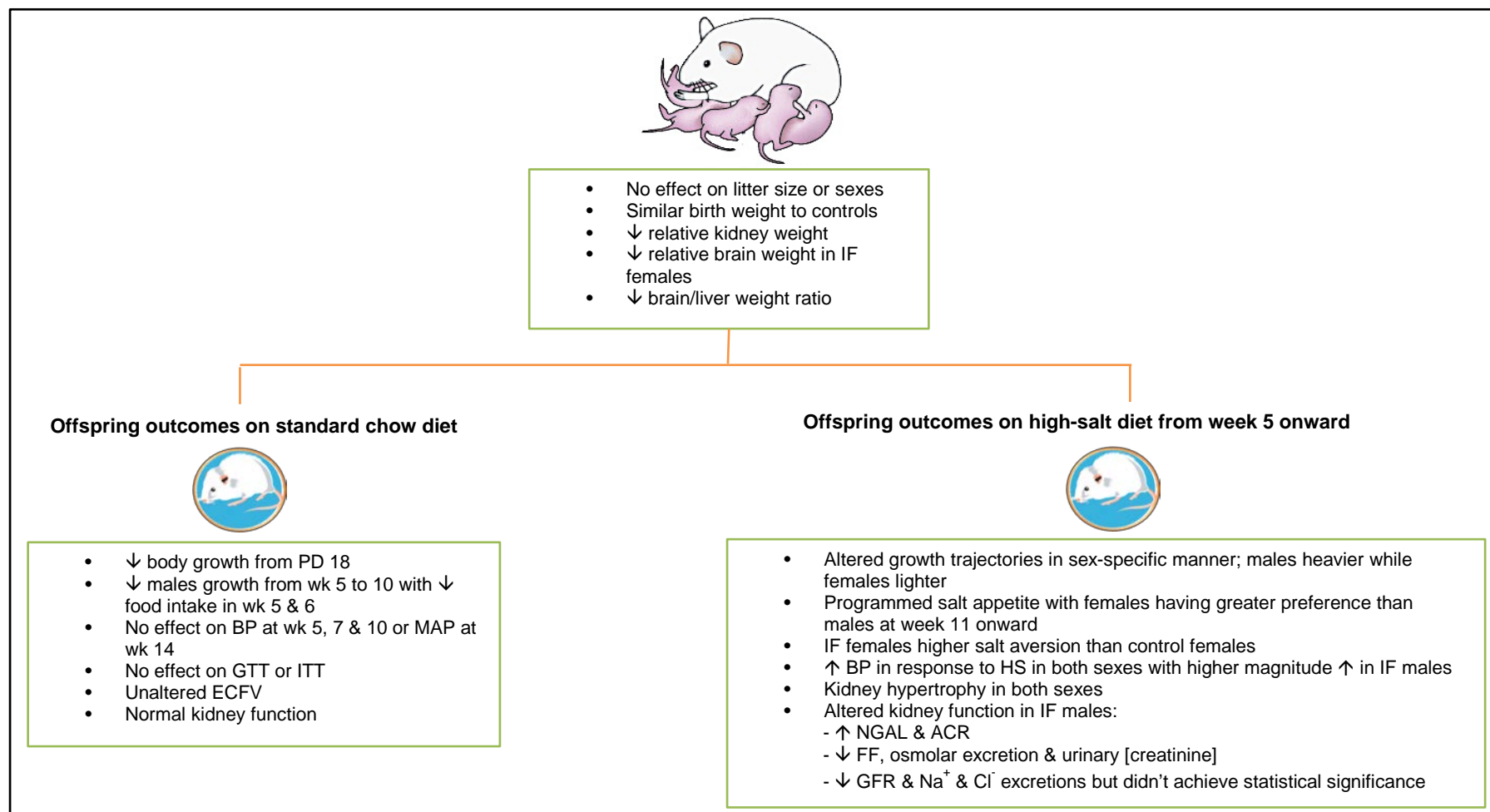
Figure 5.2 Diagram illustrates the long-term impact of prenatal intermittent fasting throughout pregnancy on offspring body growth and cardio-renal function. Increased (↑); decreased (↓); ACR, albumin:creatinine concentration ratio; BP, blood pressure; ECFV, extracellular fluid volume; FF, fraction filtration; GFR, glomerular filtration rate; GTT, glucose tolerance test; IF, intermittent fasting; ITT, insulin tolerance test; MAP, mean arterial pressure; wk, week.

5.2 FUTURE WORK

Since Ramadan fasting in humans covers only one month of the pregnancy, the broader plan is to expose the rats to a shorter period of intermittent fasting of just three days, imposed at various stages of gestation, while feeding them normally throughout the remaining pregnancy period, to recapitulate more closely the Ramadan fasting habits of pregnant Muslim women. In addition to what has been explored, further characterisation of the model and its impacts are required, with comparisons to be made between IF and control groups with respect to sex:

- Maternal environment - measurement of maternal corticosterone, the primary glucocorticoid in rats, and plasma IGF-I and leptin as hormonal markers modulated by nutrient availability.
- Maternal kidney function - using *in vivo* clearance experiments to measure the glomerular filtration rate and excretion of Na^+, K^+ and water and histological assessment to investigate the kidney hypertrophy induced by intermittent fasting.
- Placental morphology - quantitative assessment of junctional and labyrinth zones; staining of placental sections for markers of these placental zones (trophoblast specifc protein α (Tpbpa) and glial cell missing-1 protein (Gcm1) for the junctional and labyrinth zones respectively) and placental lactogen II as an endocrine marker.
- Placental function - measurement of system L amino acid transporter activity, which transports essential neutral amino acids, and expression of LAT subtypes that mediate this transporter activity using qRT-PCR, immunohistochemistry and Western blotting.
- Placental gene expression - measurement of CLOCK genes that are involved in circadian rhythms.

The second group will be allowed to proceed to term. After weaning, the pups will have free access to food. Several approaches will be used to characterise the mechanisms underlying altered growth trajectories and renal function in response to salt challenge of offspring exposed to intermittent fasting *in utero* such as:

- Performing histology to assess kidney morphological changes, and non-biased stereological assessment of nephron number, since a link between low nephron number and kidney weight is proposed in the IF group.
- Collecting renal tissue samples for histology (basic morphology, glomerular / tubular damage / collagen deposition) and quantification of key renal sodium transporters (NHE-3, NKCC2, NCC, ENaC) by Western blotting (protein) and qPCR (mRNA).
- Evaluation of the functionality of each renal tubule segment to identify the site leading to sodium retention using specific markers for each segment.
- Determining the total body sodium content.

- Identification of any alteration in the gatekeepers and microRNA profiles in the kidney and adipose tissue of IF offspring to predict the manifestation of hypertension and insulin resistance in later life.

- Determination of vascular reactivity of isolated blood vessels and measurement of vascular response to agonists, e.g. phenylephrine, acetylcholine, by myography.

- Measuring plasma leptin to outline the mechanism behind the altered growth trajectories and altered feeding behaviour in the IF offspring.

- Assessing any alteration in components of the renin-angiotensin system in the kidney or brain that may play a role in programming salt appetite and hypertension development such as renin, angiotensin II, angiotensin II receptors.

- Staining the brain for neuropeptide Y, cholecystokinin and galanin, which are the key peptides involved in appetite control (Plagemann et al., 2000).

- Measuring insulin resistance at older age of offspring rats through a glucose tolerance test and calculating the HOMA (homeostasis model assessment) estimate of beta cell function and insulin sensitivity.

CHAPTER 6

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CHAPTER 7

APPENDIX

7.1 Western blot optimisation studies for SNAT antibodies

7.1.1 Validation of SNAT antibody specificity - optimisation of conditions

(i) Sample buffer and sample preparation

Antibody binding was compared using boiled and non-boil sample buffers (Table 2.2.5) to examine optimal sample denaturation conditions. Rat placental membrane vesicles (RV) isolated at GD 21 and human placental microvillous membrane vesicles (hMVM) were prepared as described in Section 2.2.13.2.1 and used as positive controls for SNAT1 and SNAT2 expression as reported previously in rat (Rosario et al., 2011) and human placentas (Desforges et al., 2009). Protein concentration was determined by the Lowry assay (Section 2.2.13.2.2).

Aliquots of each rat vesicle and hMVM sample were diluted in dH_2O to achieve a concentration of 1 mg protein/mL. To samples, boil reducing buffer was added in a 1:1 buffer:sample ratio, heated at 100 °C for 5 min and then chilled on ice for 5 min or non-boil reducing buffer was added in a 2:1 ratio. 20 µL (20 µg protein) was loaded per lane.

Observations. SNAT1 and SNAT2 both generated immunoreactive product, with a dominant immunoreactive band of the predicted size of ~52 kDa and 56 kDa respectively, visible in both of the two lanes of rat vesicles but with less clarity in human MVM (Figure 7.1). The signals were more discrete and intense in the boiled conditions for SNAT1, and this signal was abolished by pre-absorption of antibody with pMAL fusion protein peptide. SNAT2 antibody generated more than one immunoreactive species. Apart from the band at 56 kDa, an intense diffuse species at a higher molecular weight was observed in the non-boiled condition; both signals were abolished by pre-absorption of antibody with pMAL fusion protein peptide. To gain further clarity, further optimisation steps were conducted for the SNAT 2 antibody, as detailed below.

(ii) Optimisation of buffer composition, antibody concentration and incubation conditions for SNAT 2 antibody binding

Several optimisation steps were performed for SNAT2 antibody under boiled and non-boiled conditions using rat placental vesicles: a. increasing the salt concentration in TBS from 0.15 M to 0.5 M in order to intensify a specific signal, b. reducing the primary antibody concentration (1:1000 versus 1:2000), c. comparing overnight primary antibody incubation at 4 °C followed by 2 h at room temperature with 2 h incubation at room temperature only.
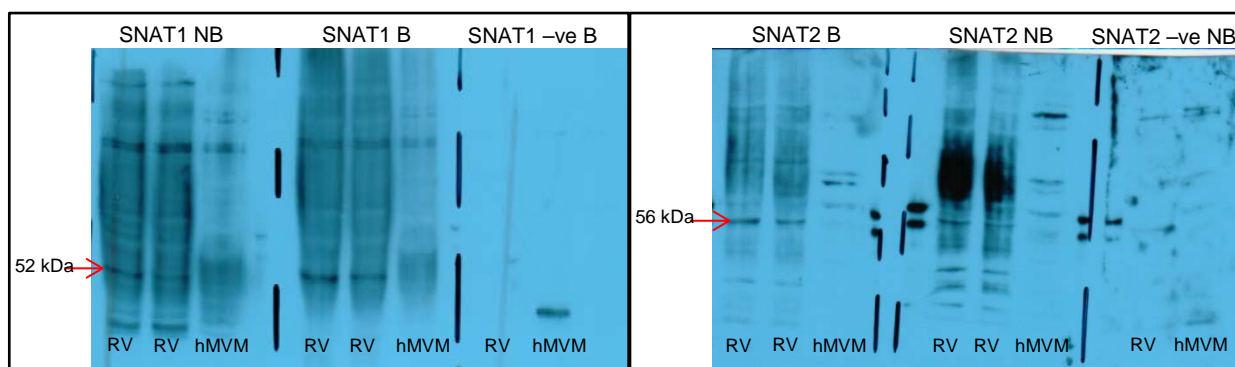
Figure 7.1 Western blot of rat placental vesicles isolated at GD 21 and human placental MVM probed with SNAT1 and SNAT2 antibodies (20 µg protein/lane). Two distinct bands at approximately 52 and 56 kDa (indicated by arrows) were observed for SNAT1 and SNAT2 (1:1000 dilution of each), respectively. Abbreviations: B, boil sample buffer; hMVM, human placental microvillous plasma membrane; NB, non-boil sample buffer; RV, rat placental vesicles; -ve, negative control using pre-absorption of antibody with blocking peptide. Exposure time 5 s for SNAT1 and 30 s for SNAT2.

Observations. Overnight incubation of the primary antibody with sample prepared in non-boil buffer generated the higher background signals, which were intensified by higher-salt TBS. As might be expected, 1:1000 dilution of antibody gave more intense signals than 1:2000 dilution (Figures 7.2 a and c). However, although signal intensity varied, banding patterns were consistent and similar across the blots irrespective of buffer salt concentration, antibody concentration or incubation condition. Multiple bands were observed, with lower ~48 kDa and higher ~140 kDa size species visible. Taken together, high-salt TBS conditions together with higher antibody concentration were considered unsuitable, and further optimization was required.

(iii) SNAT 2 antibody binding and expression – comparisons to other tissues

Due to the multiple banding pattern seen above, clarity was sought regarding the immunoreactive species identifiable in fetal rat tissues such as brain, kidney and spleen, and also rat placental lysate of the control and IF groups (a membrane-enriched fraction prepared as described by Bond et al. (2008)). From the epitope sequence, the SNAT1 and SNAT2 antibodies were predicted to be immunoreactive with mouse SNAT1 and SNAT2 respectively and so mouse placental lysate (wild-type) as well as mouse [SNAT1 + SNAT2] knockout placentas (both at GD 19 and the kind gift of Dr M Constância, University of Cambridge) were also employed to validate antibody binding specificity. Further refinements were made as regards sample and probing conditions, which were as follows: a. Samples were boiled at 60 °C and loaded with two different protein loadings (20 and 30 µg protein/lane), b. membranes were blocked with 5% non-fat dried milk in TBS with 0.1% or 0.4% Tween and c. SNAT1 and 2

antibodies were diluted 1:1000 in TBS (0.15 M NaCl) with overnight antibody incubation at 4 °C followed by 2 h at room temperature.
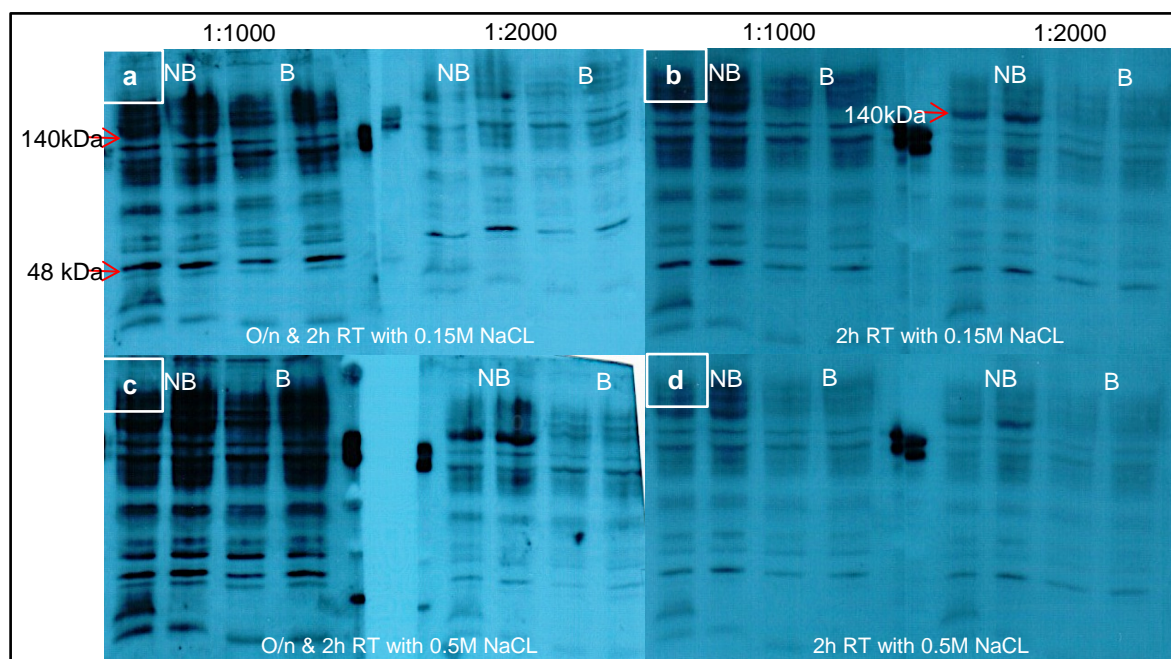

Figure 7.2 Western blots of SNAT2 expression in rat placental vesicles isolated at GD 21. Boil and non-boil conditions were used with two antibody dilutions of 1:1000 and 1:2000. **a. & c.** Primary antibody was incubated overnight at 4 °C followed by 2 h at room temperature with 0.15 M NaCl and 0.5 M NaCl TBS, respectively. **b & d.** Primary antibody was incubated for 2 h at room temperature with 0.15 M NaCl and 0.5 M NaCl TBS, respectively. A multiple banding pattern was observed with two distinct bands at approximately 48 and 140 kDa (indicated by arrows) visible. Abbreviations: B, boil sample buffer; NB, non-boil sample buffer; o/n, overnight; RT, room temperature. First two lanes are non-boiled samples, and second two lanes are boiled samples (20 µg protein/lane). Exposure time was 40 s.

Observation. For SNAT1, there was a consistent detection of a distinct band at 52 kDa in the rat placental vesicles at both protein loadings, which co-migrated with the intense and prominent band in the mouse placental lysate (Figure 7.3). However, some higher molecular weight bands were also observed in the rat placental vesicles. For the brain, a single band was seen at a slightly lower molecular weight (~48 kDa) with no band detected in the spleen, which was included as a negative tissue (Varoqui et al., 2000; Yao et al., 2000). These species were abolished in the [SNAT1 + SNAT2] knockout mouse placenta, thus providing confidence regarding antibody specificity.

For SNAT 2, a common banding pattern was broadly seen between boil and non-boil conditions and between 0.1 and 0.4% TBS-T (Figures 7.4 a and b) and the patterns of the immunoreactive species in rat placental vesicles and lysates were again broadly similar (Figure

7.4 b). However, SNAT2 showed multiple bands that did not match with the predicted size for SNAT2 (56 kDa), with some dominant higher molecular weight bands of 87 and 140 kDa visible, and a lower molecular weight of 43 kDa. Interestingly, the major species in rat placental vesicles and lysates was strikingly distinct to the signal in mouse placental lysate, which generated a very intense band at 65 kDa (Figures 7.4 a and b). Rat fetal brain demonstrated an immunoreactive product that co-migrated with that in the mouse lysate (Figures 7.4 a and b). In the rat fetal kidney, the pattern of immunoreactive species again appeared distinct to other tissues, although it appeared that it expresses the 140 kDa species in common with rat placenta (Figure 7.4 b). These complex patterns of immunoreactive species detected are likely to reflect SNAT2 proteins as in the [SNAT1 + SNAT2] knockout placenta, where both genes *Slc38a1* and *Slc38a2* genes were ablated, signals were abolished, and this occurred irrespective of buffer condition, confirming the specificity of antibody binding. Also, in order to reduce the background, the blocking time was increased from 1 h to overnight, and the washing time was extended after the primary antibody. However, no apparent differences were seen following these treatments (data not shown).
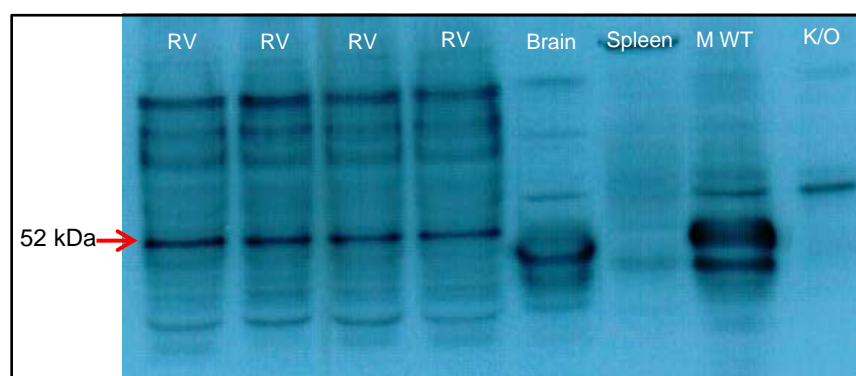

Figure 7.3 Western blot of SNAT1 immunoreactive species in various rat tissues and mouse placenta. Under boil conditions, probing with SNAT1 antibody (1:1000 dilution) showed an intense immunoreactive species at 52 kDa in rat vesicles (RV) and mouse placental lysate (M WT). The spleen, considered to be a SNAT1-negative tissue, did not show an intense band, while the SNAT1-expressing brain lysate showed an intense band at lower molecular weight. Comparison between wild-type mouse placenta (M WT) and the [SNAT1 + SNAT2] knockout mouse placental tissue (K/O) confirms abolition of SNAT1 immunoreactive species. (20 µg protein/lane for fetal tissues, M WT and K/O). Exposure time was 5 s.
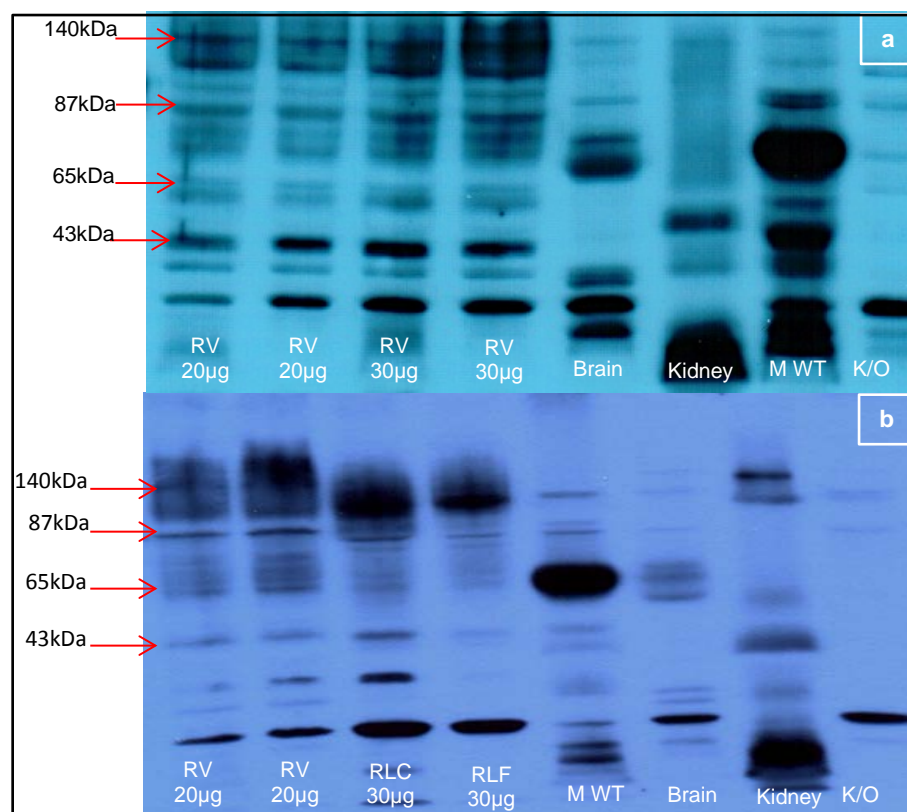
Figure 7.4 Western blots of SNAT2 in various rat tissues and mouse placenta. **a.** Under boil sample buffer conditions with 0.1% TBS-T. **b.** Under non-boil sample conditions with 0.4% TBS-T. SNAT2 antibody (1: 1000 dilution) showed multiple immunoreactive species at higher and lower molecular weights in rat placental vesicles and placental lysates that did not co-migrate with signal in mouse placental lysates or fetal brain. Rat kidney showed an intense band at a higher molecular weight that co-migrated with rat placental vesicles and lysates. All the immunoreactive species were abolished in the [SNAT1 + SNAT2] knockout placental tissue (K/O). Abbreviations: K/O, [SNAT1 + SNAT2] mouse knockout placental lysate; M WT, wild-type mouse placental lysate; RLC, rat placental lysate of control group; RLF, rat placental lysate of intermittent fasting group; RV, rat vesicles. (20 µg protein/lane for fetal tissues, M WT and K/O). Exposure time was: **a.** 45 s and **b.** 30 s.

7.2 Western blot using Li-COR system

With the persistence of multiple banding patterns observed with the SNAT2 antibody using ECL detection, efforts were shifted to the Li-COR system in order to try and get improved clarity of detection. Several optimization steps were performed (Figures 7.5 a and b) using different tissue sources (20 µg protein/lane): rat fetal brain, rat placental lysates and rat placental vesicles from tissue that had either been placed in a Belzer preservative solution before isolating the vesicles (Section 2.2.13.2.1) or isolated immediately from the harvested placenta (fresh vesicle).

Observation. The results clearly showed a distinct band at 52 kDa for SNAT1 (Figure 7.5 a) in placental lysate and vesicles that co-migrated with the brain species and two distinct bands at 60 kDa (predicted size) and 150 kDa for SNAT2 that were of higher molecular weight than the brain species (Figure 7.5 b).
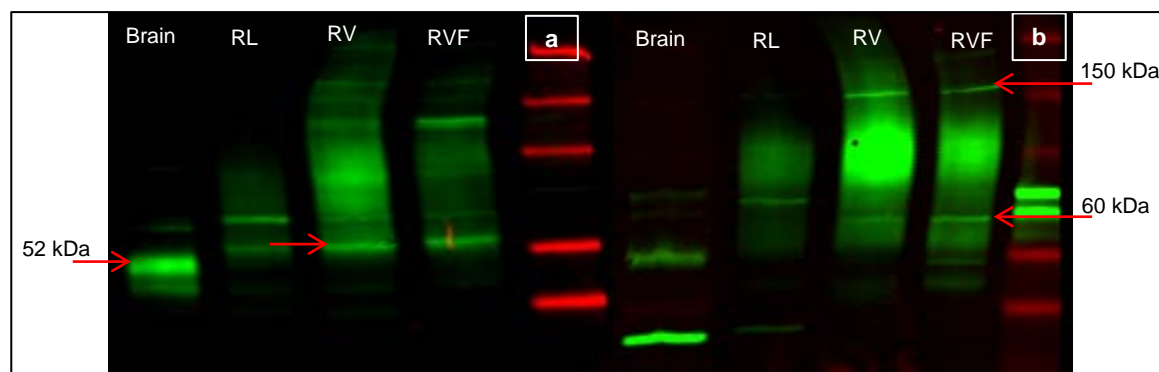

Figure 7.5 Western blot for SNAT1 and SNAT2 protein expression in fetal brain and rat placental lysates and vesicles of GD 21. **a.** Under the boil buffer condition, SNAT1 (1:1000 dilution) showed a distinct band at the predicted size of 52 kDa in brain and placental lysate and vesicles (red arrow). **b.** Under the non-boil condition, SNAT2 (1:1000 dilution) showed two distinct bands, with species at higher molecular weights compared to brain, visible at 60 and 150 kDa. Abbreviations: RL, rat placental lysate; RV, rat placental membrane vesicles; RVF, rat fresh vesicles. (20 µg protein/lane).

In summary: (i) Specificity of each SNAT antibody was confirmed both by the abolition of signal in the presence of excess blocking peptide and the clear absence of immunoreactive signal in the placentas of [SNAT1 + SNAT2] knockout mice. (ii) Different molecular weight species were consistently observed between SNAT1 and SNAT 2 expression in rat placental lysate or vesicles. (iii) SNAT2 appeared to be expressed as different molecular weight species in different tissues. (iv) Background was reduced, and detection of signal clarity improved with application of Li-COR technology, and this was employed further to compare SNAT1 and SNAT2 expression in the placentas of control and IF groups (Chapter 2, Section 2.3.14).

7.3 Fetal malformation in IF group

Several malformations were observed in IF female fetuses at GD 21 such as death *in utero* (example shown in Figure 7.6).
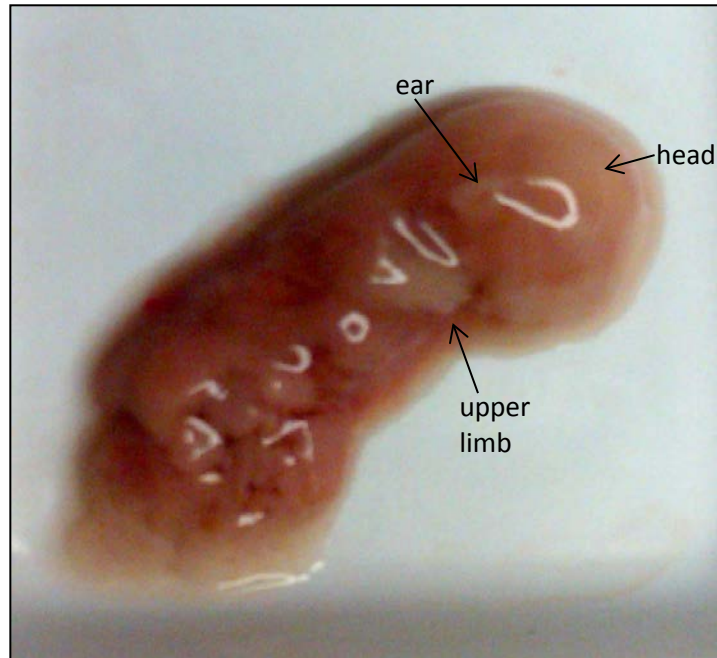

Figure 7.6 Stillbirth of female fetus exposed to intermittent fasting.