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Perinatal programming of appetite regulation and metabolic health

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for the degree of Doctor of Philosophy

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Abstract

According to the concept of perinatal programming environmental factors during fetal development and early postnatal life can influence phenotype in later life by modifying organ and tissue development and the epigenetic information of specific genes which, in turn, induce alteration in gene expression. Global nutrient restriction is a well-established intervention to study fetal programming but choline, a micronutrient essential for tissue growth and development, has not been extensively studied.

The aim of this thesis is to investigate long term effects of modifications in maternal macro and micronutrient intake on the offsprings' appetite regulation and metabolic health.

Twin-pregnant sheep were fed to requirements until 110 days of gestation and then randomised to stay on the same diet (R) or be restricted to 60% of caloric requirements (N) until term (~145 days). Offspring were subsequently subject either to a standard early postnatal growth rate as both twins remained with the mother (S) or to an accelerated growth rate resulting when only one twin remained to be mother fed (A). After weaning, offspring were reared in either a lean (L) or an obesogenic environment (O) until 17 months of age. These interventions gave rise to 4 groups: RAO, NAO, NSO and NSL. There were no differences in body weight, composition or adipocyte size with perinatal nutrient restriction but insulin response to a glucose tolerance test was increased in offspring born to N mothers. Measurement of hypothalamic gene expression in the latter offspring suggested a more orexigenic and cortisol-sensitive regulatory phenotype.

During lactation, rats were fed a diet that was either choline-devoid (D), or contained a standard amount of choline either as bitartrate (C) or as phosphatidylcholine (PC). After weaning, female offspring were maintained on a standard choline diet until 11 weeks of age. D mothers had a substantial decrease in food intake and offspring were smaller at weaning but had similar glucose tolerance. Adult offsprings' brain phospholipid concentrations were reduced, which may suggest changes in brain development, but food intake and hypothalamic protein expression were unchanged. Intake of different forms of choline, i.e. bitartrate versus PC, during lactation had no long term effects on offspring.

Both maternal dietary interventions had long term effects on offspring. Sheep developed the most adverse metabolic phenotype when the offspring were subjected to slow growth in late gestation followed by rapid growth and obesity, with the onset of insulin resistance mediated through changes in peripheral tissues. Maternal choline intake during lactation is essential for the health of the offspring as it alters brain composition.

In conclusion, both studies produced results which are consistent with the concept of perinatal programming as adult metabolic health was affected in the sheep study and organ development was affected in a long term manner in the rat study.

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For my family.

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Declaration

The work in this thesis was performed within the Academic Child Health Division, School of Clinical Sciences, University of Nottingham and within the Division of Nutrition and Metabolism, Department of Agricultural, Food and Nutritional Science, University of Alberta between January 2009 and July 2012.

This thesis illustrates my own work, completed under the supervision of Dr. Helen Budge, Professor Michael Symonds and Professor Catherine Field. This report is an accurate representation of the work performed and no other study reproducing this work, to my knowledge, has been carried out within the University of Nottingham or the University of Alberta.

Neele Dellschaft

July 2012

Abbreviations

3V	third ventricle
5-CH_3-THF	5-methyl-tetrahydrofolate
18S	ribosomal RNA 18S
α-MSH	α-melanocyte stimulating hormone
AB	antibody
ACC	acetyl-CoA carboxylase
ACh	acetylcholine
ADIPOR	adiponectin receptor
AgRP	agouti-related peptide
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ARC	arcuate nucleus of the hypothalamus
AS160	Akt substrate with molecular weight of 160 kDa
AUC	area under the curve
BAT	brown adipose tissue
BDNF	brain-derived neurotrophic factor
BHMT	betaine-homocysteine methyltransferase
BMI	body mass index
bp	base pairs
BSX	homeobox transcription factor
C	mothers fed a diet containing 1 g choline per kg diet as free choline during the lactation period and their offspring (rat study)
CART	cocaine and amphetamine related transcript
cDNA	complementary deoxyribonucleic acid
ChoCl	choline chloride, identical to free choline
CHT1	choline transporter 1
CpG islands	repetition of cytosine-phosphate-guanidine sequence
CPT1	carnitine palmitoyltransferase
CREB	cAMP-responsive element binding protein
CRH	corticotropin releasing hormone
ct	cycle threshold
D	mothers fed a choline-devoid diet during the lactation period and their offspring (rat study)
DAB	3,3'-diaminobenzidine
DAG	diacylglycerol
DHA	docosahexaenoic acid
DMG	dimethylglycine
DMN	dorsomedial nucleus
DMNV	dorsal motor nucleus of the vagus
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase

DXA	dual-energy X-ray absorptiometry
ELISA	enzyme-linked immuno-sorbent assay
FA	fatty acid
FoxO1	forkhead box O1
FTO	fat mass and obesity associated [gene]
GCR	glucocorticoid receptor
GhSR	growth hormone stimulating receptor
GLP	glucagon-like protein
GPC	glycerophosphocholine
GTT	glucose tolerance test
Hba1c	haemoglobin a1c (glycated)
Hcy	homocysteine
HOMA-IR	homeostatic model assessment for insulin resistance
i.c.v.	intracerebrovascular
i.p.	intraperitoneal
IGF	insulin-like growth factor
IL	interleukin
IMS	industrially modified spirit
IRS	insulin receptor substrate
IUGR	intra-uterine growth restriction
JAK	janus kinase
LC-HRMS	liquid chromatography-high resolution mass spectrometry
LDL	low-density lipoprotein
LGA	large for gestational age
LHA	lateral hypothalamic area
LPC	lyso-phosphatidylcholine
m/z	mass-to-charge ratio
M3R	muscarinic acetylcholine receptor 3
MC3/4R	melanocortin receptor 3 and 4
MCH	melanin concentrating hormone
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MUFA	mono-unsaturated fatty acid
n	number of animals per group
N	mothers nutrient restricted to 60% in late pregnancy; applies to mothers and offspring at birth (sheep study)
NA	mothers nutrient restricted in late pregnancy and offspring subjected to accelerated early postnatal growth; applies to offspring before weaning (sheep study)
NAO	mothers nutrient restricted in late pregnancy, offspring subjected to accelerated early postnatal growth and an obesogenic environment after weaning; applies to offspring after weaning (sheep study)
NEFA	non-esterified fatty acids

NFκB	nuclear factor κB
NHS	National Health Service
NO	nitrogen oxide
NPY	neuropeptide Y
NS	mothers nutrient restricted in late pregnancy and offspring subjected to standard early postnatal growth; applies to offspring before weaning (sheep study)
NSL	mothers nutrient restricted in late pregnancy, offspring subjected to standard early postnatal growth and an active environment to induce a lean phenotype; applies to offspring after weaning (sheep study)
NSO	mothers nutrient restricted in late pregnancy, offspring subjected to standard early postnatal growth and an obesogenic environment after weaning; applies to offspring after weaning (sheep study)
NTS	nucleus tractus solitarius
OCT	organic cation transporter
OSC	orthogonal signal correction
PC	phosphatidylcholine; also mothers fed a diet containing 1 g choline per kg diet as PC during the lactation period and their offspring (rat study)
PCho	phosphocholine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine methyltransferase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1α
PI	phosphatidylinositol
PI3K	phosphoinositide-3-kinase
PLS-DA	partial least squares discriminant analysis
POMC	pro-opio melanocortin
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
PTP1B	protein tyrosine phosphatase, non-receptor type 1
PUFA	poly-unsaturated fatty acid
PVN	paraventricular nucleus
PYY	peptide YY
qPCR	semiquantitative polymerase chain reaction
R	mothers fed to requirements; applies to mothers and offspring at birth (sheep study)
RA	mothers fed to requirements and offspring subjected to accelerated early postnatal growth; applies to offspring before weaning (sheep study)
RAO	mothers fed to requirements, offspring subjected to accelerated early postnatal growth and an obesogenic environment after weaning; applies to offspring after weaning (sheep study)

RAPTOR	regulatory-associated protein of mTOR
RPO	RNA polymerase
RT	reverse transcription
rt	retention time
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	standard deviation
SEM	standard error of mean
SFA	saturated fatty acid
SGA	small for gestational age
SHP2	protein tyrosine phosphatase non-receptor type 11
SM	sphingomyelin
SNS	sympathetic nervous system
SOCS3	suppressor of cytokine signalling 3
SR-B1	scavenger receptor class B type 1
SREBP	sterol regulatory element-binding protein
STAT3	signal transducer and activator of transcription 3
T2DM	type 2 diabetes mellitus
T_3	triiodothyronine
T_4	thyroxine
THF	tetrahydrofolate
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TRH	thyroid releasing hormone
TSH	thyroid stimulating hormone
UCP1	uncoupling protein 1
VLDL	very low-density lipoprotein
VMN	ventromedial nucleus
WAT	white adipose tissue
WHO	World Health Organisation
YWHAZ	tyrosine 3-monooxygenase activation protein, zeta polypeptide

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1. Introduction

1.1. General study overview

My thesis presents two models for investigating long term outcomes in appetite regulation and metabolic health after perinatal interventions of maternal food intake. In the first study, carried out at the University of Nottingham, sheep were subjected to global nutrient restriction during late pregnancy and growth rate manipulation in early postnatal life. Outcomes assessed from this study were growth pattern, metabolic and endocrine characteristics into young adult life, including assessment of glucose tolerance and appetite regulation, and post mortem analysis of the hypothalamus to gain further insights into regulatory effects of the interventions. The second study was carried out at the University of Alberta in Canada, for which the influence of maternal dietary intake of choline, an essential micronutrient, during lactation was investigated. Maternal and offspring development, body composition, appetite regulation and the offsprings' glucose tolerance were assessed in this model.

In this Chapter, I will summarise and discuss current knowledge on the interactions of obesity, insulin sensitivity and appetite regulations together with the epidemiological and animal research studies underlying the concepts of perinatal programming of long term health outcomes.

1.2. Obesity

1.2.1. Definition and incidence

Obesity is defined as excessive storage of triglycerides in adipose tissues and is one part of a cluster of metabolic risk factors, the metabolic syndrome, which are associated with increased risk of Type 2 diabetes mellitus (T2DM) and cardiovascular disease [1, 2]. To assess obesity in adults, an individual's height and weight are used to calculate their body mass index (BMI):

$$BMI = \frac{weight\ (kg)}{height\ (m^2)}$$

Even though the BMI is only an approximate measure of adiposity as it does not distinguish between lean and fat mass, it is a standard measure in epidemiological studies and is often used in combination with other measures of obesity including waist circumference, waist-to-hip-ratio and body composition measurements. Internationally used cutoff points for overweight is a BMI ≥ 25 and for obesity a BMI ≥ 30.

In England in 2010, 26% of both adult men and women were estimated to be obese, as defined by a BMI ≥ 30, and an additional 42% and 32% of adult men and women, respectively, were overweight with BMIs between 25 and 30 [3]. This was a slight increase from 24% obese and 41% and 32% overweight in 2007 [4]. This trend is consistent with the worldwide development of obesity with 1.5 billion, i.e. about 25% of the world population being overweight and, of these, 200 million men and 300 million women were obese in 2008 [5].

In childhood, the BMI is not a valid method to determine obesity without an adjustment for age. Instead, children are compared to the World Health

Organisation's (WHO) growth standard medians. Two standard deviations (SD) over the median is considered overweight and 3 SD over the median is considered to represent obesity [6]. Worldwide, 6.7% of preschool children were estimated to be overweight in 2010 and 9.1% of preschool children are expected to be overweight in 2020 [6]. For the UK, the National Health Service's National Child Measurement Programme defined childhood obesity as a BMI over the 95th percentile on the British growth reference from 1990 [7]. For 4 year old children, obesity prevalence was 10.1% in boys and 8.8% in girls in 2010/2011 and, in 10 year old children, the prevalence is considerably higher with 20.6% in boys and 17.4% in girls, which are increasing annually [8].

1.2.2. White and brown adipose tissue

Although adipose tissue exists both as white (WAT) and brown adipose tissue (BAT) in mammals, only WAT increases significantly with obesity whilst BAT decreases [9]. Both tissues are made up of adipocytes but also immune cells and endothelial cells from blood vessels. WAT adipocytes mainly consist of triglycerides in a lipid droplet and the nucleus, whilst in a brown adipocyte lipids take up less space and cells have more mitochondria. Whilst WAT is mainly storing energy, BAT releases energy from glucose and free fatty acids as heat by activating its unique protein, uncoupling protein 1 (UCP1) [10], and plays an important role in the survival of the newborn. Recent findings showed that brown adipocytes do not exclusively turn into white adipocytes soon after birth, as had been assumed. Adult humans still have a small depot of brown adipocytes in the supraclavicular region and UCP1-positive cells are interspersed in WAT as well [10-12]. Sheep have a similar region of high UCP1-expressing adipose

tissue [13]. In contrast to humans and sheep, however, rodents have long been known to have a distinct interscapular depot of BAT. UCP1 is, at least in rodents, activated by leptin [14] and the sympathetic nervous system [15] and is reduced in energy-scarce situations like starvation [16] and lactation [17].

1.2.3. Endocrine functions of white adipose tissue

Whilst BAT has no known significant endocrine function, WAT expresses and secretes into circulation several hormones, including leptin [18]. The plasma concentration of leptin is positively correlated with obesity and body fat mass [19] whilst adiponectin concentrations decrease as fat mass increases [20]. Both hormones have been directly linked to the regulation of food intake. If fat mass increases, circulating leptin concentrations increase, which, acting on the arcuate nucleus (ARC) of the hypothalamus, decrease food intake [21]. If fat mass decreases, circulating adiponectin concentrations increase, with contradicting findings about the effects on food intake [22].

Overall, WAT is known to secrete numerous compounds into the circulation, including pro-inflammatory cytokines, most prominently tumor necrosis factor (TNF) and interleukin (IL)6, the hormone resistin and non-esterified fatty acids (NEFA) and circulating concentrations of these are higher in obese subjects [23-26], and this increase in inflammatory molecules is associated with adverse health outcomes.

1.2.4. Characteristics of white adipose tissue

With increasing fat storage, adipocytes can grow by hypertrophy and/or hyperplasia. Plasma leptin concentrations are influenced more by hypertrophic adipocytes than hyperplastic adipocytes [27] since leptin production and secretion increases with adipocyte size, as does the production and secretion of inflammatory cytokines [28]. Koenen et al. hypothesise that this increased inflammatory activity of hypertrophic adipocytes also leads to decreased insulin sensitivity [29].

WAT is generally divided into visceral adipose tissues such as those surrounding inner organs including kidney, intestine, gonads and heart and subcutaneous tissues. These tissues differ in characteristics associated with higher replication rates in subcutaneous adipose tissues [30]. Adipose tissue characteristics and depot size are also dependent on sex, age, nutrition and level of adiposity [31]. There are distinct differences between human and rat visceral and subcutaneous fat with regards to adipocyte size and metabolic activity. In rats, visceral depots have bigger cells [32] and higher rates of lipogenesis and lipolysis compared to subcutaneous depots [33] but in non-obese humans subcutaneous fat has larger adipocytes [34], and higher rate of lipolysis and leptin production as compared to omental fat [35]. Feeding high-fat diet increases cell size in visceral adipose tissues sooner than in subcutaneous adipose tissues and visceral, rather than subcutaneous, adipocyte size seems to be specifically correlated with hepatic and systemic insulin resistance [36].

1.3. Metabolic syndrome

In 2007, the cost of treating obesity-related conditions in the UK through the National Health Service (NHS) was estimated as £4.2 billion per year [37], i.e. about 5% of the overall 2007 NHS budget of £80 billion [38]. Morbidity and mortality rates, especially of T2DM and cardiovascular diseases, are significantly higher in obese as compared to lean individuals and are even further increased in obese individuals who develop the metabolic syndrome [2, 39].

The metabolic syndrome is defined as meeting at least three of a group of risk factors, most importantly visceral adiposity as measured by waist circumference and insulin resistance demonstrated as elevated fasting glucose. New, standardised criteria for the metabolic syndrome were agreed by several international societies in 2009 [40] and are summarised below (Table 1.1).

Table 1.1: Criteria to define the metabolic syndrome.

Marker	**In men**	**In women**
Elevated waist circumference	≥94 / ≥102 cm	≥80 / ≥88 cm
Elevated triglycerides[1]	≥1.7 mM	
Reduced HDL cholesterol[1]	<1.0 mM	<1.3 mM
Elevated blood pressure[1]	Systolic ≥130 mmHg and/or diastolic ≥85 mmHg	
Elevated fasting glucose[1]	≥5.5 mM	

HDL: high density lipoprotein; [1] or drug treatment against this criterion.

1.4. Insulin resistance

1.4.1. Pathogenesis: influence of obesity

Insulin resistance is defined as an inadequate response of skeletal muscle, liver and WAT to the physiological effects of insulin. The incidence of insulin resistance and, subsequently, T2DM is much higher in overweight and physically inactive individuals than in lean counterparts [41, 42]. There are several reasons for this. With obesity, plasma NEFA concentrations are increased [25], with visceral adipose tissues being specifically lipolytically active [43, 44]. NEFA induce insulin resistance in the major metabolic organs, including skeletal muscle and WAT itself [45], by direct inhibition of the insulin receptor signalling cascade [46] and induction of lipotoxicity and a proinflammatory cytokine profile. Furthermore, they induce insulin resistance in the liver, disturbing the inhibition of gluconeogenesis and further contributing to hyperglycemia [47]. Since insulin also regulates the storage of NEFA in muscle and WAT, a lack of insulin signalling increases lipolysis [48], again increasing plasma NEFA concentrations. Furthermore, adiponectin is a promoter of insulin sensitivity [49, 50] by decreasing inflammatory signalling and by promoting uptake of NEFA into WAT [51], and its concentrations decrease with increasing body fat mass [20].

A third factor is the recruitment of macrophages to excessive WAT [52], which causes a proinflammatory profile of circulating cytokines. Hypertrophic adipocytes and hepatocytes in obese individuals secrete pro-inflammatory cytokines as well, both, therefore, contributing to the systemic chronic, low-grade inflammatory state observed in obese subjects [52-54]. Finally, with excess adiposity, ectopic adiposity also increases,

especially in men [55], which is the excessive deposition of adipocytes in non-adipose tissue sites including heart, pancreas, liver and skeletal muscle. Hepatic lipid content is linked with hepatic hyporeactivity to increased plasma glucose concentrations, i.e. a disturbed ability to stop gluconeogenesis and glycogenolysis postprandially [56, 57]. Pancreatic steatosis, together with chronic hyperglycemia, may contribute to increased apoptosis of ß-cells and decreased insulin secretion [58, 59].

Insulin-dependent control of lipolysis is stronger in subcutaneous compared to visceral fat tissues, even in nonobese subjects [60] and visceral fat secretes more pro-inflammatory cytokines than subcutaneous fat [61], both at least partially explaining the increased insulin resistance in individuals with a high waist circumference [62].

The resulting hyperinsulinemia is, at first, sufficient to regulate blood glucose to normal concentrations until the secretory capacity of β-cells declines [63, 64], so that hyperglycaemia follows, which can progress to T2DM [65].

1.4.2. Insulin secretion and effects

From the diet, macronutrients are taken up into circulation. As pancreatic ß-cells take up glucose through an insulin-independent transporter, glucose is metabolised and the increased ratio of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) induces secretion of insulin from vesicles within pancreatic ß-cells into draining blood vessels. This effect is increased by the presence of NEFA but a chronic increase in NEFA concentrations leads to oxidative stress and ß-cell damage [66]. Insulin is released into the portal vein and, therefore, has a direct effect on hepatic glucose homeostasis. Therefore, insulin concentrations rise postprandially

and, additionally, there is an increase of blood insulin concentrations in obesity due to the onset of insulin resistance [67-69].

Insulin has anabolic effects as it induces uptake and storage of glucose as glycogen and lipid in muscle and liver [70], and of NEFA and triglycerides in WAT and muscle [71, 72] and inhibits gluconeogenesis in the liver [73]. Glucose uptake into peripheral tissues is induced by insulin-dependent translocation of the glucose transporter GLUT4 to the membrane [74] whilst hepatocytes take up glucose through GLUT2 and neurons through GLUT3, both of which are not influenced by circulating insulin [75].

1.4.3. Measuring insulin sensitivity and glucose tolerance

In humans, insulin resistance can be screened for by measuring fasted plasma glucose, glycated hemoglobin (Hba1c) and/or by performing an oral glucose tolerance test (GTT) [76]. The most reliable test of insulin resistance is the application of a hyperinsulinemic euglycemic clamp, during which time the glucose infusion necessary to maintain stable plasma glucose concentrations under high insulin concentrations is measured. This directly equals the amount of glucose taken up into metabolic tissues during the time of the clamp. Both the GTT and clamp are accompanied by frequent blood sampling and measurement of plasma glucose and insulin concentrations and are, therefore, rarely used to screen populations for T2DM or in large epidemiological studies [76, 77].

The homeostatic model assessment for insulin resistance (HOMA-IR) is a measure calculated from fasted plasma insulin and glucose concentrations and correlates very well with the euglycemic clamp [78], therefore making the much more expensive and time-intensive method of the hyperinsulinemic euglycemic clamp superfluous if the only outcome of

interest is insulin resistance. The clamp is necessary as a tool to distinguish between hepatic glucose production and peripheral glucose uptake [79].

1.5. Appetite regulation

As excess adiposity is the result of a misbalance of food intake and energy expenditure, appetite regulation and the ways it can be disturbed is central to the understanding of the manifestation of overweight. Appetite is a very complex system regulated by various regions in the brain in response to metabolic and endocrine signals from the periphery. The part of the brain involved in appetite regulation which is most thoroughly investigated to date is the hypothalamus. Even though an understanding is emerging how peripheral signals influence central regulation, the afferent side of food intake and physical activity are less well understood and may involve the reward centres of the brain, hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. Appetite regulatory networks are a very active field of research but many mechanistic details remain unknown. For a graphic overview of the main pathways and nuclei involved, see Figure 1.1.

Most of this research is conducted in rodents and is, therefore, not necessarily the same as in higher mammals. However, specific mutations occuring in humans, e.g. of leptin, the leptin receptor or melanocortin receptor, show symptoms which are consistent with the networks described in rodents. Differences between species have been found in coexpression of neurotransmitters, e.g. cocaine and amphetamine related transcript (CART) and pro-opio melanocortin (POMC) are co-expressed in neurons in the rodent but are expressed in separate populations of the

ARC in the non-human primate whereas CART is co-expressed with neuropeptide Y (NPY) and AgRP in humans [80].

1.5.1. Primary neurons

The ARC has 2 distinctly different types of neurons, generally called the primary neurons, which directly react to peripheral signals and which project to so-called secondary neurons, further integrating those signals. The peripheral signals are transported over the blood brain barrier from the systemic circulation into cerebrospinal fluid. The most important peripheral signals are thought to be leptin, insulin, adiponectin, ghrelin and glucose. The ARC is situated on the surface of the hypothalamus near the median eminence and is, therefore, both exposed to cerebrospinal fluid in the third ventricle and lies in close proximity to blood flow.

1.5.1.1. Neuropeptides

The first of two types of primary neurons are those co-expressing neuropeptide Y (NPY) and AgRP as their neurotransmitters and which generally induce an orexigenic response. These are inhibited by leptin [81, 82] and insulin [83] and activated by ghrelin [84, 85]. The second type of primary neurons co-expresses POMC and CART, induces an anorexigenic response and is generally activated by leptin [86], insulin [87] and glucose [88] and inhibited by ghrelin [89]. POMC is a polypeptide hormone precursor, which is spliced after translation and forms, amongst other products, α-melanocyte stimulating hormone (α-MSH), which in turn binds to melanocortin receptor 3 and 4 (MC3R, MC4R) on the secondary neurons.

Adiponectin also binds to receptors on ARC neurons [90] but its effect on food intake is controversial with its orexigenic or anorexigenic effects perhaps depending on species and fed/fasted state of the animals [22]. In a study of intracerebroventricular (i.c.v.) adiponectin injections there was no effect on NPY/AgRP or POMC expression in the ARC and no effect on appetite regulation [91] but increased corticotropin releasing hormone (CRH) and thyroid releasing hormone (TRH) in the paraventricular nucleus (PVN), with subsequent positive effects on energy expenditure [92]. An anorexigenic effect after adiponectin injection was shown in fasted rats, as adiponectin increased intracellular pathways similarly to those induced by insulin and leptin [93]. On the other hand, an orexigenic effect of adiponectin could be shown by inducing AMP-activated protein kinase (AMPK) activity in the ARC of fed mice by increasing NPY and decreasing POMC expression [94].

Secondary neurons, i.e. those that primary neurons project onto, or neurons that are otherwise involved in appetite regulation, have a wide variety of functions and are found in several nuclei of the hypothalamus, including the PVN, ventromedial nucleus (VMN), lateral hypothalamic area (LHA), dorsomedial nucleus (DMN), the cortico-limbic system nucleus accumbens and amygdala, the latter of which are involved in food reward, and, in the brain stem, nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMNV) and raphe nucleus.

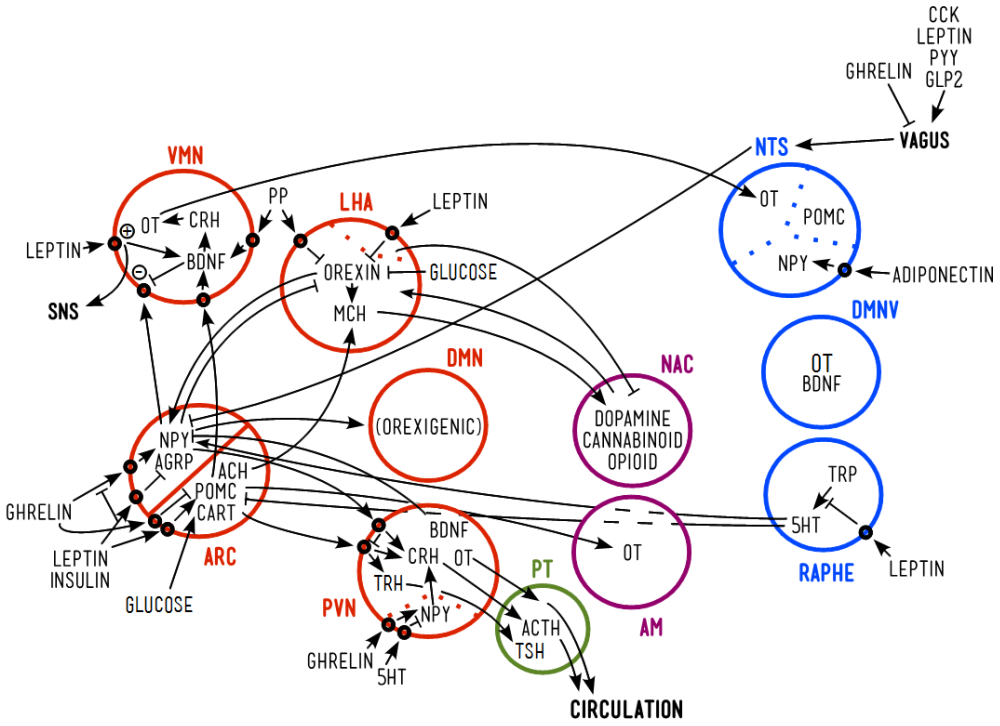

Figure 1.1: Graphic overview of appetite regulating network between hypothalamic nuclei (red), the pituitary (green, PT), the cortico-limbic system (purple), and the brain stem nuclei (blue).

Nuclei are: arcuate nucleus (ARC), ventromedial nucleus (VMN), lateral hypothalamus (LHA), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), nucleus accumbens (NAC) and amygdala (AM), nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMNV) and raphe nucleus (raphe).

NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opio melanocortin; CART, cocaine and amphetamine related transcript; OT, oxytocin; CRH, corticotropin releasing hormone; BDNF, brain-derived neurotrophic factor; PP, pancreatic polypeptide; MCH, melanin concentrating hormone; ACTH, adrenocorticotropic hormone; TRH, thyroid releasing hormone; TSH, thyroid stimulating hormone; TRP,tryptophane; 5HT, serotonin; CCK, cholecystokinin; PYY, peptide YY; GLP2, glucagon-like peptide 2; SNS, sympathetic nervous system.

This figure is based on the papers as referenced throughout this section and especially reviews [95-99].

1.5.2. Further hypothalamic nuclei

The VMN is a mainly anorexigenic nucleus, expressing oxytocin and CRH, which are both potent in inhibiting food intake [100, 101]. These neurons are excited through leptin and this excitation is inhibited by NPY [102, 103]. Brain-derived neurotrophic factor (BDNF) promotes expression of

CRH and its expression is increased by α-MSH through the MC3R/MC4R [104]. The VMN also projects back onto the ARC, increasing POMC expression in a positive feedback loop [105]. The action of NPY on the VMN is inhibited by BDNF [106], thereby increasing the anorexigenic signal.

CRH is also expressed in the PVN and is found to be decreased in response to short term [107] but not long term fasting [108]. Although NPY and POMC have opposite effects most of the time, CRH is induced by both POMC [107] and NPY [109], which may be explained by the specific nuclei projecting onto the PVN, so that fasting still induces a decrease in CRH [107]. It is also increased in stress, e.g. restraint stress [110], increasing activity of the HPA axis. CRH induces expression of oxytocin in the PVN, mediating the anorexigenic response [101]. Both oxytocin and CRH are taken into the pituitary from the PVN, going into the circulation [111] and initiating ACTH production [112], which results in adrenal release of cortisol, respectively. The PVN also includes NPY neurons, which are induced by ghrelin [89] and inhibited by serotonin [113].

Both DMN and LHA are generally orexigenic nuclei. The LHA expresses melanin concentrating hormone (MCH) and orexin [114, 115], which are reduced by leptin [114]. MCH induces activity in the nucleus accumbens [116, 117] and orexin induces NPY expression in the ARC [118]. However, in a negative feedback loop, NPY from the ARC inhibits orexin expression in the LHA [119]. Leptin also induces expression of MCH [120] and orexin [121]. Signalling in the nucleus accumbens further induces orexigenic signalling of the LHA [122]. A subset of inhibitory neurons of the LHA express obRb and binding leptin induces a reduction of dopaminergic signalling in the nucleus accumbens [123].

Hedonic regulation of food intake is thought to be regulated in the amygdala and nucleus accumbens, which has dopaminergic, opioid, cannabinoid, GABA-ergic and acetylcholine neurotransmission, which are all involved in food reward [124]. Reward value is assigned to specific foods, dependent on olfactory input, which decreases when hunger subsides during a meal [125].

BDNF is involved in several of these nuclei, mediating the anorexigenic response. It is crucial for the regulation of synaptic activity, neurotransmission and neuroplasticity [126] and is especially highly expressed in early postnatal development [127]. Expression of BDNF can be epigenetically regulated by hypermethylation and acetylation in association with perinatal restraint stress [128] and social environment [129]. Circulating BDNF is, furthermore, found to be lower in obese and Type 2-diabetic individuals [130, 131]. Chronic i.c.v. injection of BDNF decreases body weight gain [132] through several potential mechanisms: BDNF is crucial for the survival of serotonergic neurons involved in control of appetite [133]; in the VMN, BDNF increases anorexigenic signalling of MC4R-positive neurons by inhibiting the orexigenic signal of NPY and increases energy expenditure [106, 134]; BDNF induces expression of CRH [135], which is appetite-inhibiting [110]. Additionally, BDNF has been shown to be involved in regulation of the dorsal vagal complex [136], in hedonic feeding regulation [137] and in the response to leptin [138].

1.5.3. Insulin and leptin signalling

Both insulin [83, 139] and leptin [81] directly change activity of NPY/AgRP and POMC/CART neurons in the ARC by binding to their receptors expressed on both cell types. They show an anorexigenic influence by

increasing expression of POMC and inhibiting NPY expression. However, AgRP is increased by insulin whilst it is decreased by leptin [140]. Furthermore, leptin decreases orexigenic signalling of ghrelin and orexin on NPY neurons. In obesity, signalling of insulin [141, 142] and leptin [81] to the ARC is reduced despite high circulating concentrations, due to lower transportation rates over the blood brain barrier [143, 144] or due to resistance at the receptor and signalling cascade. This resistance is mediated through a higher expression of the inhibitory molecules protein tyrosine phosphatase non-receptor type 1 (PTP1B) and suppressor of cytokine signalling 3 (SOCS3) [145, 146]. Leptin resistance seems to mainly affect signalling in the ARC. However, this does not occur in the VMN [147], where leptin induces activation of the sympathetic nervous system (SNS) [148]. The SNS carries out the body's stress-related responses including increased heart rate, blood pressure, muscle catabolism and metabolic preparation for energy expenditure [149] and this may be one way through which obesity-related hypertension is mediated [150, 151].

Leptin, furthermore, inhibits the conversion of tryptophane to serotonin in the medial and dorsal raphe nucleus of the brain stem [152]. These serotonergic neurons project from the raphe nucleus to the ARC, where release of serotonin decreases expression of POMC and MC4R [153]. Serotonin has both orexigenic and anorexigenic effects, depending on the receptor type mediating the response, as serotonin shows a decrease in NPY when binding to the PVN [113].

In a pathway connecting peripheral energy status signalling with the hedonic regulation of food intake, leptin binding to inhibitory neurons of the LHA has been shown to decrease dopaminergic signalling in the nucleus accumbens [123]. A resistance to leptin seems to occur, as a

chronic increase in leptin concentrations generally decreases the reward value of food [154].

For individuals in positive energy balance, depending, in part, on leptin concentrations and induced by α-MSH signalling onto the PVN [155], the thyroid hormone system is stimulated [156], whilst it is decreased in the fasting state [157, 158]. Secretion of thyroid hormones into the circulation is induced by thyroid stimulating hormone (TSH) from the pituitary gland. TSH secretion is, in turn, induced by TRH and inhibited by somatostatin, both secreted from the hypothalamus. The thyroid hormones thyroxine (T_4), and the active form triiodothyronine (T_3), increase energy expenditure by promoting resting oxygen consumption and adaptive thermogenesis [159].

1.5.4. Signalling molecules derived from the gastro-intestinal system and the afferent vagal nerve

Ghrelin is released in the fasted state from enteroendocrine cells of the stomach [160], it decreases activation of the SNS [161, 162] and fasted concentrations are negatively correlated with body weight and fat content [163]. Circulating ghrelin has a direct effect on NPY neurons in the ARC [85]. From the stomach, it additionally suppresses firing of the afferent vagal nerve [164], which connects the gastro-intestinal system and liver with the brain stem. At the same time, CCK, an anorexigenic hormone [165, 166] released postprandially from the mucosa of the small intestine [167], increases vagal firing [164]. Similarly, other anorexigenic compounds secreted in peripheral organs including leptin [168], peptide YY (PYY) [169] and glucagon-like protein (GLP)-2 [170] can all increase vagal activity. Ghrelin induces vagal inactivity which causes inactivity of the NTS

of the brain stem and, through projections to the ARC, is associated with NPY secretion there [95].

Gastro-intestinal tract-derived peripheral signals also have direct interactions with appetite regulatory nuclei mediated through the blood circulation. Pancreatic polypeptide is released from the pancreas in response to food intake [171] and induces an anorexigenic respone in the hypothalamus by inhibiting the release of orexin from the LHA and by inducing the secretion of BDNF in the VMN [172].

1.5.5. Effects of glucose and NEFA on appetite regulation

As glucose and NEFA are taken up into cells and metabolised, they directly influence intracellular energy sensing mechanisms. Their metabolism is interrelated and influence activity of AMPK and mammalian target of rapamycin (mTOR), which are discussed in Section 1.5.6 below.

The effects of high NEFA and glucose concentrations are generally anorexigenic. Glucose concentrations in the hypothalamus and interstitial fluids are highly correlated with circulating concentrations [173], so that any effects normally occur in the postprandial state and also in metabolic dysregulation, e.g. in T2DM. During times of high glucose and NEFA concentrations, glucose is glycolysed to acetyl-CoA, converted to malonyl-CoA, which subsequently blocks fatty acid ß-oxidation, so that NEFA concentrations are not reduced in the cell [174].

These metabolic processes seem to induce regulation of appetite directly, as an increase of malonyl-CoA concentration result in anorexia and increased physical activity [175, 176] whilst a decrease of malonyl-CoA induces hyperphagia [177]. Injection of NEFA into the cerebral ventricular

system reduces hypothalamic expression of NPY and AgRP and, subsequently, food intake, but this effect is blunted in obese animals through an unknown mechanism [178, 179].

On the other hand, dietary fat and sugar increase palatability and, through the reward centre, can increase food intake and promote obesity, as has been widely demonstrated by the numerous studies feeding a high-fat diet or sucrose in the animals' drinking water to induce obesity. Dietary fat also has a direct effect on endocrine control of food intake as feeding a controlled amount of a high-fat diet, which does not induce obesity, decreases central insulin sensitivity as compared to feeding an isocaloric amount of a low-fat diet [141].

Glucose induces POMC expression in the ARC directly [88] and it may also be involved in appetite regulation as a marker of low energy levels since hypoglycemia induces release of orexin in the LHA and, with that, increased food intake [180].

1.5.6. Intracellular energy homeostatic signalling

AMPK is a kinase, which is allosterically activated by AMP and inhibited by ATP and is, therefore, generally activated in a low intracellular energy state. It is also directly activated by endocrine signals of the fasted state, including ghrelin, and is inhibited by endocrine signals of the fed state, including leptin, insulin and GLP1 [181]. For a graphic representation of intracellular energy sensing see Figure 1.2.

In fasting, ghrelin binds to its membrane-bound receptor, growth-hormone stimulating receptor (GhSR), induces phosphorylation and, with that, activation of AMPK, which in turn decreases acetyl-CoA carboxylase (ACC)

and fatty acid synthase (FAS) activity in the VMN of the hypothalamus [182]. Subsequently, the concentration of malonyl-CoA is decreased [182], which reduces glycolysis and an increase in mitochondrial ß-oxidation by induction of carnitine palmitoyl-transferase (CPT1) expression [182, 183]. How exactly this AMPK pathway induces increased expression of NPY/AgRP and reduces POMC expression is not clear but it is likely to involve induction of transcription factors in response to alterations in intracellular lipid metabolism. Ghrelin induces expression of the brain-specific homeobox transcription factor (BSX) through induction of CPT1 [184]. Both expression of NPY and AgRP are induced by this transcription factor, in combination with cAMP-responsive element binding protein (CREB) and forkhead box O1 (FOXO1), respectively [185].

Leptin, on the other hand, decreases phosphorylation of AMPK in the fed state, with opposite effects on intracellular lipid metabolism, therefore increasing intracellular malonyl-CoA, inhibiting CPT1 and inhibiting BSX-dependent expression of NPY and AgRP. Furthermore, it inhibits FOXO1 and, in this way, inhibits AgRP production [186]. POMC expression is repressed by FOXO1, an effect that is alleviated through signal transducer and activator of transcription 3 (STAT3) [186], which is induced by insulin and leptin [187]. NPY suppression by leptin acts through a STAT3-independent mechanism. At least part of the NPY suppression is signalled through a MAPK-dependent inhibition of voltage-dependent calcium channels and, with that, inhibition of firing rates of NPY neurons of the ARC [188]. FOXO1 is further deactivated by Akt, which is induced by insulin and leptin through the phosphoinositide-3-kinase (PI3K) pathway [189, 190]. However, there appears to be another, AMPK-dependent pathway to induce NPY expression, which has not been widely described [191].

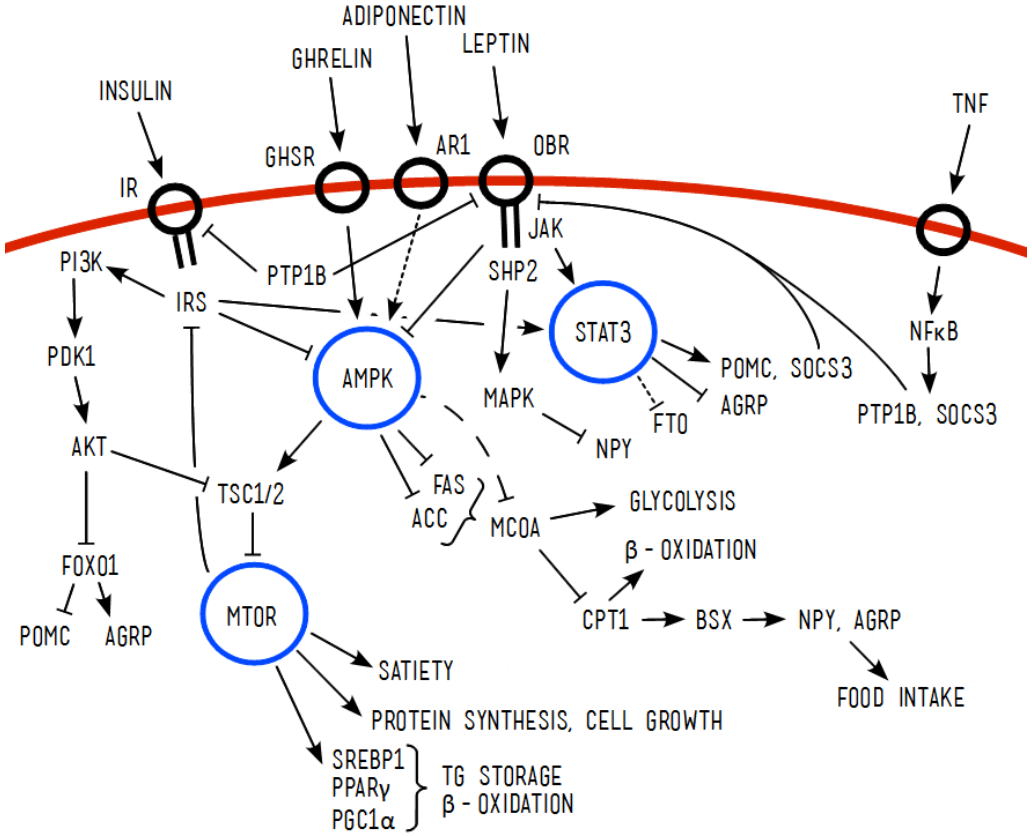

Figure 1.2: Graphic overview of intracellular energy signalling. In the fasted state, ghrelin induces AMPK activity whilst it is inactive in the fed state through insulin, leptin and ATP derived from glucose. Sensors including AMPK, mTOR and STAT3 induce or inhibit cell growth, intracellular metabolism and transcription of neurotransmitters NPY, AgRP and POMC. Signalling of insulin and leptin receptors is reduced through mTOR activity and through PTP1B and SOCS3, which are induced through inflammation and as a product of STAT3-induced gene transcription.

ACC, acetyl-CoA carboxylase; AGRP, agouti-related peptide; AMPK, AMP-activated kinase; BSX, brain-specific homeobox transcription factor; CPT1, carnitine palmitoyl-transferase 1; FAS, fatty acid synthase; FOXO1, forkhead box O1; GHSR, growth-hormone secretagogue receptor; IR, insulin receptor; IRS, insulin receptor substrate; JAK, janus kinase; MAPK, MCOA, malonyl-CoA; MTOR, mammalian target of rapamycin; NFκB, nuclear factor κB; NPY, neuropeptide Y; OBR, leptin receptor; PDK1, pyruvate dehydrogenase kinase 1; PGC1α, PPAR γ coactivator 1α; PI3K, POMC, pro-opiomelanocortin; phosphoinositide-3-kinase; PPAR, peroxisome proliferator-activated receptor γ; PTP1B, protein tyrosine phosphatase non-receptor type 1; SHP2, protein tyrosine phosphatase non-receptor type 11; SOCS3, suppressor of cytokine signalling 3; SREBP1, sterol regulatory element-binding protein 1; STAT3, signal transducer and activator of transcription 3; TG, triglyceride; TNF, tumour necrosis factor; TSC1/2, tuberous sclerosis 1 and 2.

This figure is based on the papers as referenced throughout this section and especially reviews [182, 184, 185, 192].

Induction of PI3K through insulin and leptin leads to the activation mTOR [193], which is, in complex with regulatory-associated protein of mTOR (RAPTOR, mTOR complex 1), another crucial part of cellular energy sensing. MTOR promotes protein synthesis and cell growth by inducing messenger ribonucleic acid (mRNA) translation [194] and inhibiting autophagy [195]. Importantly, mTOR also induces transcription of specific target genes by activition of transcription factors sterol regulatory element-binding protein (SREBP) [196, 197], peroxisome proliferator-activated receptor γ (PPARγ) [198] and peroxisome proliferator-activated receptor γ coactivator 1- α (PGC1α) [199], promoting triglyceride storage and mitochondrial fatty acid oxidation. It directly suppresses phosphorylation of insulin receptor substrate 1 (IRS1) and is, therefore, involved in insulin sensitivity in a negative feedback loop [200]. During fasting, mTOR is suppressed through a decrease in insulin and leptin signalling, a decrease in intracellular amino acid [201] and ATP concentration signalled through AMPK [202], therefore improving insulin sensitivity, whilst the opposite occurs in overfeeding. In the hypothalamus, mTOR activation itself induces satiety [203], although it is unclear what downstream events are responsible for this effect.

Peripheral and central insulin and leptin resistance associated with obesity are also mediated through other mechanisms. Crucial proteins involved in this process are SOCS3 and PTP1B. In a negative feedback mechanism, activation of STAT3 through leptin induces expression of SOCS3 [204], which chronically inhibits leptin signalling through its receptor when circulating leptin concentrations are elevated in obesity. SOCS3 diminishes phosphorylation and thus activation of janus kinase (JAK)2, protein tyrosine phosphatase non-receptor type 11 (SHP2) and STAT3 through the leptin receptor [205, 206] whilst PTP1B blocks both the insulin and the

leptin receptor [207], JAK2 and STAT3 [208]. Expression of both SOCS3 and PTP1B is induced by TNF through nuclear factor κB (NFκB), suggesting a mechanism through which inflammation induces insulin resistance [209]. On the other hand, muscle-specific insulin sensitivity is improved through exercise through intracellular signalling involving PI3K [210, 211], AMPK [212], Akt [210], resulting in an increase in ß-oxidation of fatty acids [213, 214].

1.5.7. Most relevant hypothalamic molecules

The key transmitters of hypothalamic appetite regulation are, therefore, NPY and POMC, as they are almost consistently signalling the increase and decrease of food intake, respectively. Further, the influence of circulating hormones on the hypothalamus can be approximated by measuring the amount of insulin, leptin, ghrelin and adiponectin receptors, and the inhibiting molecules PTP1B and SOCS3. AMPK and mTOR are key regulators of intracellular energy sensing.

1.6. Developmental programming

In the last 20 years, the concept of perinatal programming of adult health and disease has gained considerable attention. Programming is defined as a persistent change induced in early life and which will be potentially increasing the risk of developing specific diseases much later on in life. The range of diseases which are thought to be susceptible to programming is wide, including obesity, respiratory and cardiovascular diseases, plus certain types of cancer and osteoporosis. As research progresses, mechanisms mediating programming are becoming clearer. Here, I will

concentrate on the fields of most relevance to the studies presented in this thesis, i.e. programming of appetite regulation and metabolic health with focus on obesity and insulin resistance.

1.6.1. Long term effects of birth weight

Babies display a range of weight at (term) birth, with those in the lowest and the highest 10% birth weights defined as small and large for gestational age (SGA and LGA), respectively, influenced by prenatal developmental conditions. Weight at birth is dependent on many factors, including parental height [215] and ethnicity [216], placental size [215] and maternal nutrition [217], maternal health such as preeclampsia [218] and maternal nicotine exposure [219] during pregnancy. Additionally, the time period of growth restriction may be approximated by measurement of ponderal index, with a low (<10^{th} percentile) index indicating growth restriction in late pregnancy, as skeletal growth is less impaired than muscle, fat mass and organ growth, which is termed asymmetric SGA [220, 221]. The ponderal index is defined as

$$ponderal\ index = \frac{weight\ (kg)}{length\ (m^3)}$$

From 1992 onwards, Barker presented data of relationships between adult diseases and birth weight in several cohorts in the UK. These studies showed a negative relationship between birth weight throughout the whole range, i.e. not simply an effect of SGA, and death from coronary heart disease, a strong negative relationship with Type 2 diabetes and a weaker one with plasma low-density lipoprotein (LDL) concentration and blood pressure [222, 223].

Similarly, in a Swedish male cohort, low birth weight or small ponderal index was associated to a higher rate of insulin resistance at 50 years, even after adjustment for BMI at 50 years [224]. Adult development of insulin resistance has also been described as following a U-shaped relationship with birth weight [225], especially in a highly obese population [226], as heavy babies are often born to mothers with a high pre-pregnancy BMI and gestational diabetes, as will be discussed in Section 1.6.2. This U-shaped relationship is removed if offspring risk of diabetes is adjusted by current BMI when it is replaced by a negative correlation, i.e. a higher risk of developing diabetes with low birth weight [225]. An increase in factors associated with insulin resistance is seen in those of low birth weight as early as 8 years of age [227]. The negative association of birth weight and later diabetes also includes a higher risk of gestational diabetes in women, showing a non-genetic pathway through which predisposition for metabolic disease can be passed on to subsequent generations [228].

The size of a neonate is mostly determined in late pregnancy, the trimester characterised by exponential fetal growth. Using birth weight as a determinant of adult health without further definition of the timing of adverse effects is an imprecise descriptor since a baby which was growth-restricted throughout pregnancy is likely to develop differently from a baby growth-restricted in the third trimester alone. Additionally, growth restriction in early gestation has not been associated with a change of birth weight [229, 230] (and these children would, therefore, not be included in the low birth weight category) but has major impacts on adult health outcomes as described below [231].

1.6.1.1. The Dutch Famine cohort study

Differences in the consequences of the timing of adverse environment can be seen in the cohort of individuals who were born during or shortly after the Dutch Famine and who were, therefore, affected by extreme maternal nutrient shortage during specific parts of their intrauterine development. During a six month period in the winter of 1944/45, there was an extreme shortage of food supplies in the western part of the Netherlands, due to German occupation at the end of the Second World War. A railway embargo on food could not be circumvented by transportation over water because an unusually severe winter froze the water ways. This resulted in average food intake of approximately 800-1000 kcal/d for pregnant women [231] as opposed to the recommended intake of approximately 2170, 2365 and 2600 kcal/d in the 3 trimesters for women of a normal pre-pregnancy BMI [232] (based on 2100 kcal/d pre-pregnancy requirement). Individuals who had been affected by the famine during pregnancy were followed up at 50-60 years in several studies. As a comparison group, health outcomes of individuals from the same area of the Netherlands who were born shortly before the famine or conceived after the end of the famine were included. Data on health outcomes were analysed by the trimester which was most affected by the famine. Maternal famine during the second and third trimester resulted in a lower body weight whereas famine during the first trimester did not result in a change of birth weight [229]. Mothers affected in the third trimester had a low weight gain during the third trimester whilst mothers affected in both mid or in early gestation had a higher weight gain in the third trimester as compared to mothers not affected. This change in weight gain rate is likely a reaction to restoration of food supplies, making up for earlier slow growth [231].

Generally, individuals who were affected early on in pregnancy were of normal birth weight but showed the most distinct changes in adult health, with an increase in adult obesity, BMI and waist circumference [233], disadvantageous plasma lipid concentrations [229, 234], an earlier onset of coronary heart disease [231], a propensity to consume a high-fat diet [234] and were susceptible to generally poorer health [231]. These long term associations suggest the influence of environmental signals during the periconceptional period. Individuals affected in mid gestation were shorter in adulthood [233] and developed higher rates of microalbuminuria and obstructive airway diseases [231]. Male individuals affected in late pregnancy were found to weigh less in early adulthood [235] but were of similar body weight and BMI at 50-60 years of age [233]. Both male and female individuals also had disadvantageous changes in plasma lipid profile [229] and, importantly, showed a higher insulin resistance in a GTT with increased glucose and insulin at 120 minutes after glucose intake and higher fasted proinsulin concentrations [231, 236]. Based on this data, diseases of the metabolic syndrome are programmed in late pregnancy without a modification of body weight or body composition and these are the changes we assessed further in the sheep study.

1.6.2. Long term effect of maternal overweight and diabetes

Birth weight is associated with later BMI in a J-shaped relationship, which is influenced by maternal BMI to take a U-shaped relationship, i.e. both a low and a high birth weight is associated with a higher adult BMI in the offspring, independently of maternal BMI [237]. Additionally, poorly controlled maternal diabetes, gestational diabetes or impaired glucose tolerance during pregnancy increase the chance of the baby being large at

birth and the offspring to become obese and glucose intolerant [238-240]. The hypothesis is that if mothers are hyperglycaemic, more glucose passes through the placenta and chronically increases fetal plasma concentrations. The fetus then responds with a higher insulin secretion from the pancreas and this chronic high plasma insulin concentration has an anabolic effect on peripheral tissues [239, 241] and may result in higher leptin concentrations in cord blood [241, 242].

1.6.3. Long term effects of rapid early postnatal growth

Early postnatal growth can be accelerated if birth weight is low for gestational age and the infant grows up to be a normal-sized or obese child or when a normal-sized infant develops childhood obesity. For growth to be accelerated, sufficient nutrients have to be supplied and the child's appetite control plays a role in this process. Early postnatal growth is associated with a higher rate of adult diabetes. However, in both scenarios it is difficult to dissect the developmental cause of adult diabetes since low birth weight and later obesity are both associated with diabetes. Epidemiological evidence for this is important as it shows the relevance of long term programming in human populations but animal models are essential to show the isolated effect of early postnatal growth on later health. The age period used to assess early growth is very heterogeneous in the epidemiological studies, ranging from the first week of neonatal life [243] up to three years of age [244]. Subject populations commonly include both prematurely born children and those born at term, regardless of the physiological differences between these groups. In humans, studies conducted are either observational or intervention studies, i.e. by randomising infant feeding method, through which differences in early

postnatal growth are attained. Exclusive breast feeding results in a rather slow growth rate whilst feeding formula generally result in faster growth [245]. The reasons for this difference in growth rate is generally unclear but can be partially explained by higher protein content of formula milk [246, 247] and a slightly higher intake of volume when feeding is controlled by the mother through the bottle rather than by the infant itself through suckling [248]. The slower growth of breast-fed infants may also be mediated through leptin contained in breast milk, but not in formula milk, which has been shown to have acute anorexigenic effects in some studies and it has been suggested that leptin also has long term effects resulting in a suppression of excessive weight gain [249].

Children who grow rapidly in the first 6 months of life are more likely to be overweight or obese at 6-8 years of age than children who had normal or slow early postnatal growth. In the same cohort, children who show a slow growth in early postnatal life also have a higher birth weight than rapidly growing children [250]. In a observation of a shorter time period, rapid growth rates both in the first 8 days of life and in the first 4 months are correlated with adult overweight (OR of 1.28 and 1.04 per 100g weight gain in the observed period, respectively [243]), whilst other studies also show an association between growth in the first 4-6 months of life and childhood or adult overweight [251-253]. When looking at longer periods during childhood, studies found a relationship between growth in the first 3 years of life and BMI and decreased insulin sensitivity at 8 years [244] or growth throughout childhood up to 12 years and obesity and T2DM in middle age [254, 255]. Especially in the latter studies, it is less clear whether early growth is merely a predictor of growth rates in later childhood and adulthood caused by persisting environmental, social or genetic factors, or if these studies point towards a specific developmental

period which is more vulnerable for programming of later weight and metabolic health. As most of these studies [243, 244, 251-253] are large prospective studies, the risk of a systematic bias is low.

Studies concentrating on possible mediators of growth give some insight into this: SGA babies have low cord blood leptin concentrations [256] but, as they are growing rapidly in early postnatal life, have higher plasma leptin concentration at 1 year of age regardless of BMI [257]. Leptin concentrations are also higher in adulthood as compared to individuals with normal birth weight of the same adult BMI but in both these studies fat mass was not determined, i.e. leptin may still be correlated with fat mass, which is thought to be higher in later life of individuals born SGA [258]. Whether leptin has similar effects on the energy balance of fetus and newborn as it does in adult individuals is unknown. When preterm babies are fed a preterm formula high in fat and protein rather than a standard term formula or breast milk, they gain weight faster and have higher plasma leptin concentrations relative to fat mass at pubertal age although weight and fat mass, measured by impedance analysis, are not changed [259]. This, therefore, demonstrates that leptin expression is over-proportionately increased in these individuals but it is unclear whether this effect is specific for individuals born preterm or whether it could be extrapolated to rapid growth following low birth weight. In the same study, the individuals fed preterm formula also have higher fasted 32–33 split proinsulin concentration at pubertal age, a marker thought to be representative of glucose intolerance [260]. It is, therefore, feasible that a change in early growth pattern has effects both on the amount of fat mass in adulthood and on the adipokine characteristics of this adipose tissue, at least in individuals born preterm.

1.7. Perinatal development

1.7.1. Sheep and rat as models for human pregnancy and early postnatal life

To directly explore potentially disadvantageous health outcomes of nutritional interventions, especially during development, animal models need to be used for ethical reasons. Secondly, in order to understand the mechanisms behind the outcomes, tissues need to be investigated directly and the availability of histological samples from human subjects is very limited. Both rodents and larger mammals are commonly used as models for mechanisms in humans. Generally, no animal model represents perfectly all mechanisms affecting humans and different models are used depending on the research questions.

With regards to develomental stages, rodents are not directly comparable to humans and bigger mammals like sheep are a better model as they are following human development more closely. Rats have a much shorter gestation time of 21-22 days than human gestation of about 270 days whilst sheep, depending on breed, have a gestation time of 145-150 days. Both humans and sheep carry one or two fetuses per pregnancy whilst rodents carry litters of around ten pups, which are born relatively immature, i.e. some of the developmental steps that occur in utero in humans and sheep occur postnatally in rodents, including hypothalamic and pancreatic development. If the research question regards development of a specific organ, the intervention period has to be considered carefully, i.e. as pancreas development is prenatal in humans but predominantly postnatal in rodents, interventions during pregnancy cannot be compared between the two species. Therefore, conclusions have to be drawn

carefully. An abundance of studies of developmental origins of adult health and disease are carried out in rodents, also due to their obvious advantage with regards to costs and short generational time span.

In the studies in this thesis, sheep were selected to study programming of insulin resistance and appetite regulation by interventions in late pregnancy and early postnatal life. Adipose tissue mass plays a decisive role in both systems. The corresponding organs, i.e. adipose tissue, pancreas and hypothalamus, develop in similar stages of pregnancy in both humans and sheep. In the rat study of this thesis, the intervention was during the lactation period and effects on brain, adipose tissue and pancreas were assessed again. These organs predominantly develop in early postnatal life in rats whilst they are developing in late pregnancy in humans. The timing of intervention is, therefore, coinciding with the main developmental period of these organs. The evidence for developmental periods is summed up in the following sections for sheep, human and rats.

1.7.2. Development of adipose tissue

In sheep, adipose depots are first visible around day 70 of gestation (50% completion of gestation) around the kidney and heart with the appearance of brown adipose tissue, whilst subcutaneous fat is detected from day 85 (60% gestation), increases in mass and then decreases again to a very small depot at term [261]. Total lipid content at term is at about 3% but this is dependent on breed [261]. The thermogenic activity of BAT in perirenal adipose tissue increases from 140 days of gestation (97% gestation) [262], which is induced by the pre-partum rise in fetal plasma cortisol [263].

In humans, some subcutaneous and visceral fat, mostly perirenal fat, is present at birth [220], with a total body fat mass of 8-16%, highly dependent on birth weight [264] and maternal diabetes status [265]. The number of adipocytes is determined in childhood with obese children having more and bigger adipocytes than lean children [266]. Later changes in fat mass are thought to be mostly mediated through a change in adipocyte volume, not number [267] with the adverse consequences for metabolic health as described in section 1.2.4. After birth, human and sheep BAT around the central organs is replaced by WAT [268] and only specific BAT depots persist in the adult [13].

In the rat neonate, white fat is non-existant but grows rapidly after birth [269]. Total fat mass is at 5-7% at 3 weeks of age in the rat, which primarily consists of gonadal, perirenal and subcutaneous fat and only 1% of body weight as interscapular fat with predominant brown adipose characteristics [270].

Glucocorticoids have been shown to be involved in the perinatal maturation of adipose tissues [271, 272]. Human and sheep are born with a mature, functioning HPA axis and steady increase in fetal cortisol concentrations during pregnancy, especially before birth [273], as opposed to rodents, in which the offspring start producing corticosterone at 2 weeks postnatal [274].

1.7.3. Development of hypothalamus

Both in humans and sheep the main neurotransmitters, NPY, AgRP, POMC and CART, and receptors for insulin and leptin are expressed in the hypothalamus from mid-gestation, i.e. 21 weeks (52% gestation) in humans [275] and 81 days (56% gestation) in sheep [276, 277]. Even

though further developmental steps have not been investigated in humans and sheep, Grayson et al. showed in non-human primates that the number of neurons expressing neurotransmitters increases with gestational age [80]. Projections are formed between different nuclei of the hypothalamus and the brain stem as the hypothalamus goes through morphological changes [80]. An i.c.v. injection of NPY induces swallowing of sucrose solution in the ovine fetus at 130 days of gestation (90% gestation) [278], showing that at least basic regulatory mechanisms are functional antenatally.

In the rodent, low levels of NPY are detected in the hypothalamus from day 14 of gestation (67% gestation) [279] but projections from the brainstem to the hypothalamus continue to develop in the first two weeks of postnatal life [280] and projections from the ARC to the PVN and VNM are established by 16 and 21 days of postnatal life, respectively [279, 281]. Bouret et al. showed that leptin was crucial for the development of projections and that the perinatal leptin surge observed in neonatal rodents is likely to be important in brain development [282].

The hypothalamus is part of the hypothalamic-pituitary-adrenal axis. CRH and AVP from the hypothalamic PVN induce release of ACTH from the pituitary which, in turn, induces the release of cortisol into the blood stream. In a negative feedback loop, cortisol inhibits the release of both CRH/AVP and ACTH [283]. Hypoglycemia decreases CRH secretion from the hypothalamus and this effect is inhibited by leptin [284]. Consistent with this, intraperitoneal injection of leptin decreases ACTH and glucocorticoid response in a stress test in rodents [284]. Besides their other physiological stress response effects, AVP and cortisol both increase blood pressure. A normal pregnancy in human and sheep is associated with a steady increase in maternal CRH, ACTH and cortisol concentrations and,

in sheep, these increase exponentially immediately before parturition [285]. Cortisol is also involved in maturation processes, for example glucocorticoids routinely administered antenatally during preterm labour promote lung surfactant production [286]. Although substantial changes in offspring physiology can be induced by administering glucocorticoids during pregnancy, changes in nutrition in early and mid gestation only reduces maternal cortisol, which has not been shown to affect the fetus [287].

1.7.4. Development of pancreas

In development of the fetal sheep and human, the pancreas forms as a bud from the endoderm very early on in pregnancy, which then grows in size and forms islets. Single cells of the epithelium of the pancreatic duct start expressing the mature form of their secretory protein, including insulin, and migrate out of the epithelium to form islets, losing the epithelial characteristics at the same time. In sheep, insulin-positive cells can be detected from 24 days gestation (16% gestation) and islets form at about 29 days (20%) [7]. In the human fetus, secretory proteins are expressed from 52 days of gestation (20% of gestation) and primitive islets form at 12 weeks of gestation (30% of gestation) [288]. Later in gestation, the pancreas increases in mass, islets form by proliferation of secretory cells and the insulin response to a glucose challenge increases [289]. In rats, development of the pancreas is distinctly later as compared to sheep and human. Cells start producing mature secretory proteins at 17 days of gestation, i.e. in late gestation (80%) [290]. A robust insulin response to glucose is only present at 1 week after birth, following a period of ß-cell apoptosis [291], during which highly amino acid-responsive ß-cells are replaced with glucose-responsive ones [292].

1.8. Mechanistic effects underlying perinatal programming

Perinatal programming is now often attributed to different mechanisms, including differences in growth patterns of specific organs, differences in endocrine system development and epigenetic mechanisms regulating expression of specific genes. Even though detailed descriptions of mechanisms in long term programming of adult diseases are emerging, the exact contribution of each factor is not easily defined and programming of diseases is most likely an interaction of, at least, the three mediators described in the following.

Growth patterns are not similarly regulated for all tissues and organs, an effect which may be mediated by differential sensitivity e.g. to insulin and insulin-like growth factors [293]. In intra-uterine growth restriction (IUGR), low birth weight can result from reduced availability of nutrients with, at least when nutrients are not critically limited, sparing of brain, heart and adrenal gland growth. Therefore, the weight of these organs is greater relative to body weight than in neonates of appropriate birth weight [294, 295]. Other organs and tissues show a lack of development of structure and reduced numbers of specific cell types at birth, including nephrons [296], cardiomyocytes [297] and pancreatic ß-cells [298].

Endocrine systems of the offspring are also affected by changes in maternal hormones or environmental hormone-like substances, e.g. changing sensitivity of these systems in the offspring [299]. Hormones that are having long term effects in this way include insulin, glucocorticoids and bisphenol A, which has estrogenic effects [300, 301].

Long term health outcomes depend strongly on the developmental timing of environmental stimuli and the timing of pre- and postnatal development of humans is different to rodents but similar to sheep. Roughly, many of the developmental changes happening in late pregnancy in humans and sheep occur in early postnatal life in rodents. Therefore, a low growth rate in late pregnancy may have similar effects on humans and sheep as a low growth rate during suckling has on rats with regards to e.g. pancreas development and subsequent susceptibility to glucose intolerance. As species are not necessarily directly comparable, I discuss evidence of programming mechanisms in the two groups, i.e. larger mammals and rodents, separately and concentrate on the effects of differences in macronutrient intake and/or the subsequent differences in growth rates with regards to later metabolic health.

1.8.1. Epigenetic mechanisms

Epigenetic methylation refers to methylation of DNA, especially in the promoter region of genes, at CpG-islands (repetition of cytosine-phosphate-guanidine sequence) [302, 303]. The more closed conformation of methylated DNA and modified histones is associated with lower gene expression levels, i.e. with gene silencing. These modifications can vary between neighbouring genes, allowing for selective, long term regulation of gene expression and subsequent protein concentrations, which can have influence on specific phenotypes [304]. Epigenetic alterations are subject to changes in response to environmental factors during a period of sensitivity to environmental influence that is thought to range from preimplantation [305] to early postnatal life [306-308], at least in rodents. During this period of early life, stable methylation patterns of the DNA are

established [309, 310]. At least in rats, influential environmental factors can be over- or undernutrition [311], a diet high in methyl-donors [303, 312], or extremes of maternal care behaviour [313]. Epigenetic modifications can, furthermore, change during DNA replication or repair, a mechanism called epimutation [314]. Epigenetic changes occur in some, but not all, genes and results in up- or down-regulated transcription. After conception, but before implantation, all DNA is demethylated and methylation patterns are re-established after implantation [315]. This occurs similarly in imprinted genes, which make up about 0.5% of rodent genes, i.e. genes of which only one copy, maternal or paternal, is used for transcription and the other is highly methylated [310].

As DNA methylation heavily relies on the availability of methyl donors from the diet, e.g. methionine, betaine and folate, global nutrient restriction or protein restriction have direct influences on these processes. This is well established in rodents [303, 312] but evidence in humans is sparse [316]. In rodents, feeding excess methyl donors whilst restricting caloric or protein intake prevents some of these effects [317] but also induces other changes [318]. Surprisingly, both protein and global nutrient restriction during fetal development can lead to hypo- [317] and hypermethylation [311] of the same genes with corresponding effects on gene expression in the adult offspring. This implies that the regulation of promoter methylation has to be more specific than merely influenced by methyl donor shortage.

1.8.2. Programming of glucose metabolism

In IUGR babies, both the pancreatic endocrine mass and the insulin response to glucose are reduced [298, 319]. Depending on the timing of

intervention and the method used to induce IUGR in sheep models it can have quite different effects on the pancreas. Hyperthermia induced through most of the period of fetal pancreatic development, i.e. during mid-gestation, leads to a decrease of ß-cell mitosis, a lower ß-cell mass despite a normal total pancreatic mass relative to body weight [320] and islets which produce and secrete less insulin [321]. On the other hand, hypoglycemia from day 115 to 135 of gestation does not decrease pancreatic or ß-cell mass [322] but decreases ß-cell responsiveness [323]. There is little information on the long term development and physiological outcomes of these ß-cell defects in either sheep or humans. However, fasting insulin is higher in 1 year old children born SGA who catch up in weight compared to either children born of appropriate weight for gestational age or those born SGA without then showing rapid postnatal growth [324]. This suggests an over-compensatory effect, which might persist into adulthood. In a sheep model with 50% nutrient restriction in late gestation, at 12 months of age offspring have a greater insulin response to a GTT although pancreas weight is not significantly different [325].

IUGR also induces an increase in fetal cortisol, which is correlated with hypoglycemia [326] and, if the glucocorticoid betamethasone is injected into mother or fetus in late pregnancy, offspring show a stronger insulin response at 6 months and 1 year of age [327]. However, repeated administration of glucocorticoid to the mother also decreases birthweight [327], so that causality is not entirely resolved. Maternal food intake was not reported in this study but since glucocorticoids are known to suppress appetite in a dose-respondent manner [328], this effect may mediate the change in insulin metabolism observed.

Newborn sheep who experience growth restriction throughout their fetal development have a lower relative liver weight [329]. Growth restriction from mid gestation induces a higher expression due to lower promoter methylation of hepatic phosphoenolpyruvate carboxykinase [330, 331] and higher expression of glucose-6-phosphatase [332], key enzymes in gluconeogenesis. In these animals, insulin sensitivity is increased by a higher expression of insulin receptor and hepatic glycogen content is not reduced despite hypoglycemia [332]. Gluconeogenesis is normally not observed before birth because glucose is transferred from the mother through placenta [273]. However, the hypoglycemic fetus has to compensate for lacking glucose by inducing gluconeogenesis as described above. Whether these changes in hepatic gene methylation and expression persist or what compensatory effects follow is not well understood. Ectopic fat mass in liver and skeletal muscle has been linked to insulin resistance [57, 333] but, in a study with adults who were born SGA, insulin resistance, including a lack of inhibition of hepatic gluconeogenesis, has been shown to develop in the absence of changes in fat deposition in liver or muscle [334]. In the latter study, individuals born SGA had higher cortisol and growth hormone concentrations whilst insulin-like growth factor (IGF)1 concentrations were lower and these changes may be linked to hepatic and muscle insulin resistance through, as yet, unclear mechanisms.

1.8.3. Programming of body composition

Individuals born SGA have a higher fat mass at a given BMI, especially abdominal fat mass, even at a young age [335-337] and, if they grow rapidly in early life, have a higher rate of obesity than individuals with

median birth weight [237]. Body fat is dependent of birth weight [264] in humans but, in sheep, body fat is present at a low percentage and independent of birth weight [338] but is influenced by maternal caloric intake [339]. Timing of nutrient restriction has direct influence on neonatal fat mass since growth restriction in late pregnancy, detected as asymmetric SGA, leads to lower subcutaneous fat mass as compared to symmetric SGA [220].

Low birth weight sheep are also born with less muscle mass [340] and gain muscle mass slower overall, specifically slower in some, i.e. semitendinosus, extensor digitorum lateralis and extensor carpi radialis, but not all muscle groups compared to high birth weight offspring [341]. Similarly, it has been shown in humans that birth weight is negatively associated with adult lean mass [336]. As muscle is one of the main tissues to take up and metabolise glucose, this could have an impact on glucose tolerance. However, in a 50% caloric restriction in late pregnancy, expression of insulin receptor and PI3K in muscle of 1 year old offspring were not changed and insulin resistance was found in perirenal adipose tissue which had increased expression of insulin receptor and PI3K but a lower expression of GLUT4 [325]. This suggests that increased fat mass, not decreased muscle mass is the main mediator of insulin resistance after IUGR.

1.8.4. Programming of food intake

There is very little information on the long term effects of perinatal events on hypothalamic appetite control in large mammals. However, near term offspring of mothers undernourished in late gestation showed an orexigenic response as NPY was more highly expressed in the hypothalamus as

compared to controls [342], which is consistent with higher growth rate subsequent to small birth weight. On the other hand, offspring of overfed mothers had a transient higher milk intake in the first 3 weeks postnatal and at 4 weeks displayed an anorexigenic response with increased POMC and non-significantly decreased NPY expression [343]. However, interpretation of this study is complicated by the fact that the overfed group and the control group had strikingly different numbers of twin and singleton offspring.

1.8.5. Effects of modulation of postnatal growth rates in rats

In rodent models, early postnatal growth rates are often manipulated by controlling litter size. Remmers [344, 345] demonstrated that rats raised in abnormally large litters (20 pups), resulting in food restriction during suckling, showed smaller body length, weight and BMI throughout life as compared with rats raised in normal-sized litters (10 pups). A significant part of the offsprings' growth occurs after weaning and this was decreased by early postnatal life in a large litter. The effect on long term weight control through this intervention suggests that there is a critical period of body weight regulation during suckling. In this study, rats were sacrificed at postnatal days 10, 25 and 380. Analysis of hypothalamic gene expression during early life (at postnatal day 10 and 25) showed an increased ratio of orexigenic to anorexigenic signalling as compared to control rats, which was proposed to account for the higher food intake of 10-20% after weaning in males until day 35 [344, 345]. After this time, food intake did not differ between groups and no difference in the hypothalamic ratio of orexigenic to anorexigenic signalling was found at age 380 days.

In a similar study, rats were raised in litters of either 4 or 10 pups to study the effects of rapid postnatal growth and it was shown that increased food intake during suckling (mediated through reduced competition for maternal milk from litter-mates) resulted in greater rate of weight gain before weaning and increased weight and central fat mass, specifically epidydimal fat mass, throughout life [221]. At 90 days, isolated epidydimal adipocytes were less insulin sensitive after rapid early growth and had decreased abundance of glucose transporters GLUT-4 and GLUT-1. These adaptations were accompanied by reduced intracellular insulin signalling, including a lower abundance of insulin receptor and IRS-1, and a failure to activate the Akt pathway in response to insulin. Despite this, glucose tolerance tests at 90 days did not show a difference in blood glucose regulation between the two groups, perhaps suggesting that offspring can compensate for these changes at this young adult age. The results of these studies, however, clearly demonstrate that, in rats, growth in early postnatal life modulates some aspects of the long term regulation of both body weight and insulin sensitivity.

1.9. Interventions described in this thesis

In order to explore long term effects of perinatal nutritional environment on metabolic health and appetite regulation, studies of maternal macronutrient restriction in late gestation followed by variable early postnatal growth rates and of maternal choline intake during lactation were conducted. These complimentary studies were undertaken in Nottingham, UK and Edmonton, Canada to utilise the local expertise in the use of the respective models. The former study was conducted in Nottingham in a sheep model, which is a good model for human development and periods

of organ development are at equivalent stages of gestation and suckling. The latter study was conducted in Edmonton in a rat model, for which previous knowledge about choline metabolism and requirements exists and which is a good model for human development, provided differences in organ developmental periods are considered. Both models have been used before in the respective research groups and animal research staff with expertise in conducting interventions in these animals carried out each study.

1.9.1. Nutrient restriction

In the sheep study conducted in Nottingham, a 40% reduction in maternal macronutrient intake during late pregnancy was used. The main effect of nutrient restriction leading to microsomia is hypoglycemia of mother and fetus and may be accompanied by hypoinsulinemia and hypoleptinemia. As described in more detail above, nutrient restriction during late gestation has long term effects on the offspring, including a higher risk rate for disturbed glucose metabolism in adulthood. This effect is believed to be aggravated by an accelerated early postnatal growth rate. Although adiposity and especially visceral fat mass are prominent risk factors, the lower glucose tolerance observed after slow growth in late pregnancy and accelerated postnatal growth appears to not be accompanied by an increase in fat mass. Mechanisms mediating the change in glucose tolerance are not clear but may include changes in ß-cell responsiveness, hepatic glucose production and peripheral glucose uptake. Nutrient restriction during late pregnancy also coincides with development of hypothalamic networks regulating food intake in humans and large mammals but the acute and long term effects are not well established. As

nutrient availability directly affects appetite regulation, the hypoglycemia experienced in utero could change settings of the hypothalamic regulatory system as well as systemic glucose tolerance.

1.9.2. Choline

In the rat study conducted in Edmonton, mothers were fed one of 3 diets from birth to weaning, either containing no choline at all or containing an amount of choline which is recommended to meet requirements (1g/kg free choline or 2.5 g/kg choline bitartrate [346]). Choline was fed as bitartrate or as phosphatidylcholine (PC). Choline is a micronutrient with vitamin-like characteristics. It is both taken up from dietary sources, such as egg yolk and animal livers, which are rich in free choline and PC, and endogenously synthetised. PC can be derived either from attaching diacylglycerol to choline, ie. the cytidine diphosphate (CDP)-choline pathway, or from methylating phosphatidylethanolamine (PE), i.e. the PE methyl transferase (PEMT) pathway.

Choline is involved in the one-carbon cycle as betaine, in the functioning of cell membranes and vesicles as part of PC and in neural transmission as acetyl-choline, which are described in more detail below. The most important forms of choline are choline as a salt, e.g. in conjunction with a bitartrate (Figure 1.3), or as PC, a phospholipid called lecithin in the specific form when isolated from egg yolk, consistent of choline, a phosphate group and two fatty acids (Figure 1.4). Further important choline metabolites found both in the diet and tissues are sphingomyelin consisting of choline, phosphate group, one fatty acid and one sphyingosin; betaine, an oxidised form of choline; acetylcholine, choline with an acetyl group; lyso-phosphatidylcholine, PC with one fatty acid removed by

phospholipase A2; phosphocholine, choline with a phosphate group attached but no fatty acids; glycerophosphocholine, phosphocholine with a glycerol attached to the phosphate group.

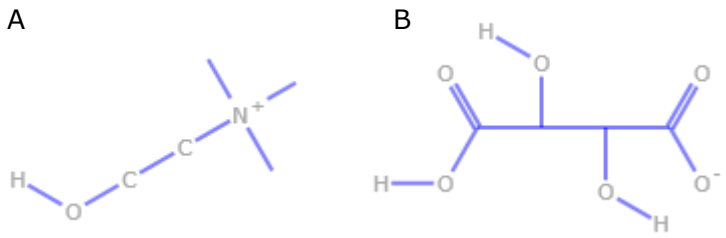

Figure 1.3: Chemical formula of choline bitartrate, with A, the positively charged choline and B, the negatively charged bitartrate.

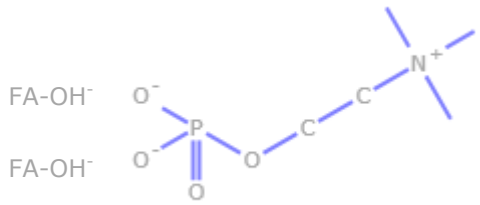

Figure 1.4: Chemical formula of PC with its 2 fatty acids attached to the phosphate group indicated as [FA-OH$^-$].

1.9.2.1. Choline bioavailability

As main dietary forms of choline are free choline and PC, this study of my PhD includes a direct comparison between consuming choline salt, i.e. the free form of choline, and PC. In human studies, ingestion of the same amount of choline as chloride, the free choline form, and as PC on separate days showed a rapid increase in serum choline concentrations and return to baseline within 8h after choline chloride ingestion and a slower increase but plateauing of choline concentration when ingestion occurs as PC [347]. In rats, ingestion of both choline chloride [348] and PC [349] results in increased brain and adrenal choline and acetylcholine concentrations subsequent to an increase in serum choline. When 15 day old rats were

intubed with formula containing radioactively labelled choline metabolites including free choline and PC, ingested free choline was found in the liver mainly as betaine, phosphocholine and PC whilst the majority of ingested PC was not converted before storage in the liver. This suggests fundamental differences in systemic metabolism but differences in effect of the 2 metabolites has not been well studied as the majority of the studies discussed below included choline as free choline alone. Both free choline and PC as well as glycerophoshphocholine and, to a lower extent, betaine and sphingomyelin are contained in vegetables, cereals, eggs and meat, especially liver, the main storage organ for choline [350].

1.9.2.2. Functions of choline

One-carbon metabolism

Various methylation processes rely on availability of methyl groups from the one-carbon cycle. In this cycle, methyl donors, mainly betaine and folate, enable methylation of homocysteine to methionine. This can then be converted to S-adenosylmethionine (SAM), subsequently transferring the methyl group to various targets including CpG islands of DNA. SAM can also be used to *de novo* synthesise PC from PE by adding three methyl groups. Whilst some of the converting enzymes of the one-carbon metabolism (Figure 1.5) are expressed in various tissues, the main conversion of homocysteine to methionine and SAM takes place in the liver [351].

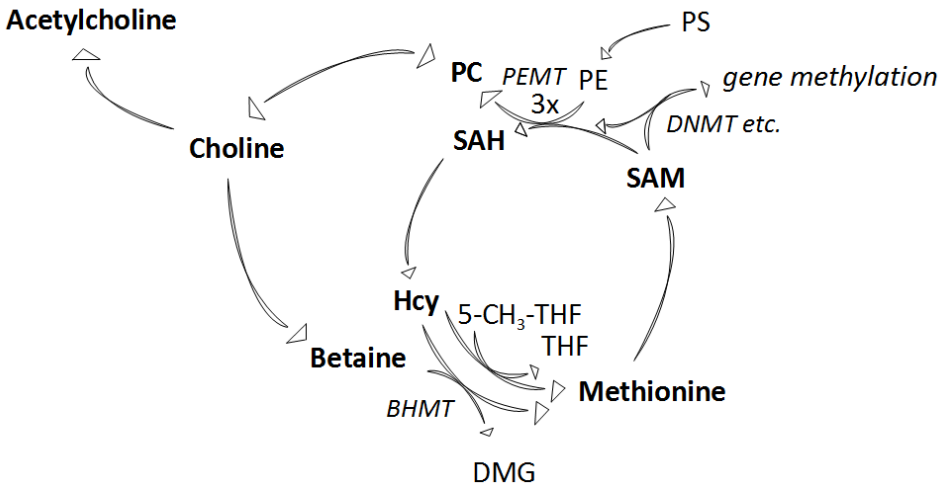

Figure 1.5: Schematic depiction of choline metabolism.

Hcy, homocysteine; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; 5-CH_3-THF, 5-methyl-tetrahydrofolate; THF, tetrahydrofolate; DMG, dimethylglycine; PEMT, phosphatidylethanolamine methyltransferase; DNMT, DNA methyltransferase; BHMT, betaine-homocysteine methyltransferase.

This figure is based on the review [351].

Cell membranes

Due to their amphipathic nature with a hydrophobic and a hydrophilic group, phospholipids make up the main part of all cell membranes in a bilayer. Sphingomyelin (SM) is preferentially deposited in the outside leaflet and PE, phosphatidyl-serine (PS) and phosphatidyl-inositol (PI) are preferentially in the inside leaflet of membranes whilst PC and cholesterol are found in both leaflets [352]. Changes in the distribution of specific phospholipids in the leaflets can alter cell signalling, the most well-studied example is PS changing from the inside to the outside of the leaflet, which occurs during apoptosis and induces phagocytosis of the cell [353]. Each phospholipid has the hydrophilic head group, e.g. choline, and 2 fatty acids on its hydrophobic side. Membrane characteristics like phospholipid and fatty acid composition can influence numerous signalling pathways and transmembrane transport [354]. In choline deficiency, membrane content

of PC is reduced whilst that of PE is increased [355]. *In vitro*, the homeostasis of PC or of the ratio of PC to PE [356] in the membrane has been shown to be crucial for cell growth and survival.

An important system relying on membranes is the transport of lipids through the blood. Triglycerides and cholesterol are transported from the liver to peripheral organs as very low-density lipoproteins (VLDL) [357]. PC is crucial for the formation of VLDL [358] and, in males, choline deficiency leads to hepatic steatosis [359].

Acetylcholine

Acetylcholine (ACh) is a common neurotransmitter, used by brain and peripheral neurons including the parasympathetic and sympathetic nervous system and in the innervation of muscles, inducing contraction through nicotinic receptors [360], whilst ACh affects contraction of cardiac muscles through muscarinic receptors [361]. It is cleared from the synaptic space by degradation through acetylcholinesterase to stop excitation or inhibition [362]. ACh plays a role during neurodevelopment [363] as a modulator of plasticity and is, therefore, involved in learning processes and memory [364].

1.9.2.3. Choline deficiency and sensitive periods

In adult men, choline requirements are 550 mg/d whilst they are 425 mg/d for women and requirements increase to 450 mg/d in pregnancy and 550 mg/d in lactation [365]. Epidemiological data suggests that actual choline intake in women is at around 400mg/d with a high interindividual variation [366]. In rodent studies, choline is given as free choline with a standard intake of 1 g/kg diet for control animals, choline-devoid diets are 0 g/kg

and treatment of supplemented animals can differ but they often receive additional choline with diet (e.g. 5 g/kg, [367]) or with their drinking water at a concentration of 25 mmol/L [368-370].

Since early development is a period associated with high demands on methylation and expansion of cell membrane surface area, choline has been widely investigated in human pregnancy and deficient periconceptional maternal choline intake increases the risk of neural tube defects in the offspring, similar to periconceptional folate deficiency [366, 371].

In rodent studies, a choline-deficient diet during pregnancy leads to altered expression of proteins involved in cell cycle regulation of the fetal hippocampus which are, in part, associated with diminished methylation of promoters of the genes of these proteins in this region [372, 373]. The adult offspring showed changes in hippocampal function [374, 375] and decreased memory performance [376] and attention to stimuli [368]. At the same time, choline-rich diets led to increased hippocampal proliferation [374, 375, 377] and enhanced memory in the adult offspring [378, 379].

Whether altered gene promoter methylation status is the driving force behind these changes is unknown. Besides its functions in forming acetylcholine, dietary choline also has a neurotrophic function [380]. A study by Garner et al. suggests that maternal supplementation with choline chloride (25 mmol/L in their drinking water) during pregnancy does not lead to higher choline or PC concentrations but higher phosphocholine concentration in the fetal brain, whilst acetylcholine concentrations were not measured [370]. This finding supports the concept that differences in brain acetylcholine abundance may be the cause of the better spatial memory in maternally choline-supplemented offspring. Other mechanisms

demonstrated are an increase in IGF2 and IGF2 receptor and IGF2-induced acetylcholine release in adult offspring of mothers choline-supplemented during pregnancy [381] and preserves hippocampal plasticity into adulthood and increases concentrations of vascular endothelial growth factor and neurotrophin 3 [367]. Even though the importance of each of these mechanisms is not entirely clarified, feeding dietary choline during pregnancy is clearly necessary for brain development of the offspring, at least for memory function in the hippocampal region [368, 374-379].

In the early postnatal period, supplementation with choline (offspring intubation with 25 mg choline chloride, day 2-21) only improved spatial memory of adult rats if they were prenatally damaged by maternal ethanol consumption [382], suggesting a critical period for adequate choline availability if brain development was disturbed during pregnancy. Although rodent brain development has not been completed by the time of birth [383-385] and could, therefore still be sensitive to choline availability after birth, a postnatal intervention of choline intake alone (offspring intubation with 0.9-1.8 mg choline chloride, day 1-24 [386]; artificial rearing on day 5-18 with formula containing 250 mg choline chloride per liter as control and 1250 mg/L as higher choline intake [387]) does not seem to affect spatial memory after a normal pregnancy [386, 387]. Studies with early postnatal choline supplementation are few and, to my knowledge, only examine spatial memory.

Feeding adult mice a choline-devoid or a high choline diet for 4.5 months influences their memory significantly as measured in an age-dependent test, with mice on the high choline diet having a better memory than both a standard choline control group and the choline-devoid group, which had the lowest score (feeding intervention diets at 8.5-13 months of age, choline-devoid diet contained less than 1g choline per kg diet, control 1.6

g/kg, high choline 12-15 g/kg [388]). Mice with dementia produced by selective inbreeding of mice with low memory scores had lower brain acetylcholine concentrations than control mice. Feeding these mice with dementia a high dose of PC (100 mg/d) for 45 days normalised their brain choline and acetylcholine concentrations and improved their memory whilst the same feeding regiment in normal mice increased their brain choline but not acetylcholine concentrations or memory performance [389]. Intraperitoneal injection with choline (saline, 50, 100, 120 mg choline chloride per kg body weight) increased brain choline and release of acetylcholine in a dose-dependent manner [390]. As mentioned above, cultured neurons have a drastically increased apoptosis rate if maintained in choline-free medium [391]. In humans, treatment with choline esterase inhibitors has been found to improve symptoms of Alzheimer's disease as acetycholine improves vasodilation and blood flow [392]. These studies suggest that choline is important for adult brain function both as the structural form, PC, and as the neurotransmitter acetylcholine.

A choline-deficient diet consumed by adults results in damages in liver and muscle [393]. As choline is necessary in the liver to secrete triglycerides and cholesterol in the form of lipoproteins [358], a choline-deficient diet leads to steatosis [355] as described above. Additionally, choline deficiency has been shown to induce apoptosis in liver and muscle and neither effect is reverted when adding other methyl donors [355, 394, 395], suggesting that PC as part of the cell membrane may be involved in the changes observed. Organ damage is mostly observed in men or postmenopausal women [396]. Premenopausal women are protected from choline depletion as oestrogen increases PEMT activity and premenopausal women have been shown to be more affected if they carry a single nucleotide polymorphism in the PEMT gene which reduces PEMT activity [396].

Choline intake and activity of biosynthetic enzymes have also been described to have effects on food intake, leptin and body weight regulation [397, 398] but the picture emerging is very incomplete. In few studies, depleting animals' diets of choline completely caused a substantial reduction of their food intake [397] although most studies do not show any effect on food intake, which might, therefore, depend on specific developmental period of intervention, e.g. the increased nutritional requirements during lactation period.

Furthermore, mice deficient of PEMT but with a normal dietary choline intake are resistant to the metabolic effects of a high-fat diet, e.g. obesity and insulin resistance, and this phenotype was reversed when increasing choline intake [398]. It is not clear from the limited amount of research how the effects of choline on appetite control are regulated and my thesis aims to contribute to this field of research.

In summary, even though choline is an essential micronutrient throughout life, both pregnancy and lactation are periods of increased choline intake requirements. This is reflected by recommendations of Adequate Intake in humans and by rodent studies observing a disadvantagous effect of feeding diets lacking choline during pregnancy and, to a lesser extent, during lactation period on spatial memory of the offspring. Present research in rodents primarily focuses on the role of choline intake during pregnancy and in adulthood but lactation period, when recommended intake is highest in a woman's life, is less well defined with regards to long term outcomes in offspring. Furthermore, there is little research with regard to the form of choline and the study presented in this thesis aims to address the differences between choline fed as free choline or as PC during lactation period.

1.10. Main hypothesis and aims

As described, the perinatal nutritional environment has been shown to have long term effects on insulin sensitivity and body composition in both epidemiological and interventional studies. However, there are limited studies describing the influence on the appetite regulatory system in appropriate animal models of obesity and insulin resistance. To further elucidate this relationship, two studies were used in the course of my PhD.

In sheep, my primary hypothesis was that sheep who were subjected to maternal nutrient restriction in late pregnancy would develop insulin resistance at an adult age, independent of changes of body mass or composition. Accelerated growth in early postnatal life would further aggrevate insulin resistance.

Since there is very little data on appetite regulation in IUGR individuals, my hypothesis with regards to hypothalamic gene expression was that insulin resistance would also develop centrally. In addition, I hypothesised that it would be consistent with central insulin resistance if appetite regulation was overall more orexigenic in offspring of mothers receiving 60% of their nutrient requirements during late pregnancy as compared to sheep not subjected to maternal nutrient restriction.

In the rat study, the aim was to determine the acute and long term effects of a maternal diet devoid in choline and of diets with phosphatidylcholine rather than free choline during lactation on maternal and offspring appetite regulation and metabolic health. Primarily, I hypothesised that feeding a choline-devoid diet would decrease maternal food intake, reduce maternal choline stores and delay offspring growth. I also hypothesised that offspring would develop glucose intolerance and changes in structure of

tissues which are highly dependent choline and/or phospholipids. Ingesting the different forms of choline may affect tissue concentrations of choline metabolites, specifically the liver, both in mothers and their offspring.

2. Methods and Materials

My thesis was designed and carried out in 2 parts, at 2 different locations. First, a sheep study was carried out in Nottingham, UK, in 2006-2007. I started my PhD in January 2009 and performed analyses on tissues as described below in Nottingham. Separate analyses of plasma metabolome was performed in Nantes, France. I then moved to Edmonton, Alberta, Canada in summer 2010 where there had been rat studies carried out when I came and the previous summer (2009/2010). I performed tissue analyses on these studies and then helped with a new rat study in spring 2011, which I also analysed tissues for. All methods are described below, first for the sheep study and then the rat study. Whenever methods overlap between the two studies, i.e. glucose tolerance test, plasma analyses and histology, they followed the same principles but details may differ, based on equipment available and standard techniques of the respective laboratory.

2.1. Sheep macronutrient restriction study

All sheep procedures were performed at the farming facilities on the Sutton Bonington Campus of the University of Nottingham. Procedures were in accordance with the UK Animals (Scientific Procedures) Act, 1986 with approval from the local Ethics Committee of the University of Nottingham (Nottingham, United Kingdom). The study was carried out as part of the Early Nutrition Programming Project of the Sixth Framework Programme of the European Union (EARNEST, project number FOOD-CT-2005-007036). The study protocol was designed by Professor Michael Symonds, Dr. David Gardner and Dr. Sylvain Sebert, at the University of Nottingham.

The study was set up with three subsequent interventional stages: during late gestation and postnatally before and after weaning (Figure 2.1, Figure 2.2).

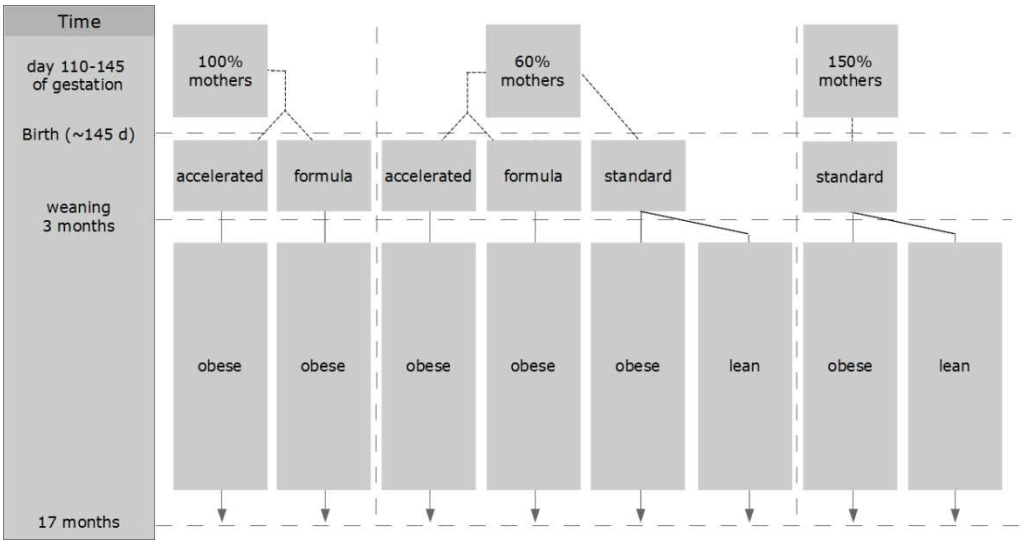

Figure 2.1: Schematic depiction of full study design.

Interventions were applied in 3 stages: mothers remained on a standard diet (100%), were restricted in macronutrients (60%) or were fed ad libitum (150%). At birth, twin were both maintained with the mother for a standard growth rate or separated for and accelerated growth rate whilst the other twin was raised on formula. After weaning at 3 months of age, offspring were maintained in an obesogenic or in a unrestricted environment.

The aim of the study is to distinguish the effects of:

1) macronutrient restriction in late gestation
2) early postnatal growth rate
3) postnatal development of body weight.

For this, only 4 groups of offspring of the study are used, i.e. only the restricted group in comparison with the group fed to requirements and not including the twins which were raised with formula (Figure 2.2).

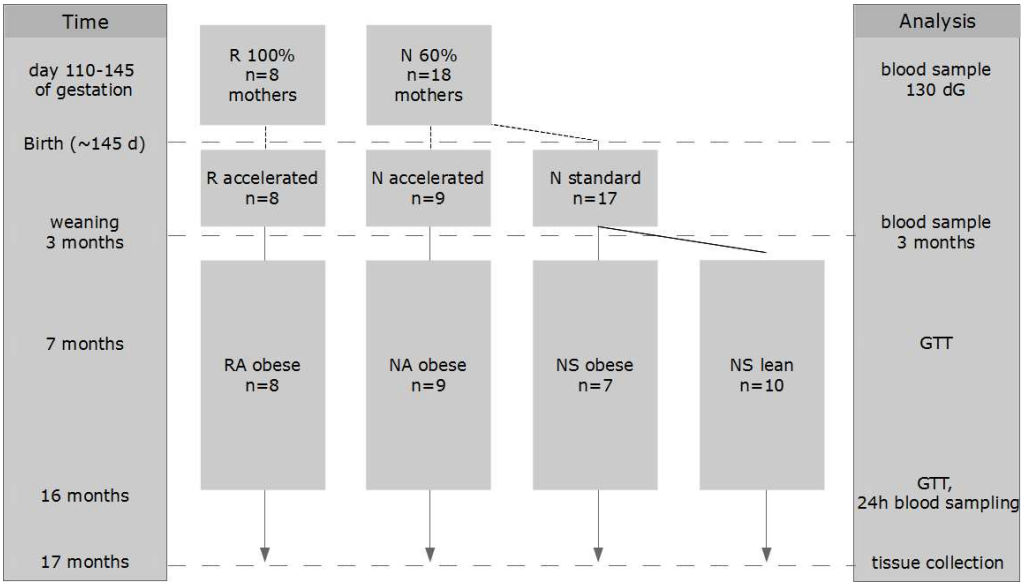

Figure 2.2: Schematic depiction of the groups used in this study.

Twin pregnant ewes were randomly assigned to one of two diets for late gestation (110-145 days): R diet meeting requirements or macronutrient restricted diet meeting 60% of caloric requirements (N). After birth twins were either both reared by their mother (standard early postnatal growth rate, S) or separated with only one twin being reared by the mother (accelerated early postnatal growth rate, A). After weaning most animals were kept in restricted space, representing a mildly obesogenic environment but part of the NS group were kept in a wider space, leading to leaner animals. Throughout the study, blood samples were taken from mothers and offspring and a glucose tolerance test (GTT) carried out at 7 and 16 months before tissue collection at 17 months of age. Discrepancies between numbers (n) of mothers and offspring are due to additional offspring intervention groups which were not included in my research, see Figure 2.1).

2.1.1. Experimental design

At 100 days of gestation, 24 Bluefaced Leicester cross Swaledale twin bearing sheep were housed individually. Mothers were randomly allocated to one of two diets from day 110 of gestation to term delivery (145 ± 2 days): a calorically sufficient diet meeting all requirements (R, n=8) or a diet only providing 60% of caloric requirements (N, n=16). Nutritional requirements were based on recognised amounts for pregnant sheep (Council AaFR 1993 Technical Committee on Responses to Nutrients. In: Technical Committee on Responses to Nutrients. Wallingford, UK: CAB International; pp 812–815), and depended on maternal body weight,

increasing with stage of pregnancy. All mothers were fed 40% concentrated pellets and 60% straw nuts (Manor Farm Feeds, Oakham, UK) and had unrestricted access to a mineral block to ensure adequate micronutrient supply. Mothers were weighed weekly before feeding and dietary requirements adjusted accordingly. Blood samples were taken from the jugular vein after an overnight fast (≥18h) on day 130 of gestation and collected into heparinised and EDTA-coated tubes. Plasma was separated by immediate centrifugation (10 min, 4000 g, 4°C).

After delivery, all mothers were fed to their full requirements throughout lactation. Offspring were separated and only one offspring stayed with the mother in order to accelerate postnatal growth (A) whilst the other offspring was formula-reared (not analysed for this thesis). Some N offspring were also both reared by their mother as twins for a standard postnatal growth rate (S). These interventions gave rise to three groups during lactation: RA (n=8, 4 males and 4 females), NA (n=9, 2 males and 7 females) and NS (n=17, 7 males and 10 females). All offspring were weighed twice during the first month after birth and then weekly until the end of lactation at 3 months of age. A blood sample was collected from each animal at 3 months of age as described above for the mothers.

After weaning, a majority of the offspring were raised in a mildly obesogenic environment (O) with limited opportunity for physical activity (i.e. a stocking rate of 6 animals on 19 m^2). A subgroup of the NS animals were kept in an outdoor area with a stocking rate of 6 animals on 1125 m^2 (NSL, n=10, 5 males and 5 females). Animals were weighed once a month and blood samples taken at 7 and 16 months of age, as described above, as part of the glucose tolerance test (Section 2.1.2).

2.1.2. Glucose tolerance test

A glucose tolerance test measures the plasma concentrations of glucose and insulin after administering a standardised amount of glucose. Analysis of the data allows assessment of the pancreatic β-cell function to increasing plasma glucose both by measuring the time to maximal insulin concentrations and the peak of insulin concentration in itself [399]. How quickly the plasma glucose concentrations return to a range comparable to the fasted glucose concentration assesses peripheral tissue sensitivity, mostly white adipose tissue and muscle, to insulin.

To test glucose tolerance at 7 and 16 months of age, all sheep received 0.5 g/kg glucose intravenously after an overnight fast (≥ 18h), and glucose and insulin plasma concentrations were measured at baseline and over a 120 minute period after injection (glucose tolerance test, GTT). Homeostatic model assessment for insulin resistance (HOMA-IR) is calculated by multiplication of fasted concentrations of glucose and insulin:

$$HOMA - IR = glucose\left(\frac{mmol}{L}\right)_{fasted} * insulin\left(\frac{\mu g}{L}\right)_{fasted}$$

The glucose tolerance test was carried out under supervision of Dr. Sylvain Sebert.

2.1.3. Individual food intake

At 16 months of age, 2 weeks before the end of the study, all offspring were housed individually indoors in UK Home Office designated floor pens (3 m^2) to monitor food intake. For each animal, daily ad libitum energy intake was assessed over 10 days. Feed offered was weighed in the morning and remaining feed weighed again the following morning, with the

difference representing feed eaten. Feed was based on a mix of low (i.e. 3 kg/d straw nuts; 8.5 MJ/kg) and high (800 g/d of concentrate pellets; 12.5 MJ/kg) energy dense food. NSL animals were fed only the low energy straw nuts during this food intake observational period. Coming from a lower energy density food source (i.e. grass), the feeding of an energy-dense feed would have skewed the results of regular food intake and the comparison between NSO and NSL animals would be confounded.

In a second, shorter food intake test, also conducted at 16 months of age, sheep were fasted for 18h and had then feed available ad libitum continually over a 24h period starting at 9 a.m., during which the intake was measured by weighing feed offered at the beginning of the period and left over feed weighed again at the end of the period. Periods within the 24h test were 0-2h, 2-4h, 4-8h and 8-24h relative to beginning of feeding. At 0, 2, 4, 8 and 24h blood samples were taken and analysed for glucose, non-esterified fatty acids (NEFA), insulin, leptin and cortisol as described below. Besides their absolute concentrations, plasma hormone and metabolite concentrations were also expressed relative to the fasted measurement, in order to describe their dynamics:

$$\text{e.g. } leptin\ dynamic\ (2\ h) = leptin\ (2\ h) - leptin\ (0\ h)$$

2.1.4. Physical activity

An Actiwatch accelerometer (Actiwatch, Linton Instrumentation, Diss, UK) was used to measure spontaneous physical activity of offspring at two time points, at 1.5 and at 15 months of age. The accelerometer measures intensity, amount and duration of movement by sampling acceleration at a

frequency of 32 Hz and recording one value per second. Values were accumulated over each minute and these averaged over a 24h period.

2.1.5. Tissue collection

At 17 months of age, animals were fasted overnight (≥18h) and then humanely euthanased by electrical stunning and exsanguination. All visceral fat depots were dissected, weighed and samples snap-frozen in liquid nitrogen and stored at -80°C. Entire hypothalami were dissected using the rostral border of the optic chiasm and the caudal border of the mamillary bodies as landmarks on the ventral surface and the lateral hypothalamic sulci as the lateral boundaries. These were then snap-frozen and stored at -80°C.

2.1.6. Postnatal growth and fat mass

Body weights were recorded throughout the study as described above. Fat mass was measured with the use of dual energy X-ray absorptiometry (DXA) scans at 16 months of age in sedated sheep. Post mortem dissection and weight of the omental, perirenal and pericardial fat tissues in comparison with total body weight were used as an additional estimate of the metabolically most active fat mass. All of these measurements were carried out by Dr. Sylvain Sebert.

2.1.7. Plasma analyses

Plasma from fasted animals at all age points (maternal at 130 days of gestation, offspring at 3 months, 7 months and 16 months) and from all

time points during the feeding test at 16 months of age were analysed for concentrations of cortisol, insulin, leptin, glucose, non-esterified fatty acids and triglycerides. Similarly, blood samples from all time points throughout the GTT were examined for insulin and glucose concentrations. For all measurements, plasma samples were gently thawed on ice and, if necessary, reagents brought to room temperature before use.

2.1.7.1. Cortisol assay

Plasma cortisol concentrations were measured with use of a competition radioimmunoassay. Both cortisol contained in a given volume of plasma and ^{125}I-labeled cortisol in liquid form were added to tubes that were coated with an antibody specific for cortisol. The endogenous cortisol and the labelled cortisol bind competitively to the antibody. After an incubation time of 45 min at 37°C all fluids were aspirated and only the amount of labelled cortisol bound to the antibody on the tube was determined by measuring the decay of ^{125}I with a gamma counter. The higher the concentration of cortisol in the plasma, the more unlabelled cortisol was bound to the antibody and the tube would, therefore, contain less radioactive label. Solutions containing known amounts of cortisol were measured with each assay to establish a standard curve.

Coated tubes, ^{125}I-labeled cortisol solution, standards (of 0, 1, 5, 10, 20, 50 nM concentration) were provided with the kit (Diagnostic Products Corporation Coat-a-Count, Siemens, Camberley, UK). As controls, a total count (measuring the total radioactivity of the labelled cortisol added) and a non-specific binding (adding and decanting labelled cortisol to a regular, not antibody-coated tube) sample were included.

25 µl of plasma, standard or calibrator and 1 ml of the labelled cortisol solution were mixed in each coated tube and incubated on a shaker at 37°C for 45 minutes. All fluids are aspirated without disturbing the coating. Radioactive decay in tubes was measured for one minute in a gamma counter (Wizard 1470 automatic gamma counter, Perkin Elmer, Waltham, MA, USA).

Counts per minute were corrected in comparison with the non-specific binding sample, plotted against concentrations of the standards and extrapolated in a semi-logarithmic curve to determine concentrations of cortisol in plasma. The standard curve was carried out in duplicates and had an acceptable coefficient of variance (<10%).

2.1.7.2. Plasma leptin

The radioimmunoassay for ovine leptin was developed by Delavaud [400] and the analysis of our plasma samples was carried out by the laboratory of Professor Keisler at the Department of Animal Science of the University of Missouri.

In each tube of the assay, 100 µl plasma or 50 µl standard (at concentrations of 1.7, 2.5, 5, 8, 15, 40, 50, 80 ng/ml, in triplicates) were mixed with 50 µl primary rabbit anti-ovine leptin antibody (1:1500, raised to establish this assay), brought to 400 µl with incubation buffer and incubated at 4°C for 24h. Radioactively labelled ovine leptin (^{125}I-leptin, 100 µl at 20,000 cpm) was added and again incubated for 20 h at 4°C. 100 µl secondary ram anti-rabbit antibody is added and incubated for 1h. To precipitate the bigger complex of antibody-bound leptin, 2 ml 4.4% polyethylene glycol 6000 (BDH Prolabo, VWR, Radnor, PA, USA) are added. Unbound leptin was removed from tubes by aspiration. Remaining

radioactivity was measured in a Cobra II gamma counter (Packard Inc, Downers Grove, Australia). The more leptin present in the plasma sample, the less radioactive leptin will be bound in the precipitant.

2.1.7.3. Insulin assay

Sandwich Enzyme-Linked Immuno-Sorbent Assay (ELISA)

An ELISA is an immunological assay which determines the concentration of a protein in a liquid, usually plasma. Antibodies specifically binding to this protein are applied to a 96-well plate and leftover binding capacity of the plate surface reduced by blocking it with unspecific protein, which does not bind to the antibody. The antigen contained in the plasma binds to the captivating antibodies and a detection antibody is added to the well to bind to a different sequence on the same antigen. The detection antibody is conjugated with biotin, which forms a strong bond with streptavidin. Through this bond, horseradish peroxidase, a detection enzyme, is bound to the well in the same proportion as antigen was present in the plasma. All unbound detection antibody and enzyme are washed off the well. The substrate tetramethylbenzidine (TMB) is added to the wells, converted by horseradish peroxidase to a blue colour. This reaction is stopped by acidification with sulphuric acid, which produces a yellow colour. The principle of ELISAs is depicted in Figure 2.3.

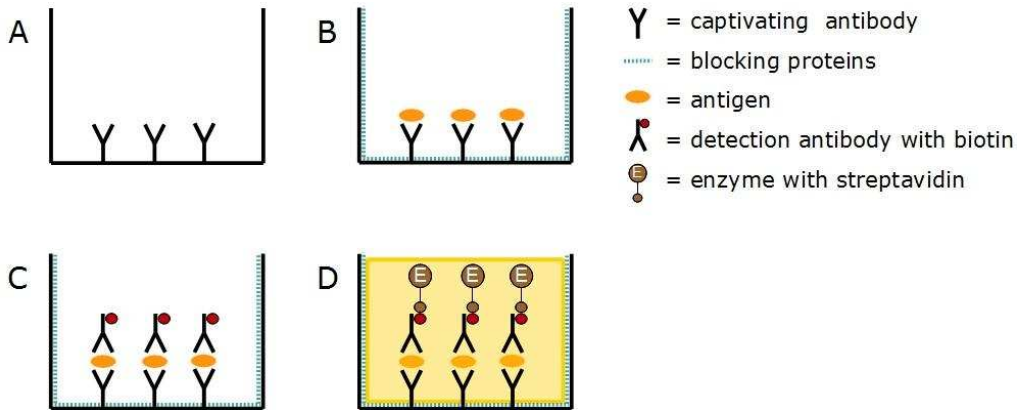

Figure 2.3: Schematic depiction of a Sandwich ELISA. A, Wells were coated with a capture antibody which was specific for the antigen; B, Wells were blocked with non-specific proteins and samples containing the antigen were added; C, Detection antibody binds to antigen; D, Horseradish peroxidase binds to the detection antibody through a strong non-covalent interaction between biotin and streptavidin, substrate was converted into a yellow compound after acidification.

Plasma insulin measurement with ELISA

Plasma insulin was measured using a Sheep Insulin ELISA (Mercodia, Uppsala, Sweden). The 96-well plate included in this kit is pre-coated with antibodies, which bind specifically to sheep insulin. To each well, 25 µl of the sample and 50 µl of a reagent containing the detection antibody and the streptavidin-conjugated enzyme were added and the mixture was incubated on a shaker at room temperature for 2 hours. The plate was washed thoroughly six times and fluid was removed from the plate by tapping it firmly onto absorbent paper. 200 µl of a TMB solution was added to each well and again incubated for 15 minutes. The reaction was stopped by adding 50 µl sulphuric acid (0.5 mol/L) and the optical density was measured on a plate reader (µQuant, Bio-tek Instruments Inc, Potton, UK) at 450 nm wavelength. Results were corrected in comparison to a blank (all reagents with distilled water as sample). Standards are included in the

kit and a linear standard curve with 5 points (0.1-2.5 µg/L) was used to determine the insulin concentration of samples.

2.1.7.4. Plasma metabolites

TG, NEFA and glucose concentrations were analysed by colorimetric tests, in part manually on 96-well plates and in part on a Randox RX Imola auto-analyser (Randox Laboratories, Crumlin, UK). Reagent and plasma volumes used for the auto-analyser were as specified by Randox. The principles between both methods are very similar but for the automated analysis all reagents were provided by Randox Laboratories whilst in the manual analysis reagents from Wako (Neuss, Germany) were used for NEFA. In both methods samples were compared to standards as described and whilst it cannot be guaranteed that both methods would produce the exact same result, samples of a set, e.g. all GTT samples, or all 3 months plasma samples, would be run with the same method and not compared to samples assessed with the other method.

All measurements were corrected in comparison with a blank (all reagents with distilled water as sample). All samples and standards were run in duplicate and samples with a variation coefficient of <5% were considered valid.

Plasma triglyceride measurements

For the measurement of plasma TG concentration, 2 µl plasma was mixed with 200 µl reagent containing the enzymes lipase, glycerol-kinase, glycerol-3-phosphate oxidase and peroxidase. The mixture was incubated at room temperature for 10 minutes and colour intensity measured on the plate reader (as before) at 500 nm wavelength. Intensities were then

compared to a standard curve derived from a standard in a twofold serial dilution to five standard points (12.2-195 mg/dl).

During the incubation, TG were broken down to glycerol and fatty acids by the lipases in the reagent mix. Glycerol kinase then phosphorylated glycerol to glycerol-3-phosphate, which was in turn turned to dihydroxyacetone-phosphate and hydrogen peroxide under the presence of glycerol-3-phosphate oxidase and oxygen. Hydrogen peroxide was subsequently reacting with 4-aminophenazone and chlorophenol, which were also contained in the reagent mix, to form a quinone imine of pink colour.

Plasma non-esterified fatty acid analysis

For measurement of NEFA concentrations, 2 µl plasma were mixed with 100 µl reagent A, containing acyl-CoA-synthetase and coenzyme A. The mixture was incubated at room temperature for 15 minutes. After that, 100 µl reagent B was added, containing acyl-CoA-oxidase and peroxidase. After another 15 minute incubation, the colour intensity was measured on a plate reader (as before) at 550 nm wavelength. The results were compared to a linear standard curve of three points at NEFA concentrations of 0.25, 0.5 and 1 mmol/L, which were run on the same plate.

During the first incubation, NEFAs were converted to acyl-CoA by the Coenzyme A and acyl-CoA-synthetase included in reagent A. During the second incubation, acyl-CoA was oxidised to 2,3-trans-enoyl-CoA under production of hydrogen peroxide by the acyl-CoA-oxidase contained in reagent B. The hydrogen peroxide then reacted with 4-aminophenazone and 3-methyl-N-ethyl-N-(ß-hydroxyethyl)aniline to form a pink-coloured quinone imine.

The automated measurement with the kit produced by Randox differed from this method only in so far as the hydrogen peroxide was used to convert 4-aminoantipyrine and N-ethyl-N-(2hydroxy-3-sulphopropyl)-m-toluidine to a purple adduct.

Plasma glucose analysis

For glucose measurements, 2 µl were mixed with 200 µl reagent, containing glucose oxidase, 4-aminophenazone, phenol and peroxidase. The plate was mixed, incubated at room temperature for 25 minutes and the optical density measured on a plate reader (as before) at 500 nm wavelength. Results were compared to a single standard of 5.49 mmol/L in a linear relationship.

During the incubation, glucose was oxidised to gluconic acid and hydrogen peroxide. This was subsequently reacted with 4-aminophenazone and chlorophenol to form a quinone imine of pink colour.

2.1.8. Adipose tissue histology

Histology analyses were carried out both in the sheep study and the rat studies as described in Section 2.2.9. For histology, tissues are preserved, dehydrated and blocked in paraffin to enable very thin sectioning. Sections are transferred onto glass slides and immunohistochemistry is performed by binding a primary antibody to the protein of interest. Antigens need to be prepared for the primary antibody binding as preservation with formalin can change the structure of proteins. This antigen retrieval is often done by heating the sections in buffer. Primary antibody is then bound with a

secondary antibody bound to either a peroxidase resulting in a change of colour where antibodies are bound to antigen, or to a fluorescent marker.

At dissection, omental adipose tissue samples were immediately submerged in 10% formalin (Sigma-Aldrich, Gillingham, UK) and remained in formalin for 7 days. Samples were then transferred to a tissue processor (Shandon Excelsior, Thermo Scientific, Wilmington, DE, USA) with 6 subsequent baths in ethanol (industrially modified spirit, IMS, Sigma-Aldrich, as before) for 1 hours, 3 baths in xylene (Fisher Scientific, Loughborough, UK) for 1 hour and 3 baths in paraffin wax (Tissue Tek II embedding wax, Sakura, Tokyo, Japan) at 60°C for 80 minutes. Tissue samples are blocked in paraffin, sectioned to 5 μm on a rotary microtome (AS200, Anglia Scientific, Cambridge, UK) and transferred to slides (Superfrost plus, Thermo Scientific, as before).

For analysis of adipocyte size, sections stained for tumour necrosis factor (TNF) were used. Methods used were as previously described [401]. In brief, in an automated immunohistochemistry processor (Bond-Max, Leica, Milton-Keynes, UK), slides were treated with a primary antibody binding TNF and a secondary antibody bound to a horseradish peroxidase complex for imaging. Shortly, sections were dewaxed in 2 baths of xylene for 5 minutes, then immersed in 2 baths of ethanol for 5 min. Peroxidase activity within the sample was quenched for 45 min by immersion in 3% hydrogen peroxide to reduce background staining. Samples were then washed 3 times with a wash solution (containing surfactant and methylisothiazolinone in Tris-buffered saline, all standard reagents used in the Bond-Max provided by Vision Biosystems, Newcastle Upon Tyne, UK) and a 1:150 dilution of the primary antibody (mouse anti bovine, AbD serotec, Oxford, UK) applied and incubated for 15 min. Samples were washed 3 times again and treated with a solution containing

methylisothiazolinone and animal serum in Tris-buffered saline for 8 minutes, which enhances sample penetration of the subsequent polymer. After further 3 washes, the polymer, i.e. the complex of secondary anti mouse antibody and horseradish peroxidase, was applied and incubated for 8 min. Samples were washed twice with wash solution and once with deionised water and 3,3'-diaminobenzidine (DAB) was applied and incubated for 10 min. After 3 washes with deionised water, haematoxylin was applied for a 5 min incubation for visualisation of nuclei. Finally, samples were washed in deionised water 3 times, dehydrated in IMS twice for 2 minutes and in xylene twice for 2 and 3 minutes each. A drop of DPX mounting medium (Fisher Scientific, as before) was applied onto the sample and covered with a cover glass. All chemicals except for xylene, IMS and the primary antibody but including the secondary antibody were provided by Leica (as before) as Bond Polymer Refine Detection.

Slides were viewed on the brightfield setting of a microscope (Leitz DMRB, Leica, as before) at 20x magnification, imaged (camera C4742-95, Hamamatsu, Welwyn Garden City, UK) and the area of adipocytes analysed using Volocity software (version 6, PerkinElmer, as before). For each sheep, an average of 33 adipocytes were cropped and area and circumference of cells was measured and averaged. Samples were processed and analysed by Dr. Vivek Saroha.

2.1.9. Gene expression analysis

The hypothalamus is a very heterogeneous tissue, with the expression of neurotransmitters restricted to certain cell types and nuclei within the hypothalamus. Examining the frozen hypothalami by eye it was not deemed possible to isolate nuclei of interest like the arcuate nucleus (ARC)

and the ventromedial nucleus (VMN). Hypothalami were, therefore, homogenised as a whole. Therefore, expression of a gene within an aliquot of the homogenate reflects expression of this gene in any cells within the hypothalamus.

From each sample, messenger ribonucleic acid (mRNA) was isolated, quantified and 4 µg reversely transcribed to complementary DNA (cDNA). For the semiquantitative polymerase chain reaction (qPCR), primers were designed, from which standards were produced. Both were used for the quantification of cDNA in the qPCR, which represented the amount of mRNA of the gene of interest present in the original hypothalamus sample.

2.1.9.1. Principle of PCR

Primers are short oligonucleotides of approximately 20 base pairs (bp). When annealing to DNA, a pair of primers bind to complementary sequences on the gene they are designed for. Primer design is successful if the primers only bind to this specific locus and not to any other part of the genome. The two primers in a pair each bind to one strand of double-stranded DNA, called the forward and reverse primers, respectively, and their binding sites are mostly between 100 and 200 bp apart from each other on the gene. The part of the gene between the primers is the sequence that is amplified during PCR, called the amplicon or PCR product. The bound primer is elongated by binding free phosphonucleotides in the 5’ to 3’ direction by a thermostable polymerase. Subsequently, both strands – the original template and the newly synthesised strand including the primer – are denatured and the primers can bind to both of them again. If, for example, PCR is repeated for x cycles, the final amount will ideally be 2^x the number of original copies of DNA present. If the sample used is

cDNA, the final amount of amplicon copies is representative of the number of intact mRNA molecules for this gene present in the tissue of interest at the time of RNA isolation.

2.1.9.2. Isolation of messenger RNA

Offspring hypothalami which had been dissected and snap frozen immediately after humane euthanasia were homogenised in a buffer containing β-mercaptoethanol (RLT buffer, Qiagen, Hilden, Germany). RNA was extracted from homogenised hypothalami with the use of a phenol-based method (TRI reagent, Ambion, Applied Biosystems, Foster City, CA, USA) before removing DNA and washing and eluting RNA with the use of the RNeasy Plus kit (Qiagen, as before).

100 mg of tissue homogenised in 200 µl RLT buffer was mixed with 1 ml TRI reagent and centrifuged for 3 minutes at 12,000 g to remove cell debris. 200 µl chloroform was added to the supernatant, mixed thoroughly and incubated at room temperature for 10 minutes. Cooled centrifugation at 12,000 g for 15 minutes separated the aqueous phase that contains nucleotides from the phenol phase. The aqueous phase was transferred onto a gDNA column of the RNeasy Plus kit and centrifuged for 30 seconds at 12,000 g to filter out genomic DNA. 570 µl 70% ethanol was added to the flow-through, mixed and transferred onto the RNA column provided in the RNeasy Plus kit. By a rapid centrifugation, RNA was filtered from the solution onto the membrane of the column, which was then washed with RW1 and RPE buffers provided with the kit. RNA was then eluted from the membrane by adding 30 µl RNAse-free water and centrifugating for a short time.

Concentrations and purity were measured with NanoDrop 1000 (Thermo Scientific, as before). RNA concentration was measured spectrometrically at 260 nm wave length in a 1.5 µl aliquot of the eluted RNA solution. A second measurement of optical density at 280 nm was used to detect any contamination with protein as a ratio of optical density at 260 nm/280 nm≥ 2 is considered uncontaminated. Complete removal of genomic DNA gDNA) and integrity of the RNA were ensured by gel electrophoresis of 1 µg of RNA of each sample.

2.1.9.3. Reverse Transcription

RNA were reverse transcribed (RT) to cDNA with use of the High Capacity RNA-to-cDNA kit (Applied Biosystems, as before). 4 µg of RNA in a total volume of 18 µl was added to 2 µl reverse transcription enzyme and 20 µl buffer, both included in the kit. After mixing, samples were incubated at 37°C for 60 minutes to transcribe RNA into double stranded DNA. An incubation at 95°C for 5 minutes followed for denaturation of double stranded to single stranded DNA, which could then be used for PCR. Both incubations were carried out in a thermal cycler (Touchgene Gradient, Techne, Burlington, NJ, USA).

Controls included are a 'no template control' (every reagent excluding the sample) and 'no RT controls' (every reagent and sample excluding the reverse transcription enzyme) for each sample to ensure that reagents were not contaminated and that the RNA was free of gDNA, respectively.

2.1.9.4. Primer design

If there were no primer sequences published for the gene and species of interest, I designed primers with the use of web-based databases and programmes.

The sequence of the gene of interest was searched for in the *Ensembl* data base [402]. This database has the added value of showing the exon borders for each gene. Designing the primers to be on opposite sides of an exon border gives another opportunity to check for contamination of the isolated mRNA with genomic DNA because, if gDNA is amplified, it will produce a bigger amplicon, i.e. including the exon sequence that was spliced out to form mRNA. This bigger amplicon would show alongside the smaller mRNA-derived amplicon when running the PCR products on a gel (see Section 2.1.9.6). Sequences are searched for specifically for ovine species. If there are no known sequences for this species, sequences of cow or pig often work successfully in sheep samples.

From the original sequence, primers are designed using the *Primer3* algorithm [403]. A product size of 100-200 bp and a primer size of 18-27 bp are chosen. *Primer3* presents four possible primer pairs, which are then analysed for their probability to form hairpins or primer dimers using *NetPrimer* software (http://www.premierbiosoft.com/netprimer/; Premier Biosoft International, Palo Alto, CA, USA). The primer pair with the best *NetPrimer* rating was chosen, produced (Sigma Aldrich, as before) and tested in a PCR (see section entitled Real-time semiquantitative PCR below), including a no-template control. PCR products were run on a gel. If the negative control showed a band of the same product size as the desired amplicon, this may point to use of nucleotide-contaminated water. If the gel of the sample PCR product showed multiple bands or if the

amplicon appeared to be of an unexpected size as compared with the DNA ladder included in the gel this primer pair was dismissed and primers had to be designed again for this gene. The identity of the amplicon was confirmed by sequencing and comparison with the original gene sequence (as described below in the Section 2.1.9.7).

2.1.9.5. Production of standards for the qPCR

For each gene of interest, regular PCR was carried out to produce the amplicon defined by the specific primer sequences in a high purity and high concentration. The concentration was measured using the NanoDrop (as above) and the amplicon was diluted. These dilutions were used as standards during the qPCR. Double-stranded cDNA was denatured to its single-stranded state at 94°C. At 60°C primers annealed to the single-stranded cDNA and nucleotides were added during the elongation step at 72°C. At the denaturation step of the next cycle, the strands were separated again so that both the original strand and the new copy could serve as templates.

For the PCR, 10 µl master mix including nucleotides and a buffer (Thermo-Start PCR master mix, Thermo Scientific, as before), 1 µl of both forward and reverse primers and 6µl water were added to 2 µl cDNA, equivalent to 0.2 µg mRNA before reverse transcription. PCR cycles were carried out in a thermal cycler (as before): 45 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds. In the last cycle, 72°C was held for 7 minutes before cooling down to 4°C.

2.1.9.6. Gel electrophoresis and gel extraction

PCR products were separated on an agarose gel to ensure that the primer pair selected generated only one product. Nucleic acids are negatively charged at the phosphate group, resulting in a migration towards the anode when electrophoresis is applied. PCR products of a smaller size migrate through the gel faster than a larger size amplicon, therefore separating into bands [404]. The size of the product in each band can be determined by comparing it to a DNA ladder which contains DNA fragments of known sizes and which has been run alongside the PCR products on the gel.

Agarose was melted at a 2% (w/v) concentration in TAE buffer (0.04 mol/L Tris hydroxymethyl amino-methane (Sigma Aldrich, as before) 0.02 mol/L glacial acetic acid (Fisher Scientific, as before) and 0.001 mol/L EDTA (pH 8.0; VWR, as before) in distilled water) and 0.004% ethidium bromide (v/v), a dye that fluoresces under UV light when intercalated into DNA, were added. The mixture was poured onto a tray with a comb inserted to create pouches and let to cool to room temperature to form a gel. Tray and gel were placed in an electrophoresis chamber filled with TAE buffer. 20 µl PCR products with 5 µl gel loading buffer (12.5% Ficoll 400 (Qiagen, as before), 0.1% bromophenol blue in 5x concentrated TAE buffer) were inserted into the wells alongside a DNA ladder (5 µl, 100 bp DNA marker, Bioron, Ludwigshafen, Germany). A voltage of 100 V was applied for 30-60 minutes. Product bands were viewed and photographed with a camera chamber at -26°C (LAS-3000, Fujifilm, Tokyo, Japan). Bands were excised and DNA was extracted from the gel as described by the manufacturer (QIA quick gel extraction kit, Qiagen, as before). Gel was dissolved in a buffer at 50°C, isopropanol was added and the mixture was filtered through a column. After washing, PCR products were eluted from the

column membrane using an elution buffer. Concentrations and contamination with protein were determined with the NanoDrop as described before.

2.1.9.7. DNA sequencing

Extracted DNA and corresponding primers were submitted to the DNA Sequencing Laboratory in the School of Biomedical Sciences at the University of Nottingham. Amplicon sequences were compared to a nucleotide collection database using the basic local alignment search tool for nucleotides (*BLASTn*) algorithm [405] to confirm identity.

2.1.9.8. Real-time semiquantitative PCR

Measurements of expression of specific genes were performed with a SYBR green system (Thermo Scientific, as before) on a Quantica machine (Techne, as before) which enables measurements of specific mRNA by incorporating fluorescent molecules into the RNA sections that are amplified during the PCR. SYBR green is a molecule included in the qPCR reaction that increases its fluorescent capacities when bound to double stranded DNA. Overall fluorescence is measured in every cycle after the elongation phase but before denaturation of double stranded to single stranded DNA. Therefore, the measured fluorescence increases with the amount of amplicons in the sample over the course of the qPCR run. The cycle during which fluorescence can be distinguished from the background noise is called the cycle threshold (ct). The qPCR programme also often includes a gradual increase of temperature at the end of its run, i.e. a 'ramp', to determine the melting temperature of the amplicons generated during the qPCR. This is a way of ensuring that only one product is formed

during the amplification because the melting temperature is product-specific depending on the length of the amplicon and its distribution of G/C and A/T nucleotides contained.

RNA samples were measured in comparison with a standard curve of serial dilutions of cDNA amplicon of the measured gene (10^{-4} to 10^{-9} ng/µl), which was produced with the same primer set as described above (in the section Production of standards for the qPCR). For all measurements, the same Quantica machine was used to ensure comparability of results. A standard curve was produced by plotting the log concentration of the standards against their Ct. Measurements were only deemed successful if the standard curve showed an R^2 of >0.95 and an efficiency of between 1.95 and 2.05. An efficiency of 2 indicated the ideal amplification, i.e. a doubling of the number of amplicon molecules with each thermocycle [406]. Housekeeping genes considered for normalisation were the ribosomal RNA subunit 18S, the ribosomal phosphoprotein gene RPO and the gene for tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein YWHAZ. Gene expression measurements were carried out in 3 separate batches, each a new 1:10 dilution of stock cDNA and expression of housekeeping genes was measured again in each batch.

Normalisation assumes an efficiency of 2 (based on the ΔΔCt method developed by Pfaffl [407]) and was carried out using the equation

$$gene\ expression = 2^{-(ct\ target - ct\ reference)}$$

(ct: cycle threshold of the target gene and of the reference gene).

Genes of interest and housekeeping genes and their respective primers are listed in Table 2.1. The genes of interest selected are involved in appetite regulation and the insulin system, as explained in Chapter 1.

Table 2.1: Primers used for measurements of hypothalamic gene expression.

Gene	Forward and reverse sequence	°C	Amplicon length
NPY	TCATCACCAGGCAGAGATACGG	59	140
	GAGCAAGTTTCCCATCACC		
PTP1B	GGAAGAAGCCCAGAGGAGTT	60	132
	CGAGCCTTTCTCCATCACTC		
SOCS3	AGCTCCAAGAGCGAGTACCA	60	176
	ACGCTGAGGGTGAAAAAGTG		
GCR	ACTGCCCCAAGTGAAAACAGA	59	150
	ATGAACAGAAATGGCAGACATTTTATT		
MC3R	CGGTGTGGTGTTCATCGTCTAC	59	82
	TGAGCAGCAACATGGCAAGG		
MC4R	GGTGTCGGGCGTCTTGTTC	59	64
	CGGTGATGAGGCAGATGATGAC		
IR	CTGCACCATCATCAACGGAA	59	161
	CGTAACTTCCGGAAGAAGGA		
obRb	TGAAACCACTGCCTCCATCC	60	130
	TCCACTTAAACCATAGCGAATCTG		
ADIPOR1	CTTCCCTGGAAAATTCGACA	59	154
	TCAGAGGAGGGAGTCGTCAG		
ADIPOR2	GGCAAGTGTGACATCTGGTTTC	59	107
	GAAACGGAACTCCTGGAGGTT		
AMPKA2	GCTGGATTTTGAATGGAAGG	59	153
	CAGCACCTCATCATCAATGC		
GHSR	CTCGCTCAGAGACCAGAACC	59	178
	GACGAAAGAGACGAGGTTGC		
BDNF	CATGGGACTCTGGAGAGCAT	58	198
	CAAAGGCACTTGACTGCTGA		
CRH	CATCACAGCACCCCCAGCC	59	227
	GCAGCGCTCGGAAAAAGTT		
mTOR	GCCTTCCGACCTTCTGCCTTC	59	96
	CCGCTGTCCGTTCCTTCTCC		
AVP	CGACCTGGAGCTGAGACAGT	59	143
	GGCAGGTAGTTCTCCTCTTGG		
FTO	ACACATGGCTTCCCTACCTG	60	220
	GAGGATGCGAGAGACTGGAG		
AGRP	TGAAGCGGATAATGGAGGAAC	58	148
	GAGAGGGTCAAGTAGAGATAG		
18S	GATGCGGCGGCGTTATTCC	59	125
	CTCCTGGTGGTGCCCTCC		
RPO	CAGCAAGTGGGAAGGTGTAATCC	59	74
	CCCATTCTATCATCAACGGGTACAA		
YWHAZ	TGTAGGAGCCCGTAGGTCATCT	59	102
	TTCTCTCTGTATTCTCGCCATCT		

For each gene, the nucleotide sequence is stated, together with the ideal annealing temperature (°C) and length of the amplicon (in base pairs).

NPY, neuropeptide Y; PTP1B, protein tyrosine phosphatase, non-receptor type 1; SOCS3, suppressor of cytokine signalling 3; GCR, glucocorticoid receptor; MC3/4R, melanocortin receptor 3/4; IR, insulin receptor; obRb, leptin receptor, long form; ADIPOR 1/2, adiponectin receptor 1/2; AMPKA2, AMP-activated protein kinase α2; GHSR, growth hormone secretagogue receptor; BDNF, brain-derived neurotrophic factor; CRH, corticotropin releasing hormone; mTOR, mammalian target of rapamycin; AVP, arginine vasopressin; FTO, fat mass and obesity associated; AGRP, agouti-related peptide; 18S, ribosomal RNA 18S; RPO, RNA polymerase; YWHAZ, tyrosine 3-monooxygenase activation protein, zeta polypeptide.

qPCR relies on the same reactive principles as a regular PCR. The programme starts with a denaturation at 95°C for 15 minutes followed by 45 cycles of 95°C for 15 seconds and a gene-specific annealing temperature of 58-60°C for 30 seconds. qPCRs were carried out without an extra temperature step for extension of the primers because the polymerases used in qPCRs are less temperature-sensitive than the polymerase used in regular PCR. In the sample mix, the extension of short amplicons occurs in the time the machine (Quantica, Techne, as before) needs to heat up from the annealing temperature to 72°C. During the cycling, fluorescence is measured at the end of each annealing step. After cycling is complete, a 'ramp' is carried out with the temperature steadily rising in 1°C increases from 65°C to 95°C with a 10 second hold and a measurement of fluorescence on each step.

2.1.10. Analysis of the plasma metabolome

Plasma samples from mothers at 130 days of gestation and from 7 month old offspring were analysed for a wide spectrum of metabolites with the use of liquid chromatography-high resolution mass spectrometry (LC-HRMS). This work and the subsequent statistical analysis was carried out by Dr. Marie-Cécile Alexandre-Gouabau at the Unité Physiologie des Adaptations Nutritionnelles in the Department Alimentation Humaine of the Institut National de la Recherche Agronomique, Nantes, France.

2.1.10.1. Metabolome analysis with LC-MS

Plasma was gently defrosted on ice and filtered by centrifugation (Nanosep Omega, Pall, Port Washington, NY) at 6000 rpm for 30 min at 10°C to remove proteins of high molecular weight (over 10kDa).

For the metabolome analysis, reverse phase HPLC with positive electrospray mode was used, because it is known to have a large application range and because it has been used with success under the same conditions in previous metabolomic studies in the laboratory at the University of Nantes [408, 409]. The instrument used was a Finnigan Surveyor Plus HPLC system, coupled with an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). All solvents and reagents were of analytical or HPLC grade quality and were purchased from Solvent Documentation Synthesis (Peypin, France). Fifteen mL of filtered plasma samples were injected onto a 150 x 2.1 mm Uptisphere HDO-C_{18} column with 3 µm particle size (Interchim, Montluçon, France). Elution was performed with a mobile phase consisting of water containing 0.1% acetic acid (A), and acetonitrile containing 0.1% acetic acid (B). The elution gradient with increasing concentration of acetonitrile used was as follows: 0-4 min, 0% B; 4-15 min, 10% B (v/v); 15-25 min, 50% B; 25-30 min, 100% B. The flow rate was 0.2 mL/min and the temperature of the column was maintained at 37°C. For mass spectrometry, the HPLC column was connected without splitting to the electrospray interface operating in positive mode with nitrogen. Spray voltage was set to 5 kV. The temperature of the heated transfer capillary was maintained at 350°C. Full scan mass spectra were acquired from mass-to-charge ratio (m/z) 50 to 800 at a mass resolution of 30,000 (full width at half maximum) in centroid mode. Two types of quality control (QC) samples were used, which were injected 4 times in randomised order in each batch of injection. QC1 was a Milli-Q water sample and QC2 was a standard mixture solution consisting of methyltestosterone, stanozolol, medroxyprogesterone acetate, triamcinolone and ponasterone A at 1 ng/µL. To identify the peaks in the chromatograms of samples, standards were used including amino

acids, carnitine and its derivatives, which were run on the HPLC in the same way as the samples (Sigma Aldrich, St Quentin-Fallavier, France). Standards were dissolved in 98% ethanol to a concentration of 0.1 mg/mL before LC-MS analysis.

2.1.10.2. Metabolomic fingerprint processing

The open-source *XCMS* software [410] was used for non-linear alignment of the data and automatic integration and extraction of the peak intensities for each mass-to-charge and retention time ([*m/z*; *rt*]) features (ions). *XCMS* parameters for the R language were implemented in an automation script with the algorithm "match-filter" by default except the interval of *m/z* value for peak picking that was set to 0.1, the noise threshold set to 6, the group band-width set to 10 and the minimum fraction set to 0.5. After pre-processing, *XCMS* compares the signal abundances observed for identical ions in two groups of samples using a Student t-test, to identify metabolites presenting intensities significantly different between two groups and ranked these metabolites according to the associated statistical confidence level (p-value). A p-value lower than 0.05, and a fold change higher than 1.5, were used as selection criteria to identify the potentially most relevant metabolites.

All multivariate data analyses and modelling were performed using *SIMCA-P+* software (version 12, Umetrics Inc., Umeå, Sweden) on log-transformed data [411] submitted to a Pareto scaling procedure of common use in the field of MS-based metabolomic, due to the wide dynamic range typically observed in MS-based metabolomic data. A supervised method, partial least squares discriminant analysis (PLS-DA) was applied for each transformed data set analysed separately (mothers

and offspring) to maximise the discrimination between sample groups focusing on differences according to metabolic variations in mothers and in offspring in response to perinatal nutrition and to identify the variables (i.e. ions) which best explained this discrimination. In our study, the PLS-DA model was performed with two principal components, t[1] and t[2].

PLS-DA was combined with a multivariate pre-processing filter, orthogonal signal correction (OSC). By removing within-class variability and confounders which might interfere with chemometric analysis such as LC-MS technical variability, OSC can significantly improve PLS-DA performance, yielding a better discrimination of the clusters [412]. Due to the wide dynamic range typically observed in MS-based metabolomic data, these analyses were performed on log-transformed data, submitted to a Pareto scaling procedure commonly used in the field of MS-based metabolomics. The quality of the generated OSC-PLS-DA models was evaluated by several goodness-of-fit parameters: R^2 (X), the proportion of the total variance of the dependent variables that is explained by the model; R^2 (Y) defining the proportion of the total variance of the response variable (i.e. the class of the samples) explained by the model and the predictive ability parameter Q^2 (Y), which was calculated by a seven-round internal cross-validation of the data. In addition, a permutation test (n=100) was carried out to validate and to test the degree of over fitting for OSC-PLS-DA models. The correlation coefficient between the original Y and the permuted Y is plotted against the cumulative R^2 and Q^2 and a regression line is calculated. Generally, the model was successfully validated when R^2(Y) and Q^2(Y) are above 0.6 and the R^2- and Q^2-intercept limits (when the correlation coefficient is zero) for a valid model should be less than 0.4 and 0.05, respectively. The score values from OSC-PLS-DA were subjected to ANOVA to test the OPLS-DA model and the validation

was considered successful with $p<0.05$. The variables which most significantly discriminate the metabolic signatures were pinpointed by their loadings on PLS-DA.

The monoisotopic masses responsible for the metabolic differentiation between the intervention groups were used to make queries in the online Metabolite and Tandem MS Database (http://metlin.scripps.edu/) and Human Metabolome Database (http://www.hmdb.ca/) for obtaining corresponding possible chemical structures (raw formulas). The identity of annotated compounds was confirmed by analysis of the corresponding commercially available standard compound on the same analytical system (i.e., LC-LTQ-Orbitrap) under the same conditions (validation based on both retention time and mass spectra, i.e. the time point of the metabolite washing off the column relative to injection and the mass and charge of the metabolite).

2.2. Rat choline study

Animals were treated in accordance with protocol, which had been approved by the Committee of Animal Policy and Welfare of the Faculty of Agricultural, Life and Environmental Sciences at the University of Alberta. All husbandry and animal work was carried out at the Health Sciences Laboratory Animal Services at the University of Alberta.

Primiparous Sprague-Dawley rats (n=12) were obtained from Charles River Laboratories (Montreal, QC, Canada) on day 14 of gestation and caged individually. After giving birth (term at 21 days of gestation), mothers stayed in individual cages with their offspring and were maintained on one of three interventional diets as follows until postnatal day 21. Two days after birth, all litters were culled to 11 offspring. One male and one female offspring were dissected at 21 days of age, 7 offspring culled without dissection and 2 female offspring from each mother were kept until 11 weeks of age. In this postweaning period, the 2 littermates were housed together (Figure 2.4). As 1 of the 4 D mothers (fed a choline-devoid diet, as explained below) died during giving birth it was decided to keep 3 offspring of each of the remaining D mothers after weaning to have comparable numbers (n) to the offspring of PC and C groups (mothers fed a diet containing phosphatidylcholine (PC) or free choline (C)).

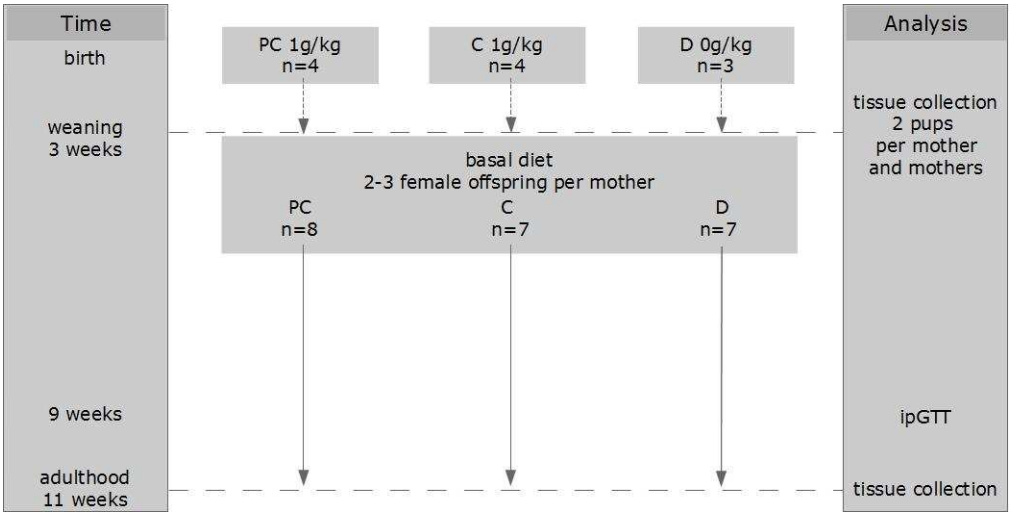

Figure 2.4: Schematic depiction of the rat choline study.

After giving birth, rat mothers were fed one of three diets: containing 1g choline per kg diet as PC (PC), containing 1g choline per kg diet as choline bitartrate (C) or a choline-devoid diet (D). Offspring were weaned at 3 weeks of age, when mothers and 2 offspring per mother were euthanased and tissue dissected. Two to 3 female offspring per mother were maintained on a basal diet with 1g choline per kg diet as choline bitartrate. Insulin sensitivity was measured in a glucose tolerance test (ipGTT) at 9 weeks of age and animals were euthanased and tissue dissected at 11 weeks of age.

2.2.1. Diets

Before giving birth, mothers were fed standard rodent chow (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA). The different isocaloric choline diets were introduced on day 20 of gestation (term 21 days of gestation). Intervention diets were fed ad libitum to the mothers until the offspring were three weeks of age. After weaning at three weeks of age, the remaining offspring were fed the basal diet containing the standard choline concentration.

Diets were designed by Professor Catherine Field based on AIN-76 high fat diet as previously published [413] and made specifically for this experiment (Table 2.2). Mothers were randomised to one of three isocaloric, isonitrogenous diets which differed only in the choline source and content: a choline-devoid diet (D, n=3 due to one mother dying from

dystocia) containing no choline, a standard choline diet containing the recommended choline concentration as a bitartrate (C, 1.2 g choline per kg diet, n=4) and a diet containing choline in the form of PC (PC, 1.0 g choline per kg diet, n=4). Choline bitartrate was obtained from Harlan Teklad, Madison, WI, USA. Lecithin was isolated from chicken egg yolks, provided by Dr. Jonathan Curtis, University of Alberta, analysed for PC content and added to the diets accordingly. The amount of vegetable fat added to the diet was reduced and modified, so that the final PC diets were similar in fat content and fatty acid composition to the diets not containing PC. All diets contributed 25% of energy as protein, 34% of energy as carbohydrates and 41% of energy as fat (Table 2.2).

Table 2.2: Composition of experimental diets used during the lactation period and of the postweaning diet, per kg diet.

Component (g/kg)	D	C	PC
Casein [1]	270	270	270
Starch [2]	244	241	244
Sucrose [1]	126	126	126
Vitamin mix (AIN 93) [1]	19	19	19
Mineral mix [1]	50	50	50
Calcium phosphate dibasic [3]	3.4	3.4	3.4
Inositol [3]	6.3	6.3	6.3
Cellulose [1]	80	80	80
Marinol [4]	2.4	2.4	2.1
Flax oil [5]	7	7	6.2
Olive oil [2]	48	48	42.5
Sunflower oil [2]	67	67	59.3
Canola tallow [6]	75.6	75.6	66.9
Cystine [7]	1.8	1.8	1.8
Choline bitartrate [1]	0	2.5	0
Egg lecithin	0	0	23.8
Energy density kcal/g	4.3	4.3	4.3
% of energy from protein	25	25	25
% of energy from carbohydrate	34	34	34
% of energy from fat	41	41	41
Choline g/kg diet	0	1.2	1.0

D, choline-devoid diet; C, standard choline bitartate diet; PC, standard phosphatidylcholine diet. 1, Harlan Teklad (Madison, WI, USA); 2, Safeway (Calgary, AB, Canada); 3, MP Biomedicals (Solon, OH, USA); 4, Lipid Nutrition (Channahon, IL, USA); 5, Gold Top Manufacturing and Distributing Ltd (Edmonton, AB, Canada); 6, Richardon Oilseed Ltd (Lethbridge, AB, Canada); 7, North American Mogul Products (Chagrin Falls, OH, USA).

The basal diet fed after weaning in the longer study has a lower energy density of 3.1 kcal/g and a lower fat content of 31% of energy and has a concentration of 1 g choline as bitartrate per kg diet (product number 84172, Harlan Teklad, as above, with 20% vegetable fat mix added, see Table 2.3).

Table 2.3: Composition of the basal diet fed to offspring after weaning. All components are listed as g per kg diet.

Component (g)	Basal diet
Basal diet mix [1]	800
Fish oil [4 2]	4
Flax oil [5 3]	5
Olive oil [2 4]	46
Sunflower oil [2 4]	70
Canola tallow [6 5]	75
Energy density kcal/g	3.1
% of energy from protein	25
% of energy from carbohydrate	44
% of energy from fat	31
Choline g/kg diet	1.0

1, Harlan Teklad (Madison, WI, USA); 2, Lipid Nutrition (Channahon, IL, USA); 3, Gold Top Manufacturing and Distributing Ltd (Edmonton, AB, Canada); 4, Safeway (Calgary, AB, Canada); 5, Richardon Oilseed Ltd (Lethbridge, AB, Canada).

Initially n=8 offspring were maintained per group but two offspring died in the C and D group, respectively, resulting in n=7 for these groups and n=8 for the PC group.

Mothers were weighed and food intake was recorded weekly until euthanasia. If mothers lost more than 15% of their original post partum weight during the lactation period, they were switched onto the standard diet, C, until weaning. After weaning, mothers were kept for another week for tests, which were not part of my study. This consisted of a fat challenge, i.e. feeding the mothers a pellet of dietary fat after an overnight fast and blood samples for 12 hours afterwards to assess their lipid absorption and metabolism. At the end of that week, all mothers were euthanased and dissected as described below. During this week, all diets continued as during lactation, except for mothers with weight losses over 15% as described above, which were maintained on a modified diet. This modification is further described in Chapter 5. Before weaning, every week three randomly selected offspring of each litter were weighed. Growth rate

during suckling was determined by subtracting the weight of different ages, e.g.:

$$growth\ rate\ 1-2\ weeks = weight(2\ weeks) - weight(1\ week)$$

Similarly, the two remaining of each offspring were weighed and measured weekly until sacrifice in postnatal week 11. Food intake was measured as detailed below weekly until postnatal week 10.

2.2.2. The 2009/2010 studies

In a previous study, conducted in 2 cohorts in 2009 and 2010, 26 similar animals were used from the same source. The same diets were fed during lactation as above except for the PC diet, for which the lecithin used was isolated from soy, not egg (USB Corp., Cleveland, OH, USA). Animal numbers were higher for this study with n=8 mothers fed PC diet, n=11 fed C and n=7 fed D. In this study, mothers' diet of D animals was not changed as the mothers did not lose weight as rapidly as they did in the 2011 study. A similar but slower weight loss was observed in D mothers of this cohort. From each mother 2-3 offspring were euthanased at 21 days of age without being fasted and stomach content was collected as described below. Livers were dissected from all animals and their fat content analysed. Maternal small intestine was dissected and 1 cm pieces of duodenum, jejunum and ileum prepared for histology as described below (2.2.10. Intestine histology).

It was intended to combine data from the 2009/2010 studies with the 2011 study in order to 'establish' robust n-numbers. However, since several key measures including maternal weight, food intake, gonadal fat mass and offspring weight differed between years we decided that pooling

of the studies was not appropriate. Therefore, the 2011 study described here had to hold up to statistical tests despite low n-numbers. The cause of the difference in mother's weight between the studies is not clear, as source, strain and age at breeding was the same between years.

2.2.3. Food intake tests

Food intake of mothers and of the offspring after weaning were measured by providing a known amount of their assigned diet and weighing the remaining amount of uneaten food 48h later. Results were calculated to represent food intake over 24h and, although their intake was not necessarily assumed to be similar, divided by the number of sharing a cage in the case of the post-weaning offspring. Some of the littermates sharing a cage would differ quite substantially in weight and their food intake would be assumed to be quite different but, since food intake was expressed relative to body weight, the impact of averaging food intake in this way is thought to be small.

2.2.4. Intraperitoneal glucose tolerance test

This glucose tolerance test follows the same principles as described in Section 2.1.2 of the sheep study. Each animal was fasted overnight, weighed and a fasted blood sample collected from the tail vein by clipping off the tip of the tail, which is painless for the animals as nerve endings do not extend into the tip of the tail. For each subsequent blood sample the wound is re-opened rather than clipping the tail again. Glucose solution was administered intraperitoneally at a dose of 1 g glucose per kg body weight, diluted to 25% glucose in sterile water. Tail blood samples were

then collected at 10, 20, 30, 60, 90 and 120 minutes after the injection. Blood was immediately applied to a strip of a blood glucose testing device (Accu-Chek Compact Plus, Roche, Laval, QC, Canada) from each time point. This device has been used in rats in other studies [414] and, during the GTT, our glucose measurements were comparable to the outcomes of other studies with non-diabetic rats using colorimetric assays (5-13 mmol/L measured in our study, equivalent to 100-260 mg/dL) [415, 416]. Blood was additionally collected into heparinised capillaries (Fisher, Pittsburgh, PA, USA) and transferred into microcentrifuge tubes, kept at 4°C. To obtain plasma, blood was centrifuged (10 minutes at 10000 rpm), the supernatant plasma transferred into new tubes and stored at -80°C for subsequent insulin measurements by ELISA as described below. This work was carried out with the help of Ms Nicole Coursen and Mr. Michael Wadowski.

2.2.4.1. Plasma insulin concentrations

To determine plasma insulin concentrations, the Insulin Rat Ultrasensitive ELISA (80-INSRTU-E01, Alpco, Salem, NH, USA) was used following the manufacturer's instructions. Samples were compared to seven standards ranging in concentration from 0.15 to 5.5 ng/ml. Controls were included in all 96-well plates to correct for variance between plates.

In each well, 5 µl of standards, controls and samples were pipetted in duplicate. A streptavidin-horseradish peroxidase conjugate was added and left to react with the insulin for 2h (with 700-900 rpm shaking, at room temperature). The plates were washed six times and tapped thoroughly to remove excess liquids. For detection, 100 µl TMB solution was added and incubated for 30 minutes under the same conditions as before. The

reaction was stopped by adding 100 µl of an acidic stop solution and the intensity of yellow colour was read on the plate reader at 450 nm wave length. Plasma insulin concentrations were calculated using a 4 parameter logistic fit in comparison to the known concentrations of standards. Each 96-well plate contained, besides the standards, a duplicate of one same sample with unknown concentration to allow for observation and correction of inter-assay variability.

2.2.4.2. Tissue collection

Weanling offspring were fasted for at least three hours whilst adult animals were fasted overnight before tissue collection. Animals were euthanased by exposure to CO_2 gas and death ensured by testing for pedal response and subsequent cervical dislocation.

Blood was collected by cardiac puncture, transferred into an EDTA-coated tube (Vacutainer, BD, Franklin Lakes, NJ, USA) and kept at 4°C. Tubes were centrifuged at 3000 rpm for 10 minutes and plasma transferred into a clean tube. Blood from weanling offspring was pooled between littermates as the treatment n was the mother and this enabled to gain a higher tissue sample for analyses.

Whole brains were removed from the skull and separated along the interhemispheric fissure. Each half of the brain was separated into three sections coronally, with the middle section containing the hypothalamus. This middle section was either prepared for immunohistochemistry by embedding in paraffin or was frozen for later lipid extraction. The left brain section was inserted into a tissue cassette and submerged in a mixture of 10% formaldehyde (original concentration of 40% in water, resulting in a final concentration of 4% formaldehyde), 10% acetic acid and 80%

methanol (v/v) for 48h before transfer to 70% ethanol until further treatment as described in section Brain immunohistochemistry. The right brain section was embedded in OCT medium (Optimal Cutting Temperature medium; Shandon Cryomatrix, Thermo Scientific, Nepean, ON, Canada), snap frozen on dry ice and stored at -20°C until lipid extraction.

Livers, gonadal and perirenal white fat and interscapular brown fat were dissected by an experienced technician (as below), weighed and snap-frozen in liquid nitrogen. Tissues were stored at -80°C until further analysis. From all animals, the small intestine was removed between the pyloric sphincter and the ileocecal valve, the full length measured and a short section each of duodenum, jejunum and ileum immediately immersed in 20% Z-Fixx in formalin (v/v, Thermo Fisher Scientific, Edmonton, AB, Canada) for later histology. This work was carried out with the help of Ms Nicole Coursen, Ms Susan Goruk, Ms Kelly-Ann Leonard and Ms Howe-Ming Yu.

2.2.5. Stomach contents

For this, stomach contents of the earlier study (Section 2.2.2.2), carried out as described above, were analysed. Stomach contents of offspring were collected after euthanasia and stored at -80°C. They were then pooled in offspring from the same mother to ensure sufficient amounts for all analyses and ground together to a powder in a frozen ceramic mortar on dry ice. Total weights were recorded and 200 mg aliquoted for analysis of choline metabolites.

2.2.6. Choline metabolite and phospholipid analyses

Brains were analysed for concentrations of phospholipid classes and maternal livers and stomach contents for choline metabolite classes. Lipids were extracted following the method of Bligh and Dyer [417]. Both liver and stomach content samples were carried out in duplicate. However, this was not undertaken for brain samples as these were dissected from brain halves in a block including the hypothalamus, in order to standardise the anatomical regions contained in each sample.

The procedure of lipid extraction was the same for all of these samples: 100 ± 0.5 mg tissue sample were weighed and homogenised with a polytron homogeniser in 2 ml of a chloroform : methanol : water mixture (in the proportions of 1 : 2 : 0.8 by volume). The homogenate was centrifuged at 3000 rpm for 5 minutes and the supernatant transferred into a new glass vial. Another 2 ml of the same solvent mixture was added to the tissue pellet and homogenised, centrifuged and transferred to the same vial as before. This step was repeated one more time to yield a total extract of 6 ml. Before analysis, 1 ml of this extract was filtered using a 0.22 µm syringe filter (with PTFE membrane, catalogue number 28145-491, VWR International, Edmonton, AB, Canada) and stored at 4°C. The remaining 5 ml lipid extract from the brain were dried down under nitrogen at 45°C and taken into solution again in 1 ml hexane for isolation of PC and PE and subsequent fatty acid analysis as described below.

Phospholipid concentrations and choline metabolites were measured in these extracts based on the methods of Zhao et al. [418]. In summary, samples were analysed for choline metabolites with liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (AB Sciex; Concord, ON,

Canada) and compared against standards. Data were acquired and analysed with the use of Analyst 1.4.2 software. Samples were eluted from an Ascentis Express 150 x 2.1 mm HILIC column, 2.7 µm particle size (Sigma, St. Louis, MO, USA) in a gradient elution, increasing the concentration of 10 mmol/L ammonium formate in water at pH 3.0 (phase B) in acetonitrile (A) over time: 0-0.1 min, 8% B; 0.1-10 min, from 8% to 30% B; 10-17 min, 95% B; and then back to 8% B at 17.1 min for column re-equilibrium prior to the next injection. Cycle time was 30 min per injection of 2 µl volume. The flow rate of the mobile phase was 400 µl/min for the period from 20 min to 27 min and 200 µl/min for all other periods. For mass spectrometry, a turbospray ion source was used in positive ion mode with nitrogen. The handling of samples on the LC-MS and quantification of metabolite and phospholipid concentrations was carried out by Mr. Yeping Xiong.

2.2.7. Analysis of fatty acid moieties in brain phospholipids

As described above, brain lipids were extracted and dissolved in 1 ml hexane. 100 µl of this extract were applied to an activated H plate using a glass syringe in a line of small droplets and allowed to dry (Uniplate silica gel H, Analtech Inc., Newark, DE, USA). These silica-coated glass plates are used for thin layer chromatography to separate lipid extracts into the different classes of phospholipids. Plates were run in glass chambers that accommodated two plates standing up, leaning against the upper edge of the chamber wall. To the bottom of the chamber was added 1 cm high solvent (34.1% chloroform, 10.2% methanol, 28.4% 2-propanol, 20.5% triethylamine and 6.8% KCl (0.25% in water) by volume) to act as a

mobile phase and a sheet of filter paper which coats the wall of the chamber to improve saturation with the solvent.

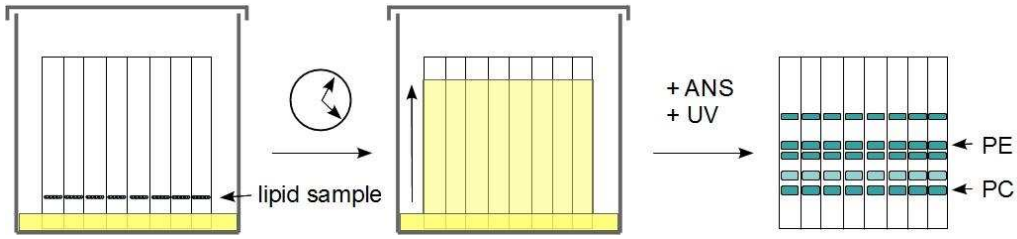

Figure 2.5: Thin layer chromatography.

Eight lipid extract samples were applied to an H plate, the plate was positioned in a closed chamber containing a solvent mixture. By capillary forces, the solvent moves up the plate, spreading the lipids into the different phospholipid classes. The plate was dried, sprayed with 0.1% 8-anilino-1-naphthalenesulfonic acid (ANS) and viewed under UV light to reveal the position of phospholipid classes. The bands containing phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were used in further analyses.

The plate was then placed in the chamber, ensuring the samples were on the lower end of the plate but not touching the solvent and the chamber closed ensuring saturation of air with the solvent. During the 2h incubation the plate was saturated with the solvent and lipid components of the sample spread across the plate. The plate was then removed from the chamber, dried and then sprayed with a thin layer of 0.1% 8-anilino-1-naphthalenesulfonic acid, a hydrophobic fluorescent molecule. Under UV light, the location of the different phospholipid classes can be determined as bands and relevant areas of silica containing PC and PE were scraped off and placed into separate glass vials. Two ml hexane and 1.5 ml boron trifluoride methanol (both from Thermo Fisher Scientific, as above) were added, the vials closed, vortexed thoroughly and boiled (110°C) in a heating block for 1h. They were then allowed to cool and 1 ml deionised water was added, mixed and left at 4°C overnight. The hexane layer was transferred into a new glass vial and was dried down under nitrogen gas. To achieve a concentration suitable for analysis with the GC, samples containing PC were redissolved in 50-100 µl hexane. Samples were applied

to the GC (Agilent Technologies 7890 A GC System, Santa Clara, CA, USA) and run on a 100-m CP-Sil 88 fused capillary column (Varian Inc., Mississauga, ON, Canada).

2.2.8. Liver total fat content

Total fat was isolated from mother and offspring livers using a modification of the Folch method [419]. After defrosting, 300 mg of liver tissue were homogenised in 1 ml 0.025% CaCl solution for 30 seconds at 6.5 m/s using a FastPrep-24 homogeniser (MP Biomedicals, as above). In glass tubes, 12 ml of 2:1 chloroform:methanol and 1ml 0.025% CaCl solution was added to the liver homogenate. These tubes were vortexed thoroughly and left overnight at 4°C. This treatment results in two layers and the lower, chloroform phase was transferred into a new, pre-weighed, glass tube and the remaining phase washed once more with 2:1 chloroform:methanol. After one hour, the chloroform phase of the second wash was added to the first aliquot. The chloroform was evaporated under nitrogen until only the fat remained in the tube that was reweighed, with the difference between the two weights being the weight of the fat extracted. Total liver fat was expressed as mass of fat adjusted for original sample weight (mg/g).

2.2.9. Brain immunohistochemistry

2.2.9.1. Tissue preparation and sectioning

After 48h immersion of brain tissue in FAM as described in Section 2.2.2.4.2, samples were transferred to 70% ethanol until further processing using the Leica TP 1020 tissue processor. In the processor, the

tissues were left for one hour each in the following solutions: 70% ethanol, 3 baths of 100% ethanol, 3 baths of xylene and 3 baths of paraffin wax. Tissues were subsequently blocked in paraffin wax. All materials were sourced from Thermo Fisher Scientific (as above).

Brains were cut coronally and sections were designated as including the ARC of the hypothalamus if the hypothalamic region protruding into the third ventricle and the hippocampal arcs close to the interhemispheric fissure were visible at about 2 mm width. These two landmarks were observed to be present at the same depth as the arcuate nucleus with the aid of a rat brainmap (brainnav.com, Elsevier Inc.) and to be corresponding to a depth of -2.7 ± 0.3 mm Bregma (Figure 2.6). The appropriate depth was then cut into 6 µm thick sections using a rotary microtome (Leica RM2125RT, Nussloch, Germany) and transferred onto slides (Fisher Superfrost microscope slides, catalogue number 12-550-016, as above) with 2-3 sections per slide and at least 5 slides per sample to allow for repeats. Slides were left at 60°C overnight to improve adherence of tissue to the slide.

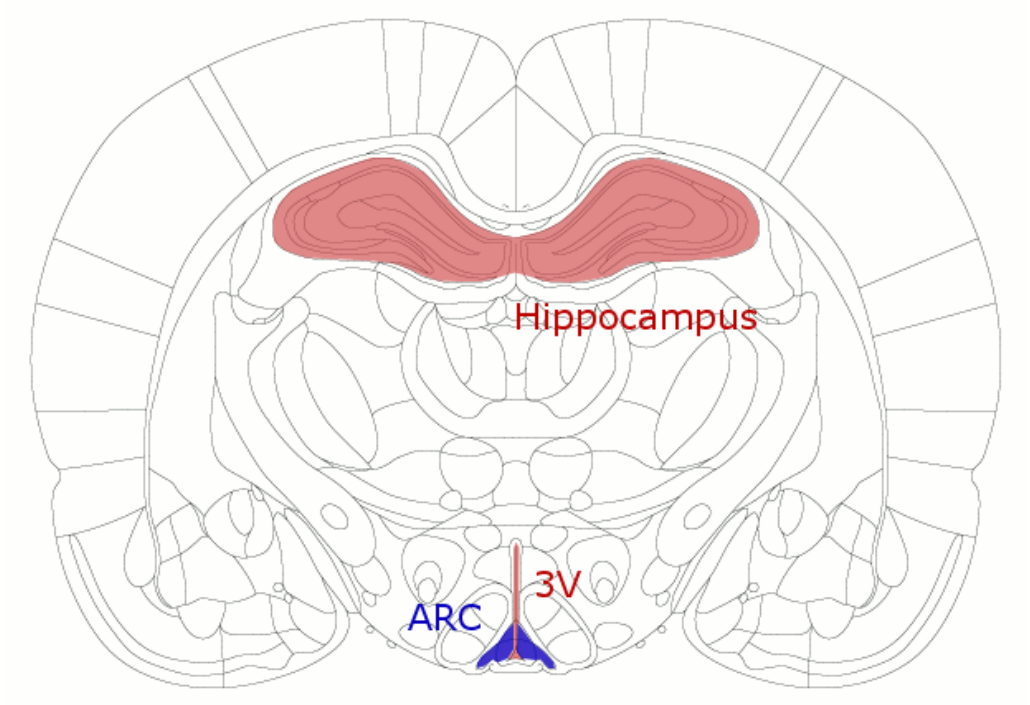

Figure 2.6: Schematic drawing of brain anatomy at coronal section Bregma -2.76 mm. ARC (blue) shows the location of the arcuate nucleus in relation to the third ventricle (3V) and hippocampus (both in red). Brainmap used from brainnav.com (Elsevier Inc.).

2.2.9.2. Immunohistochemistry

First, paraffin was dissolved by placing the slides in two 10 minute xylene washes. The tissues were then rehydrated in 3 minute graded ethanol baths of 100%, 95% and 75% ethanol in deionised water (v/v). Samples were then kept in tap water until antigen retrieval. After rehydration and until dehydration at the end of the staining process, the tissues were not allowed to dry in order to reduce non-specific staining. Antigens were retrieved in 1mM EDTA in water (pH 8) at 95-100°C for 10 minutes (for NPY (neuropeptide Y) staining) or 15 minutes (for α-MSH (α-melanocyte stimulating hormone) staining) in a vessel placed in a boiling water bath and then samples cooled slowly for 20 minutes. Slides were washed 3 times for 3 minutes in deionised water.

Tissues were then blocked with PBS containing 4% goat serum and 0.5% Triton-X (v/v) for 1h and drained shortly. Primary antibodies were diluted in PBS containing 1% BSA (wt/wt) to 1:1000 (for NPY staining) or 1:12000 (for α-MSH staining) and 60 µl was applied per section. As a negative control 60 µl PBS with 1% BSA (wt/wt) was applied to one section per slide. The slides were incubated overnight at 4°C.

The next day, slides were washed 3 times in PBS for 3 minutes and the diluted secondary antibody (AB) was added at a dilution of 1:100 (anti-rabbit antibody (AB) for NPY staining) or 1:250 (anti-sheep AB for α-MSH staining) and 60 µl was applied per section. To control for non-specific staining, blocking buffer without secondary AB was applied on 3-4 slides only treated with the primary AB. All steps after application of the secondary AB were performed with the slides covered in foil to reduce light exposure and thus prevent photo bleaching. After 1h (for NPY staining) or 2h (for α-MSH staining) incubation with the secondary AB, slides were washed twice for 5 minutes in deionised water. The slides were dehydrated in an ethanol bath with 20 seconds in 95% (v/v in deionised water) and then 100% ethanol and air dried. Fluorsave solution (Calbiochem, Merck, Darmstadt, Germany) was applied on the tissues to increase stability of the fluorescent markers and slides were covered with cover slips (Thermo Fisher Scientific, Edmonton, AB, Canada), the edges were then sealed with nail polish.

Primary ABs used were from Millipore (Billerica, MA, USA): rat anti-NPY raised in rabbits (AB9608) and rat anti-α-MSH raised in sheep (AB5087). Secondary ABs were obtained from Invitrogen (Burlington, ON, Canada): anti-rabbit raised in goat with AlexaFluor 532 label (A-11009) and anti-sheep raised in donkey with AlexaFluor 488 label (A-11015).

Primary and secondary antibodies were initially tested in varying concentrations to obtain optimal staining. For NPY staining, the primary antibody was tested at 1:500, 1:1000 and 1:2000 dilutions. All three dilutions showed similar results with the clearest difference between slides stained for NPY and the negative control at 1:1000 dilution (Figure 2.7). For α-MSH staining, the primary antibody was tested at 1:5,000, 1:10,000 and 1:15,000. The dilution of 1:15,000 gave the most consistent result and was used for all analyses (Figure 2.8).

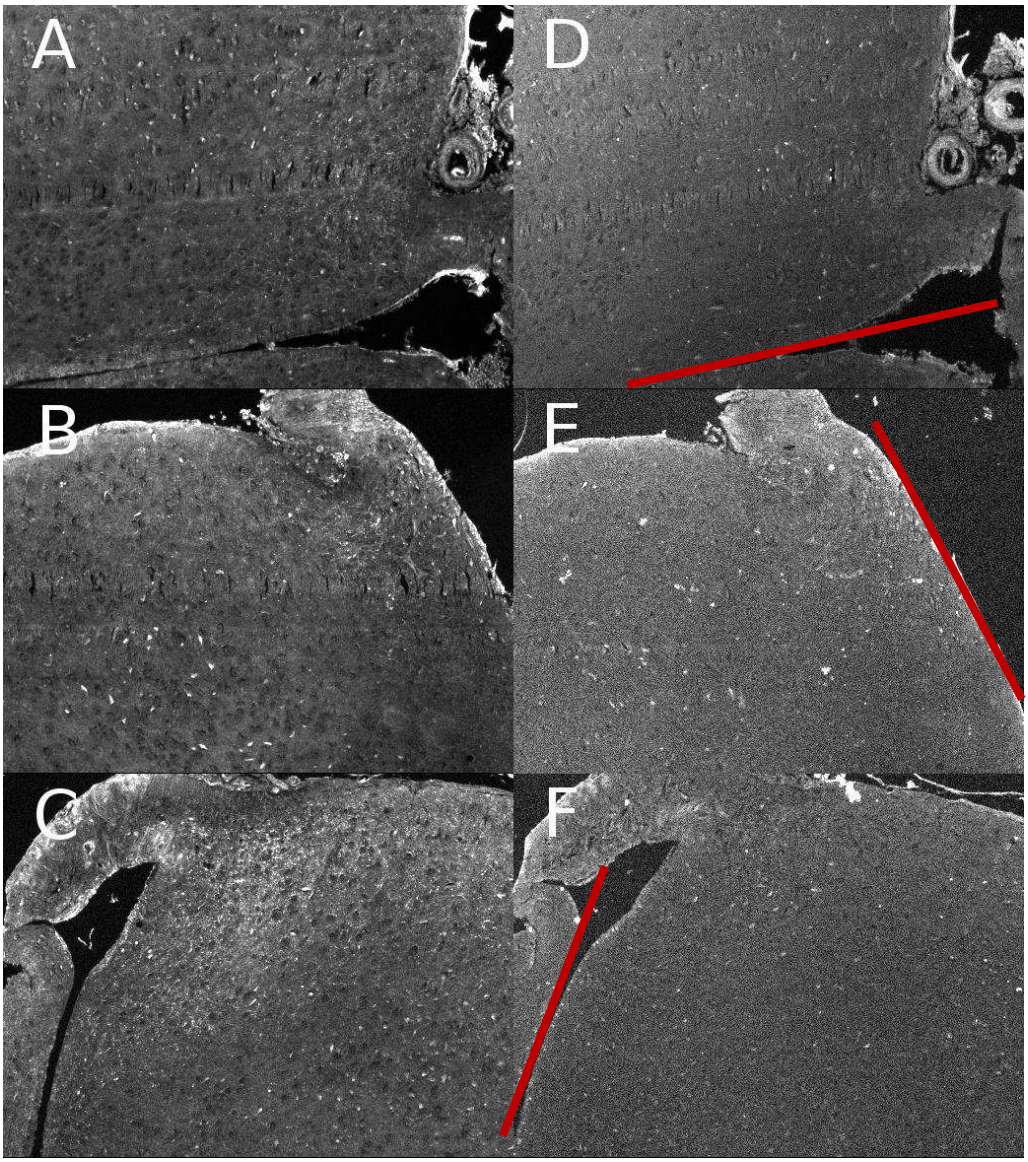

Figure 2.7: Images of arcuate nuclei stained for NPY at varying dilutions of primary antibody.

A: staining with primary antibody diluted 1:500; C: staining with primary antibody diluted 1:1000; E: staining with primary antibody diluted 1:2000. B, D, F: respective negative slides, not containing primary antibody. Red lines in negative image indicate position of the third ventricle. White scale bar indicates 100 µm.

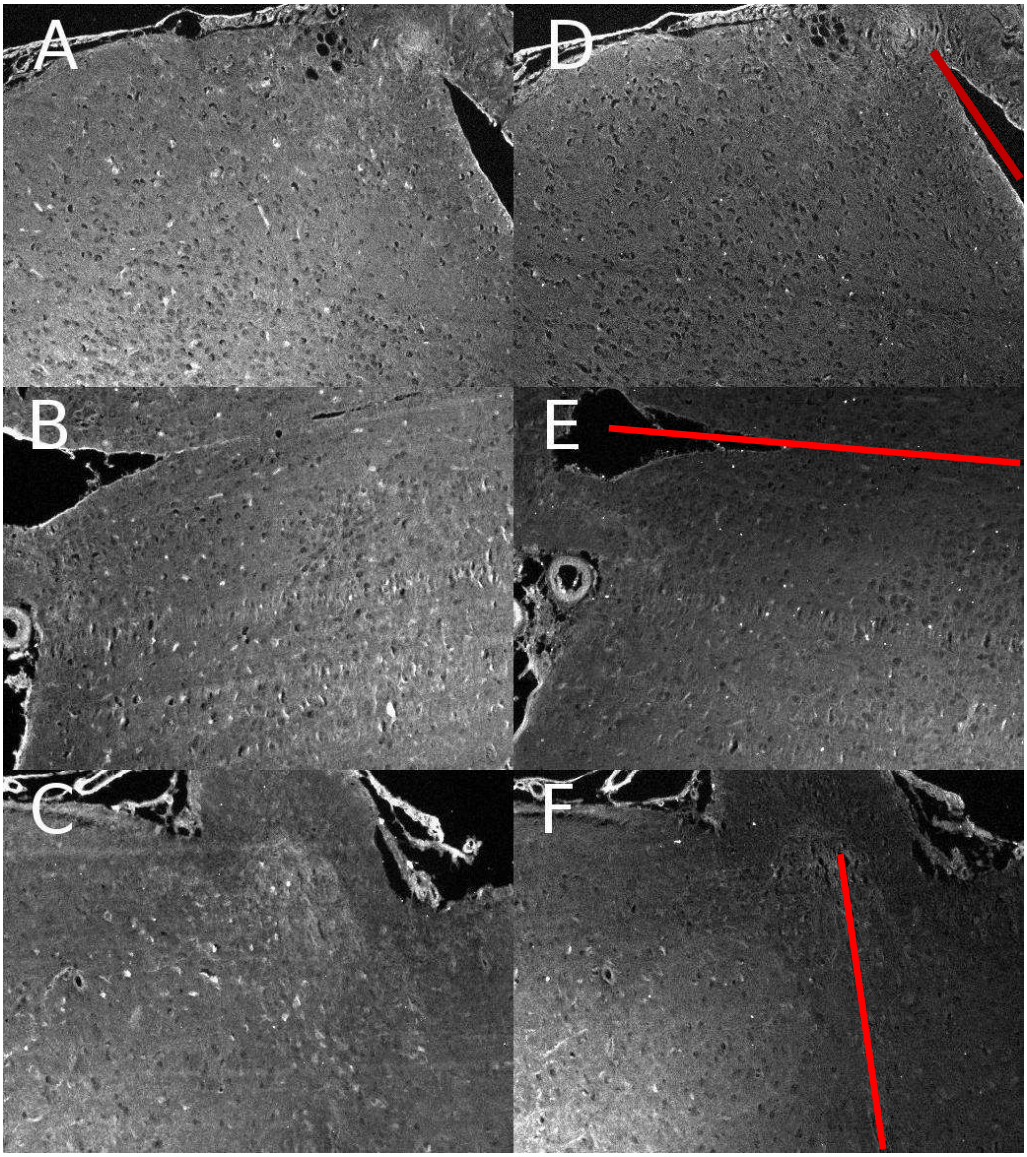

Figure 2.8: Images of arcuate nuclei stained for α-MSH at varying dilutions of primary antibody.

A: staining with primary antibody diluted 1:5,000; C: staining with primary antibody diluted 1:10,000; E: staining with primary antibody diluted 1:15,000. B, D, F: respective negative slides, not containing primary antibody. Red lines in negative image indicate position of the third ventricle. White scale bar indicates 100 µm.

All slides were viewed on an Axio Imager.Z1 microscope (Zeiss Ltd, Toronto, ON, Canada) and photographed with an AxioCam MRm camera (Zeiss Ltd, as above) and the software used for the image analysis was ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). The area corresponding to the ARC was determined in comparison with brain map images, measured and stained cells within this area counted. Images used for the analysis were taken

with a 10x objective at exposure time of 1 millisecond (ms, for NPY staining) or 15 ms (for α-MSH staining).

2.2.10. Intestine histology

Sections of duodenum, jejunum and ileum were used to analyse general morphology of the small intestine. Maternal intestinal sections were fixed in 20% w/w Z-Fixx in formalin (Thermo Fisher Scientific, as above) for 48 hours and then transferred to 10% buffered formalin (Thermo Fisher Scientific, as above) until blocking. All tissues were inserted in a tissue processor (Leica TP 1020, as above), placed in washes of 10% buffered formalin, 70% ethanol, 3 changes of 100% ethanol, 3 changes of xylene (Fisher Scientific, as above) and 3 changes of paraffin wax (TissuePrep Embedding Media, Fisher Scientific, as above) for one hour per bath. Samples were then embedded into paraffin wax and cut to 6 µm thickness as described in more detail above in section Brain immunohistochemistry. Sections were stained with haematoxylin and eosin following the producer's instructions (aqueous instant dyes, Shandon, Thermo Fisher Scientific, as above).

Samples were deparaffinised and re-hydrated with subsequent washes of 2 changes of xylene for 10 minutes each, 2 changes of 100% ethanol for 5 minutes each and 95% and 70% ethanol for 2 minutes each. They were washed briefly in distilled water, stained in Harris's haematoxylin for 4 minutes and washed under running tap water for 5 minutes. The samples were differentiated in 1% acetic acid in ethanol for 30 seconds and washed again for 1 minute. Haematoxylin was blued in a 30 second wash in 0.2% ammonia in distilled water (v/v) and washed under running tap water for 5 minutes, then rinsed in 95% ethanol. Samples were counterstained in

yellow eosin for 30 seconds. Finally, they were dehydrated in 95% ethanol, 2 changes of 100% ethanol and 2 changes of xylene for 5 minutes each and mounted with a xylene-based mounting medium (permount mounting media, Thermo Fisher, as above).

Slides were viewed and villus height and crypt depth were measured using computer-assisted microscopy (Zeiss Primo Star, Metamorpth Meta Imaging series by MDS analytical technologies).

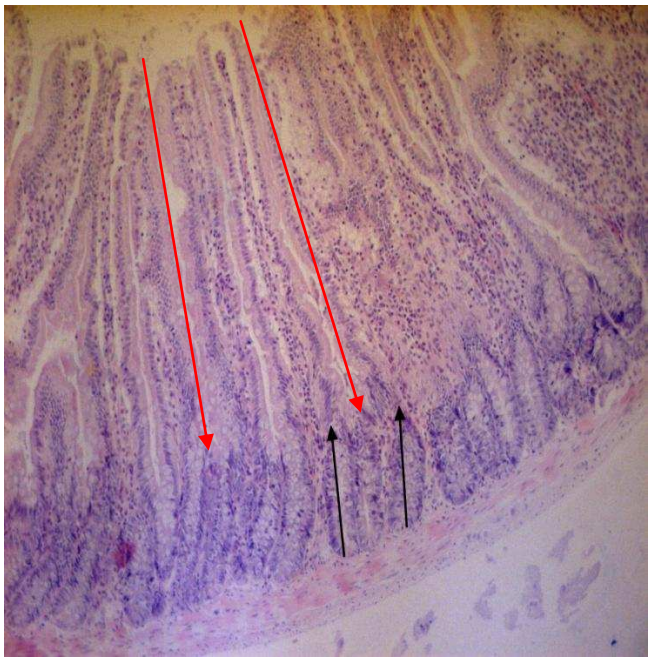

Figure 2.9: Jejunum section stained with haematoxylin and eosin showing examples of measurements for villus height (red arrows) and for crypt depth (black arrows).

Villus height was measured from the tip of the villus to the top of the crypt whilst crypt depth was measured from the top of the crypt to its base as illustrated in Figure 2.9. At least four measurements were taken for each animal and intestinal section. Whilst all intestinal analyses were carried out by undergraduate project student Ms Chisom Ikeji, I have analysed the original data for inclusion in this thesis.

2.3. Statistical analysis

Statistical analysis of data from both the sheep and the rat studies were principally the same. Data from mothers for each outcome was tested for normal distribution using a Kolmogorov-Smirnov test. If data were normally distributed, an ANOVA with an a priori comparison of contrasts between groups, i.e.

> PC vs C, C vs D, PC vs D in the choline study;
>
> RAO vs NAO, NAO vs NSO, NSO vs NSL in the macronutrient restriction study;

was undertaken and a p-value determined dependent on whether or not equal variance was given between groups [420]. An a priori comparison, also called planned comparison, is more hypothesis-driven compared to a post-hoc test, as it allows comparison of specific group pairs rather than comparing all groups to each other, therefore leading to lower p-values without increasing the error rate [421].

For the plasma concentrations measured during the 24h feeding test in the sheep study, a paired t-test was used to compare concentrations at 2 time points, i.e. to test for a difference between the fasted and the fed morning samples.

If data were not normally distributed, their logarithm (base 10) was again tested for normal distribution. If data were still not normally distributed, Mann-Whitney tests were used for the comparisons given above. For easier reading, both normally and non-normally distributed data were displayed as mean ± standard error of mean (SEM) throughout the thesis.

Offspring data collected during lactation were treated as multiple measurements of an outcome if offspring were born from the same mother. Therefore, the number (n) of offspring during lactation is the same as the number of mothers as the intervention has been applied to the mother rather than to the offspring individually/ independently. Data were averaged between siblings and then analysed as described above for maternal data.

After weaning, sibling offspring showed differing phenotypes and it was, therefore, deemed appropriate to not treat them as multiple measurements but to block data by using a hierarchical model instead [421, 422]. With this model, siblings were considered separately but it was taken into account that they are offspring from the same mothers, i.e. as parts of a group (litter) within a group (diet). The test assesses whether the maternal intervention group had an influence on the outcome, doing multiple comparisons as stated above.

All analyses were carried out on SPSS software version 17 (PASW, IBM, Portsmouth, UK).

3. Long term effects of maternal nutrient restriction on growth and insulin sensitivity

3.1. Introduction and hypotheses

This chapter investigates the acute and long term metabolic changes caused by manipulating the perinatal nutritional environment. The sheep is a good model for human intrauterine and neonatal development and the results have implications for human pregnancies, effects on children and their risk of developing metabolic disease as adults. The study was designed to examine the effects of nutrient restriction in late pregnancy, coincident with the period of maximal fetal growth. This is a period during which nutrient restriction can result in intrauterine growth retardation (IUGR). Subsequent to the manipulation of environment during pregnancy, I examined the influence of early postnatal growth rate, which has been postulated to be a separate risk factor for the development of insulin resistance.

This work is important since early postnatal life is less well established to be a period sensitive for programming. In their review paper, Bateson et al. proposed that the difference in growth during development, i.e. slow growth in late pregnancy followed by faster growth in early postnatal life, is especially detrimental to long term metabolic outcomes [423]. Finally, metabolic diseases are increasing in prevalence, most likely due to the increase in obesity mediated by changes in lifestyle, which started in Western countries and is gradually spreading across the world. The obesogenic environment to which the offspring of mothers fed to

requirements and mothers nutrient restricted in late gestation were exposed consisted primarily of a restriction in their physical activity, leading to a modest elevation in their body weight, a study intervention most relevant to the majority of people with a sedentary lifestyle.

Following data from the Dutch famine follow-up studies, described in more detail in the Introduction (Chapter 1),

1) I hypothesised that the offspring of nutrient restricted mothers would be lighter at birth but would not be different in adult body weight compared to animals not subjected to maternal nutrient restriction.
2) These offspring would, nevertheless, develop insulin resistance, which would be increased by an accelerated early postnatal growth rate.
3) A combination of accelerated early postnatal growth rate following a reduced birth weight would lead to higher mature body weight and the obesogenic environment after weaning would amplify adverse metabolic effects.

It should be noted that the offspring were only studied until they reached a young adult age. As metabolic effects only start to develop at this age, these would be expected to deteriorate further once impaired glucose tolerance was established.

3.2. Materials and Methods

The design of the study and all materials and methods used to establish the results described in this chapter are described in the Materials and Methods (Chapter 2) and Figure 3.1. Briefly, twin pregnant ewes were

either nutrient restricted from gestational day 110 to term, 145 days, (N) or kept on a diet providing all their nutritional requirements throughout pregnancy (R). After birth, twin offspring were either both raised by the mother to induce a standard early postnatal growth rate (S) or were separated with only one twin remaining with the mother so as to induce an accelerated growth rate (A). After weaning at 3 months of age, animals were maintained in an obesogenic environment with restricted opportunity for physical activity (O) or in a unrestricted environment to induce a lean phenotype (L). During gestation there were 2 groups, R and N, whilst during the lactation period there were 3 groups, RA, NA and NS, and, after weaning, 4 groups, RAO, NAO, NSO and NSL as summarised in Figure 3.1. Blood samples were taken regularly and analysed for metabolic and endocrine factors. Body weight gain and physical activity were monitored regularly and glucose tolerance tests carried out at 7 and 16 months of age. At 16 months, body composition was determined by Dual-energy X-ray absorptiometry (DXA) and all offspring dissected. Comparisons were drawn between comparable groups i.e. offspring from groups differing only in one letter of their group abbreviation.

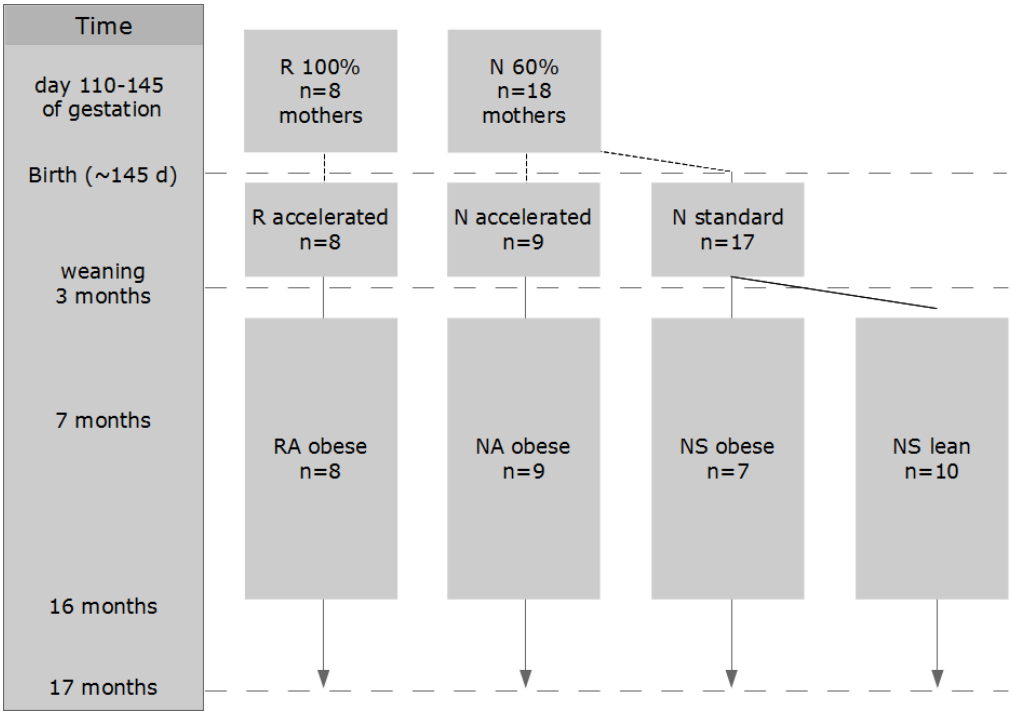

Figure 3.1: Schematic description of study groups of the sheep macronutrient restriction study.

Twin-bearing mothers are fed meeting requirements until 110 days of pregnancy. From 110 days until term (145 days) mothers are fed either to requirements (R) or to 60% of macronutrients (N). After birth, accelerated growth is induced by separation of twins and only one twin remaining with the mother (RA and NA) or offspring had a standard growth rate (NS). After weaning, most offspring were subjected to an obesogenic environment (RAO, NAO, NSO) whilst others were maintained in a unrestricted environment (NSL).

3.3. Results

3.3.1. Mothers

When adjusted for their body weight at the beginning of the nutritional intervention, N mothers gained less weight in late pregnancy as compared to R mothers (Figure 3.2).

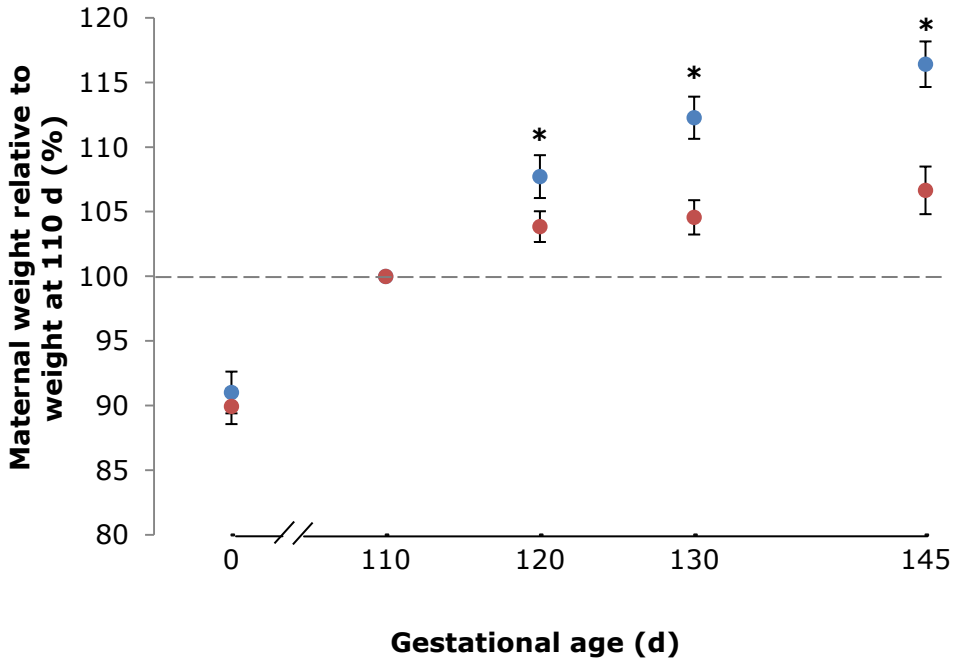

Figure 3.2: Maternal body weight throughout gestation, relative to weight at 110 days of gestation, the start of the intervention.

Mothers were fed a diet meeting requirements (R, blue, n=8) or a nutrient restricted diet delivering 60% of caloric requirements (N, red, n=18 between gestational days 110 and 145. * p<0.05 between N and R mothers. Data are means with error bars representing SEM.

At 130 d gestation plasma glucose concentrations were lower and plasma non-esterified fatty acid (NEFA) concentrations were higher in nutrient restricted mothers (Figure 3.3).

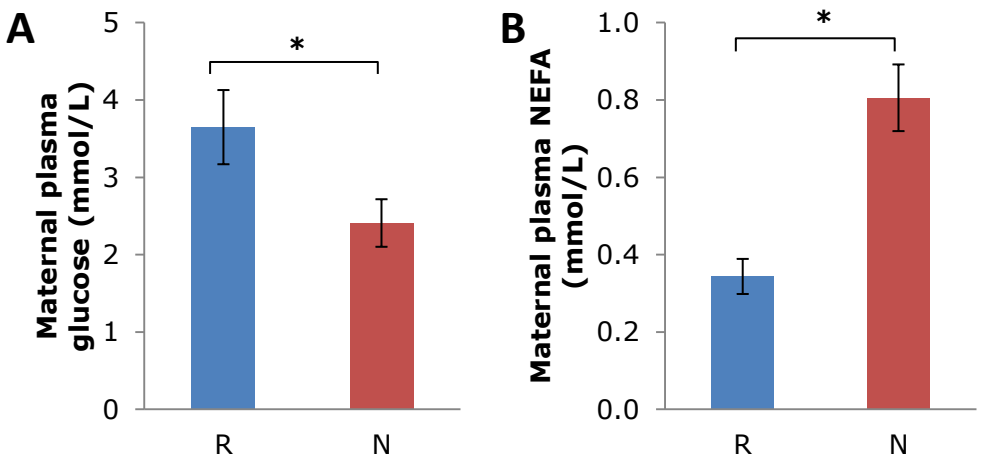

Figure 3.3: Maternal plasma glucose (A) and NEFA concentrations (B) at 130 days of gestation. Mothers were fed a diet meeting requirements (R, n=8) or a nutrient restricted diet delivering 60% of caloric requirements (N, n=15) between gestational days 110 and 145. NEFA, non-esterified fatty acids. * p<0.05 for R versus N comparison. Data are means with error bars representing SEM.

Plasma cortisol, insulin and triglyceride (TG) concentrations did not differ between R and N mothers (Table 3.1).

Table 3.1: Maternal plasma cortisol, insulin and triglyceride concentrations at 130 d gestation.

Maternal plasma concentration	R	N
Cortisol (nmol/L)	17 ± 3	22 ± 3
Leptin (ng/mL)	1.5 ± 0.3	1.0 ± 0.1
Insulin (µg/L)	0.3 ± 0.01	0.3 ± 0.01
Triglycerides (mg/dL)	29 ± 1	30 ± 1

Mothers were fed a diet meeting requirements (R, n=8) or a nutrient restricted diet delivering 60% of caloric requirements (N, n=15) between gestational days 110 and 145. Data are means ± SEM.

3.3.1.1. Maternal metabolome

The analysis of maternal metabolome resulted in a total of 2629 (mass-to-charge ration and retention time, [*m/z*; *rt*]) features, which showed a marked overall difference in metabolic patterns between R and N mothers in the OSC-PLS-DA score plot (Figure 3.4).

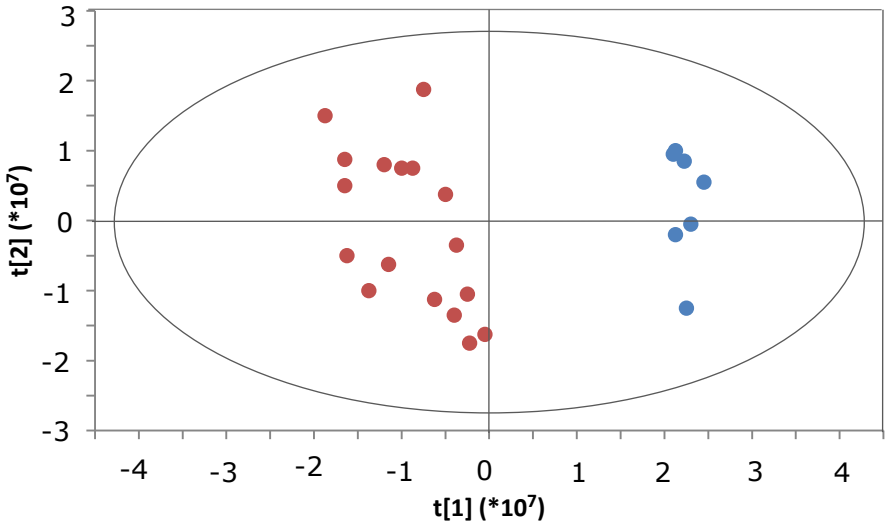

Figure 3.4: OSC-PLS-DA score plot to show differences in the overall metabolome of mothers' plasma at 130d gestation.

Mothers were fed a diet meeting requirements (R, blue, n=7) or a nutrient restricted diet delivering 60% of caloric requirements (N, red, n=17) between gestational days 110 and 145. Validation parameters: R^2X (cum)=0.429, R^2Y (cum)=0.928, permutation test (n=100) with R^2 intercept=0.371 and Q^2 intercept=-0.342, ANOVA-p-value=$2*10^{-8}$.

Of the 2629 compounds, 195 were significantly different (*XCMS* t-test treatment with $p<0.05$) between N and R mothers. However, only 8 of these compounds could be identified with standards (Table 3.2). An additional 5 compounds with a trend to be different between N and R mothers could also be identified ($p<0.1$).

Table 3.2: List of metabolites which were significantly altered in maternal plasma at 130d gestation between N and R mothers.

Compound	RT	M	$[M+H]^+$ observed	fold change	p-value
Leucine	5.78	131.0946	132.1019	1.45	0.005
Proline	2.54	115.0633	116.0676	1.35	0.035
Tryptophane	13.45	204.0898	205.0972	2.97	<0.001
Valine	2.36	117.0789	118.0862	-1.31	0.007
Pyroglutamic acid	5.02	129.0426	130.0498	1.37	0.031
o-Acetyl-carnitine	3.77	203.1157	204.1230	1.84	<0.001
Hexanoyl-carnitine	15.63	259.1784	260.1856	2.30	<0.001
Cortisone	16.36	360.1936	361.2009	1.37	0.084
Glycine	3.66	75.0320	76.0393	1.32	0.080
Phenylalanine	11.37	165.0789	166.0863	1.25	0.087
Riboflavin	6.76	376.1383	377.1455	1.43	0.067
Isobutyric acid	14.55	88.0524	89.0597	1.43	0.063

In the lower part of the table metabolites are listed which showed a trend (t-test with a $0.05<p<0.1$) to a difference between N and R mothers. A positive fold change indicates that the compound has a higher concentration in N compared to R mothers, a negative fold change indicates a lower concentration in N compared to R mothers.

Mothers were fed a diet meeting requirements (R, n=7) or a nutrient restricted diet delivering 60% of caloric requirements (N, n=17) between gestational days 110 and 145. RT, retention time; M, monoisotopic mass; [M+H]+, mass with one additional hydrogen atom; all compounds were identified using authentic standards.

3.3.2. Offspring at weaning

Mothers that were nutrient restricted in late gestation gave birth to lighter offspring as compared to R mothers (N, 3.8 ± 0.2 kg; R, 5.0 ± 0.2 kg). When raised alone by their mother after birth so as to induce an accelerated growth rate, NA offspring remained lighter than RA offspring throughout most of lactation period (and this reached statistical

significance between 5-38 d and 74-90 d of postnatal age). An acceleration of early growth rate in NA offspring led to a higher weight, which reached statistical significance in comparison with those N offspring raised by their mothers with their twin (NS) between 38-52 d and at 90 d of age. At weaning, RA offspring are significantly bigger than NA, and NA are significantly bigger than NS offspring (Figure 3.5).

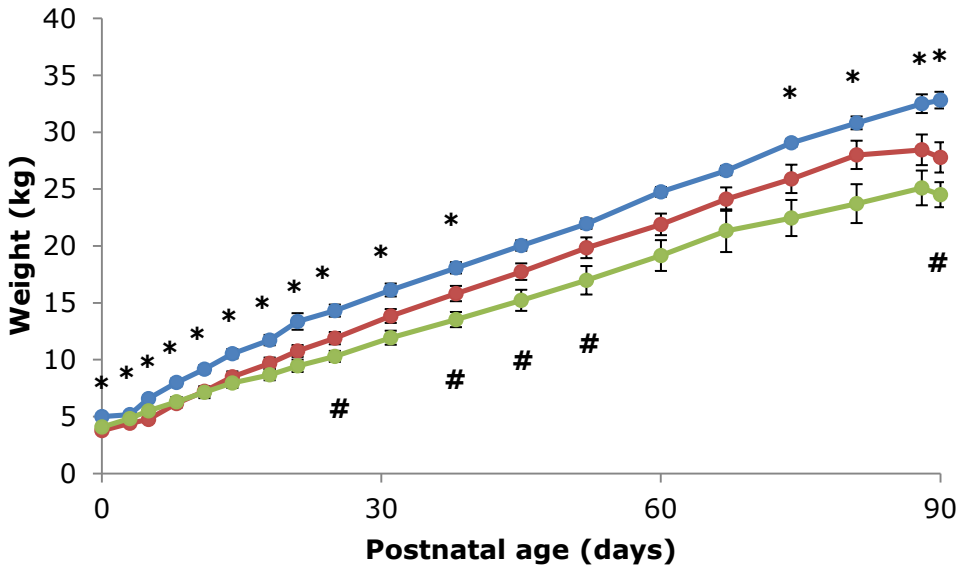

Figure 3.5: Offspring growth development between birth and weaning at 90 d of postnatal age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RA, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NA, red, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS, green, n=17). * p<0.05 for RA versus NA; # p<0.05 for NA versus NS. Data are means and error bars represent SEM.

NA offspring increased their weight compared to birth weight more than NS offspring throughout the lactation period and, after 30 days, more than RA offspring, despite the lower body weight of NA compared to RA at weaning (Figure 3.6).

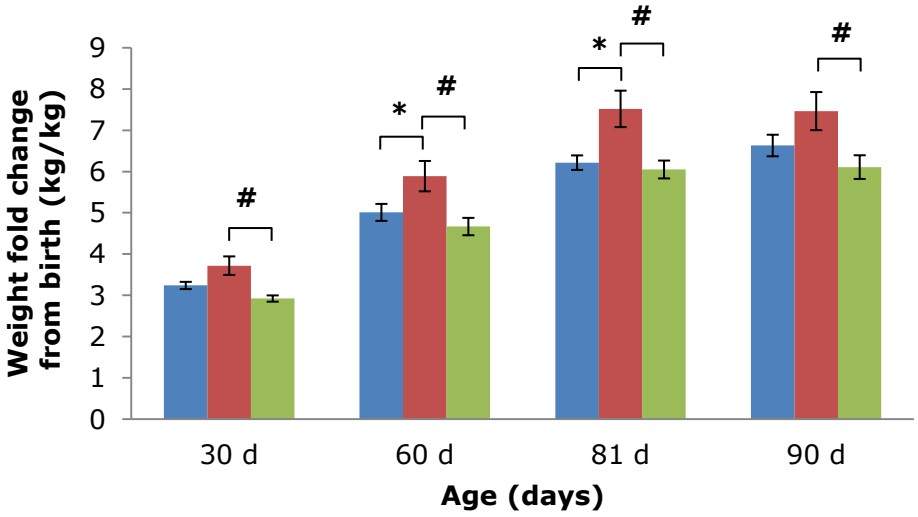

Figure 3.6: Fold change in body weight from birth weight throughout lactation.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RA, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NA, red, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS, green, n=17). * p<0.05 for RA versus NA; # p<0.05 for NA versus NS. Data are means and error bars represent SEM.

At 3 months of age plasma hormone and metabolite concentrations did not differ between intervention groups (Table 3.3).

Table 3.3: Plasma concentrations of hormones and metabolites at 3, 7 and 16 months of age divided by study group.

Plasma conc.	Age	RAO	NAO	NSO	NSL
Cortisol (nmol/L)	3	32 ± 4	42 ± 10	40 ± 5	
	7	74 ± 19	60 ± 14	54 ± 7	50 ± 12
	16	41 ± 9	56 ± 14	44 ± 10	22 ± 4
Leptin (ng/ml)	3	0.65 ± 0.10	0.46 ± 0.09	0.47 ± 0.07	
	7	1.8 ± 0.2	2.5 ± 0.5	2.2 ± 0.4	1.5 ± 0.3
	16	4.1 ± 0.6	6.2 ± 0.9 [a]	3.7 ± 0.5 [b]	3.1 ± 0.2
Insulin (µg/L)	3	0.16 ± 0.09	0.07 ± 0.01	0.07 ± 0.01	
	7	0.16 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.02
	16	0.36 ± 0.04	0.38 ± 0.02	0.43 ± 0.09	0.31 ± 0.01
Glucose (mmol/L)	3	3.0 ± 0.2	2.8 ± 0.1	2.6 ± 0.1	
	7	3.76 ± 0.14	3.79 ± 0.19	3.12 ± 0.44	3.23 ± 0.26
	16	2.93 ± 0.20	3.15 ± 0.11	2.97 ± 0.12	3.17 ± 0.10
TG (mg/dl)	3	7.7 ± 0.4	7.6 ± 0.3	7.9 ± 0.4	
	7	15.2 ± 1.0	14.8 ± 1.0	16.1 ± 3.6	20.7 ± 3.0
	16	15.9 ± 1.8 [c]	10.6 ± 2.6 [d]	10.7 ± 1.7	14.5 ± 1.3
NEFA (mmol/L)	3	0.46 ± 0.07	0.38 ± 0.04	0.32 ± 0.05	
	7	1.3 ± 0.1 [e]	1.0 ± 0.1 [f]	1.3 ± 0.1	1.6 ± 0.3
	16	0.65 ± 0.06	0.49 ± 0.07	0.48 ± 0.08	0.40 ± 0.05

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RA, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NA, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS, n=17). After weaning, animals were maintained in an obesogenic (O) or unrestricted environment (L; NSO n=8, NSL n=9). Postnatal age is given in months. Data are expressed as mean ± SEM. Comparison a versus b, p=0.029; c versus d, p=0.036; e versus f, p=0.028.

At the age of 1.5 months all offspring had similar physical activity independent of intervention group (Figure 3.7).

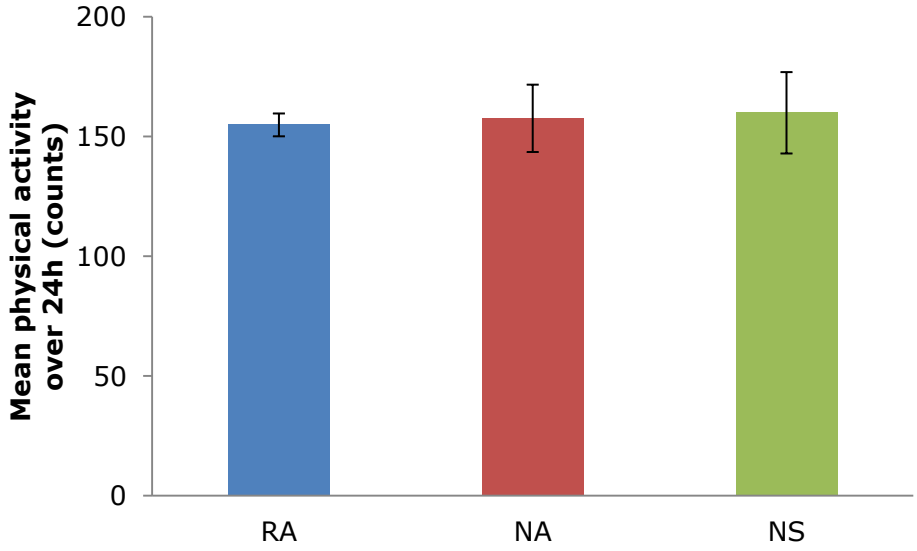

Figure 3.7: Mean physical activity measured in offspring over a 24h period at 1.5 months of age.
Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RA, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NA, red, n=7) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS, green, n=13). Data are means and error bars represent SEM.

3.3.3. Offspring at puberty

The differences in body weight, present at birth and through the lactation period, did not persist into adulthood when offspring were maintained on the same diet and in the same environment after weaning. Differences in offspring body weight that were present at weaning (3 months of age) did not persist after 5 months of age and all offspring are of similar weight between 6 and 12 months of age. Offspring raised in a restricted environment showed a higher body weight from 14 months of age than those raised in a free-living environment regardless of maternal intake in late gestation and postnatal feeding before weaning (Figure 3.8).

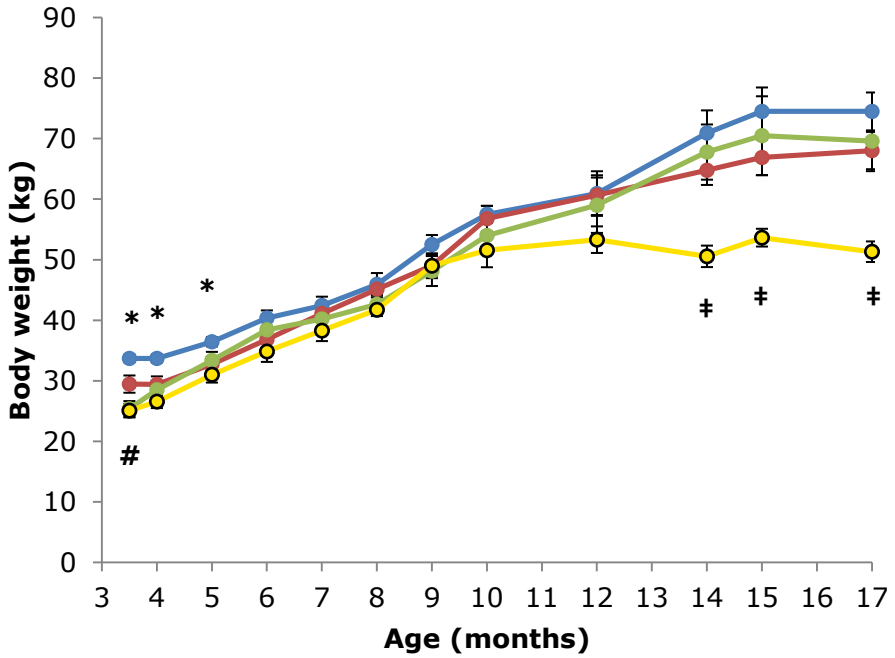

Figure 3.8: Offspring growth after weaning, divided by groups.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=9).

*** p<0.05 for RAO versus NAO; # p<0.05 for NAO versus NSO; ‡ p<0.05 for NSO versus NSL. Data are means and error bars represent SEM.**

At 7 months of age, plasma cortisol, leptin and triglyceride concentrations were not influenced by intervention group and NEFA concentrations were slightly lower in NAO as compared to RAO (Table 3.3).

At the same age, a glucose tolerance test (GTT) was carried out with the main finding that NSL offspring had a lower plasma glucose concentration as a response to the glucose injection compared to NSO offspring (Figure 3.9).

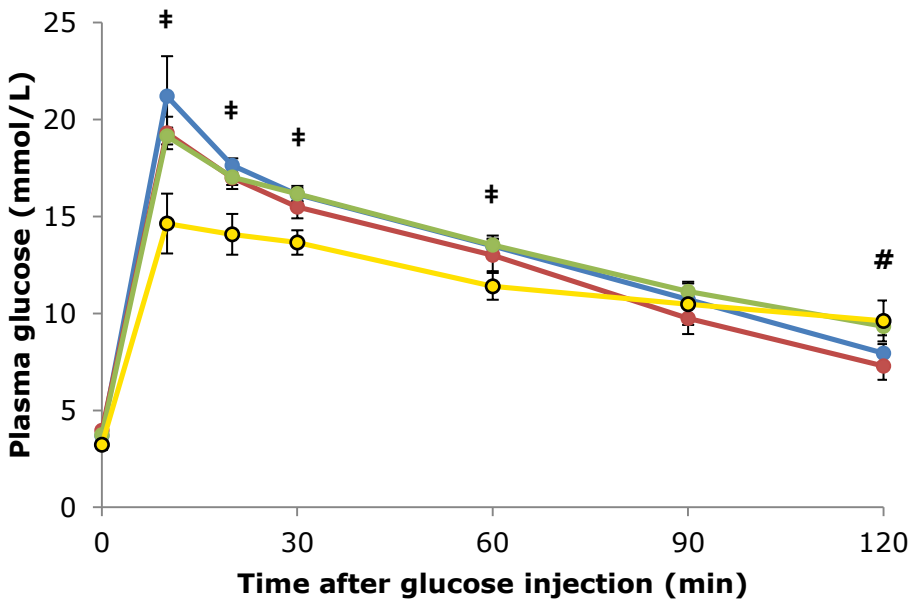

Figure 3.9: Plasma glucose concentrations during a glucose tolerance test conducted in offspring at 7 months of age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=5). # $p<0.05$ for NAO versus NSO; ‡ $p<0.05$ for NSO versus NSL. Data are means and error bars represent SEM.

Plasma insulin concentrations in the NAO group had a high inter-individual variance, but the data overall suggested that plasma insulin concentrations were highest in offspring which had experienced the NAO interventions (Figure 3.10).

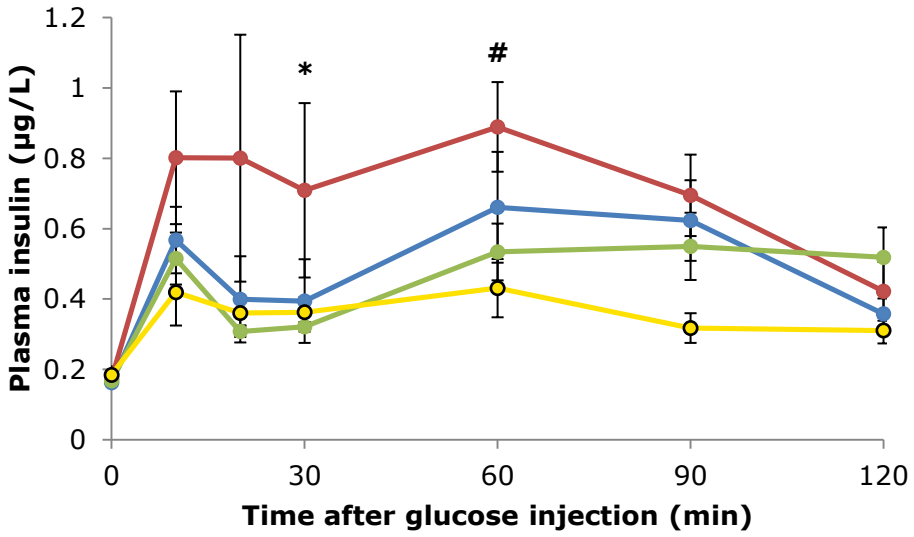

Figure 3.10: Plasma insulin concentrations during a glucose tolerance test conducted in offspring at 7 months of age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, green, n=8) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=9). * p<0.05 for RAO versus NAO; # p<0.05 for NAO versus NSO. Data are means and error bars represent SEM.

These differences in glucose between NSO and NSL and in insulin between NAO and NSO are reflected in significant differences in the area under the curve of their plasma concentrations (Table 3.4).

Table 3.4: Plasma concentrations of glucose and insulin in offspring at 7 months of age over the course of the GTT. Glucose and insulin are expressed as the area under the curve over the course of the 120 min GTT and a homeostatic model assessment for insulin resistance (HOMA-IR) calculated by multiplication of fasted glucose and insulin concentrations.

Plasma conc.	RAO	NAO	NSO	NSL
Glucose AUC (mmol/L)	1167 ± 46	1070 ± 74	1252 ± 93 [a]	1015 ± 77 [b]
Insulin AUC (µg/L)	45 ± 12	67 ± 13 [c]	37 ± 7 [d]	23 ± 6
HOMA-IR	0.61 ± 0.05	0.66 ± 0.07	0.52 ± 0.08	0.70 ± 0.21

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, n=8) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, n=9). AUC, area under the curve. Comparison a versus b, p=0.048; c versus d, p=0.040. Data are displayed as means ± SEM.

3.3.4. Adult offspring

Body weight, fat and lean mass did not differ between offspring raised in the same restricted environment. Body weight and absolute fat mass but not relative fat mass were higher in NSO as compared to NSL. Relative visceral fat mass, a sum of omental, perirenal and pericardial fat mass expressed relative to body weight, was lower in NSL but was not different between offspring raised in a restricted environment (Table 3.5).

Table 3.5: Body and organ weight and body composition of offspring at 16-17 months of age. Body composition was measured by DXA scan at 16 months of age and expressed relative to the weight at that age. Offspring were dissected at 17 months of age and visceral fat (as a sum of omental, perirenal and pericardial fat mass), liver, heart and pancreas weight recorded. Adipocyte size was measured in histological sections of omental adipose tissue.

		RAO	NAO	NSO	NSL
Weight (16 mo)	kg	67.7 ± 3.9	59.2 ± 2.7	59.2 ± 4.3 [a]	46.8 ± 1.2 [b]
Lean mass	kg	53.5 ± 5.1	42.1 ± 3.4	45.5 ± 4.4	39.4 ± 1.5
	%	78.0 ± 3.7	71.1 ± 4.3	76.2 ± 2.4	84.0 ± 1.9
Fat mass	kg	14.4 ± 2.1	17.1 ± 2.8	13.7 ± 1.1 [e]	7.5 ± 0.9 [f]
	%	22.0 ± 3.7	28.8 ± 4.3	23.8 ± 2.4	16.0 ± 1.9
Weight (17 mo)	kg	74.5 ± 3.1	68.0 ± 3.1	69.6 ± 5.0 [c]	51.3 ± 1.7 [d]
Visceral fat	kg	2.6 ± 0.3	2.6 ± 0.6	2.5 ± 0.4 [g]	0.5 ± 0.1 [h]
	%	3.5 ± 0.5	3.8 ± 1.0	4.1 ± 0.5 [i]	1.0 ± 0.2 [j]
Adipocyte size (μm^2*1000)		4.8 ± 0.4	6.1 ± 0.9	5.5 ± 1.1 [k]	2.1 ± 0.2 [l]
Liver	%	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Heart	%	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Pancreas	%	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, n=8) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, n=9). Comparison a versus b, p=0.025; c versus d, p=0.009; e versus f, p=0.003; g versus h, p<0.001; i versus j, p<0.001. Data is displayed as means ± SEM.

At 15 months of age, physical activity was similar between the groups maintained in an obesogenic environment but nearly three times higher in NSL offspring (Figure 3.11).

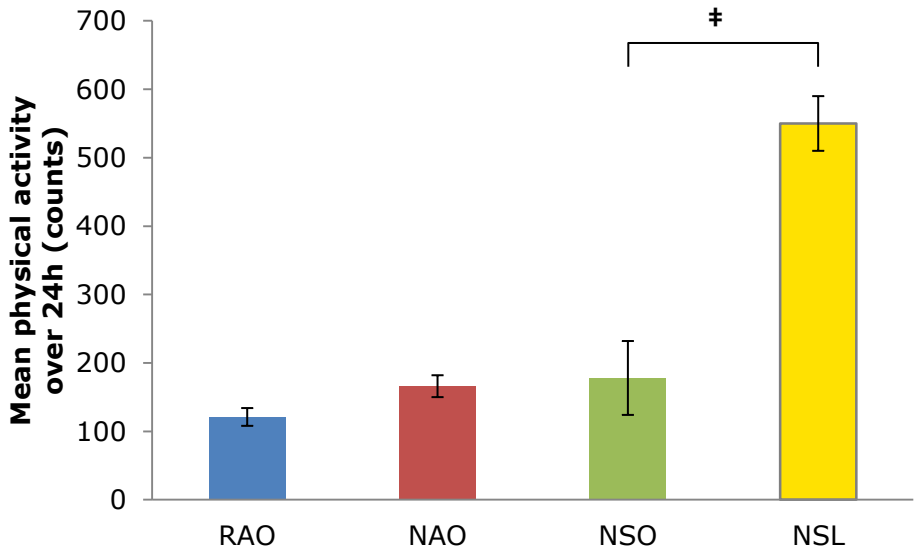

Figure 3.11: Mean physical activity measured over a 24h period at 15 months of age in all offspring.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, green, n=6) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7). ‡ $p<0.01$ for NSO versus NSL. Data are means and error bars represent SEM.

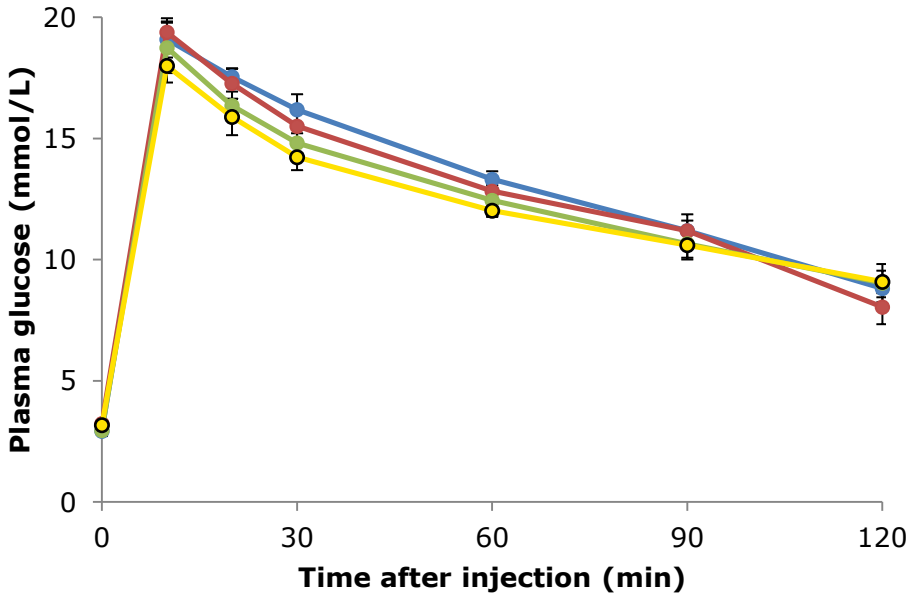

Figure 3.12: Plasma glucose concentrations during a glucose tolerance test conducted in offspring at 16 months of age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=6), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, green, n=6) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=8). Data are means and error bars represent SEM.

Plasma insulin differed during the GTT, with a higher insulin response in NAO as compared to RAO and a higher response in NSO as compared to NSL at 10 and 30 minutes after glucose injection (Figure 3.13).

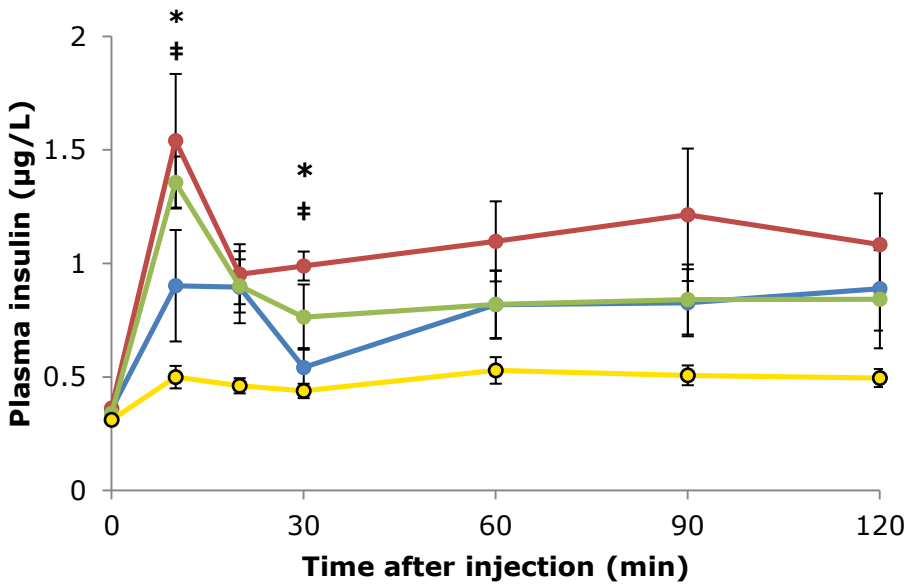

Figure 3.13: Plasma insulin concentrations during a glucose tolerance test conducted in offspring at 16 months of age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, green, n=6) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=9).

*** p<0.05 for RAO versus NAO; ‡ p<0.05 for NSO versus NSL. Data are means and error bars represent SEM.**

Over the course of the GTT, plasma glucose area under the curve (AUC) did not differ between groups, insulin AUC was higher in NAO as compared to RAO and the HOMA-IR was higher in NAO as compared to both RAO and NSO (Table 3.6).

Table 3.6: Plasma concentrations of glucose and insulin in offspring at 16 months of age over the course of the GTT. Glucose and insulin are expressed as the area under the curve over the course of the 120 min GTT and a HOMA-IR calculated by multiplication of fasted glucose and insulin concentrations.

Plasma conc.	RAO	NAO	NSO	NSL
Glucose AUC (mmol/L)	1302 ± 36	1226 ± 60	1215 ± 78	1148 ± 46
Insulin AUC (µg/L)	53 ± 14 [a]	94 ± 14 [b]	67 ± 15	22 ± 5
HOMA-IR	0.92 ± 0.08 [c]	1.17 ± 0.05 [d]	1.00 ± 0.06 [e]	0.99 ± 0.03

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, n=8) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, n=9).

Comparison a versus b, p=0.022; c versus d, p=0.001; d versus e, p=0.008. Data is displayed as means ± SEM.

3.3.4.1. Metabolome in adult offspring plasma

In adult offspring plasma, 2124 ([*m/z*; *rt*]) features were found and the three intervention groups were clearly separated on the OSC-PLS-DA score plot (Figure 3.14).

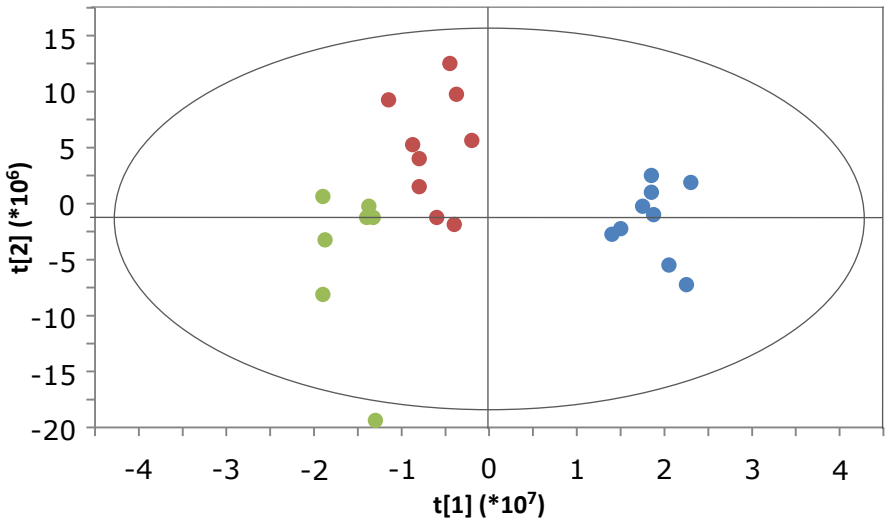

Figure 3.14: OSC-PLS-DA score plot to show overall differences between offspring plasma metabolome at 16 months of age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NSO, green, n=7). After weaning, offspring were maintained in a restricted environment. There is no data available for NSL offspring.

Validation parameters: R^2X (cum)=0.771, R^2Y (cum)=0.573, permutation test (n=100) with R^2 intercept=0.285 and Q^2 intercept=-0.272, ANOVA-p-value=0.012.

Of the 2124 compounds, 22 showed a difference (t-test with $p<0.05$) in concentration between RAO and NAO and 4 showed a difference between NAO and NSO. However, only 1 compound, citrulline, could be identified with authentic standards (Table 3.7), which had a higher concentration in NAO as compared to RAO. Valine had a trend to be higher in NAO as compared to RAO ($p=0.065$).

Table 3.7: List of metabolites which were significantly changed or had the trend to be altered (0.05<p<0.07) in offspring plasma at 16 months of age. A positive fold change indicates that the compound has a higher concentration in NA offspring compared to RA and NS compared to NA, whilst a negative change fold indicates a higher concentration in NAO compared to NSO.

				NAO/RAO		NSO/NAO	
Compound	**RT**	**M**	**$[M+H]^+$ observed**	**FC**	**p**	**FC**	**p**
Citrulline	2.26	175.0957	176.1029	1.79	0.034	1.14	0.6
Valine	2.36	117.0798	118.0863	1.53	0.065	-1.02	0.9

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NSO, n=7). After weaning, offspring were maintained in a restricted environment. There is no data available for NSL offspring. RT, retention time; M, monoisotopic mass; $[M+H]^+$, mass with one additional hydrogen atom; FC, fold change; p, p-value; all compounds were identified using authentic standards.

3.4. Discussion

Maternal nutrient restriction in late gestation has acute effects on the maternal metabolic profile and maternal and fetal growth and has long term consequences for the offsprings' glucose tolerance and insulin secretion, especially when it is followed by an accelerated growth rate in early postnatal life.

3.4.1. Nutrient restriction induces a change in maternal metabolome

The metabolic changes seen in mothers at 130 days gestation, 20 days into the intervention, are consistent with mild food restriction. During food withdrawal, energy is provided from muscle, liver and adipose tissue and proteolysis enables continuance of synthesis of essential proteins [424]. This catabolic state is mediated through a decrease in insulin and increase in plasma glucagon and glucocorticoid concentrations as plasma concentrations of nutrients are reduced [425, 426]. Amino acids are broken down from liver and muscle and used for ATP production and to synthesise essential proteins to keep up physiological functions [426, 427]. Triglycerides from adipose tissue are lysed to NEFA and glycerol, which are utilised in ß-oxidation and transformed to ketone bodies and glucose. Hepatic glycogen is transformed to glucose and released into the blood stream. As the brain is dependent on the availability of glucose and ketone bodies [428], these metabolic adaptations are crucial for survival. These changes are indeed seen in N mothers as concentrations of amino acids (leucine, proline, tryptophane, pyroglutamic acid, glycine and phenylalanine), fatty acids (total NEFA and isobutyric acid) and the mitochondrial fatty acid transporter carnitine are increased whilst glucose

concentrations are decreased. For technical reasons, plasma concentrations of ketone bodies were not assessed in the metabolome analysis but it is likely that they were also increased. A decrease in plasma insulin was not observed but this may be due to the relatively mild form of nutrient restrictions used, which does not induce metabolic changes as markedly as stronger, or more prolonged, reduction in food availability would be expected to do.

N mothers did not undergo significant weight gain in late gestation during the intervention and offspring were smaller at birth. Since nutrients pass the barrier between maternal and fetal blood stream in the placenta, it can be assumed that their fetuses were also subjected to a decrease in plasma glucose and increase in NEFA and amino acids. Hormones, with exception of cortisol, do not pass from the mother to the fetus but are produced by the placenta and the fetus itself. The decrease in maternal plasma glucose could, therefore, cause a lower secretion of insulin in the fetus, induce gluconeogenesis [330, 332] or alter development of ß-cells [298, 323] as these changes have been described in other studies with decreased fetal glucose as an intervention to induce IUGR. These acute changes could, therefore, have long term effects on the offsprings' glucose tolerance.

3.4.2. Offspring born to nutrient restricted mothers have a higher growth rate in early postnatal life but perinatal growth rates do not influence adult weight

Early postnatal growth is influenced by food availability, which explains the difference in growth rates from birth through lactation between NA and NS offspring. As was aimed for with this intervention, growth rates in offspring

having to share maternal milk were not as high as in those offspring who were separated from their twin (i.e. were raised singly). Offsprings' milk intake, although not measured in this study, can be assumed to be higher in NA offspring as compared to NS as they grew faster.

The difference in fetal growth rates in late gestation also influenced early postnatal growth rate, as NA offspring show a higher fold change in body weight from birth through lactation as compared to the RA group. This suggests that the low fetal growth rate in the period of late pregnancy established potential for a faster postnatal growth, when permitted by food availability. However, adult obese N offspring did not become heavier than R offspring, indicating that they "caught up" in body weight but did not overshoot the expected growth trajectory as represented by RAO offspring. After weaning when all offspring were maintained in the same, restricted environment, body weight was the same between groups within 3 months. From studies of the Dutch famine birth cohorts, it would not have been expected that maternal undernutrition in late pregnancy would lead to higher obesity rates or higher body mass index (BMI) in adult offspring as subjects affected by the famine in late pregnancy were lighter at 19 years of age as compared to unaffected individuals [235] and had normal rates of adiposity at 50 [233] and 58 years of life [229]. However, these individuals were not subject to an obesogenic environment and results cannot be directly related to my study. However, sheep studies inducing nutrient restriction in late pregnancy showed a normal adult weight in the offspring when raised in an obesogenic environment [429].

It would have been consistent with human studies [243, 250-253] if an accelerated early postnatal growth rate had been associated with a higher adult body weight, i.e. for NAO offspring to be heavier than NSO at 17 months, but this was not the case. In my study, neither growth rate in late

pregnancy or in early postnatal life resulted in changes in body weight or body composition of the resulting adults, further suggesting that offspring were well adapted to these challenges.

At a late pubertal age of 10 months, animals maintained in the unrestricted environment kept a steady lean body weight of about 50kg until tissues were sampled at 17 months whilst all animals in the restricted environment continued gaining weight until about 15 months of age and then maintained weights of 65-75kg, i.e. 1.3-1.5 fold the weight of lean animals. Even allowing for the fact that there is no precise definition of sheep obesity, this difference seems large enough to designate these animals 'obese'. In existing literature, sheep ranging from 1.2 [429, 430] up to 2 fold [431] the body weight of their lean control groups are considered 'obese'. The 'obesity' induced in this study was deliberately limited by restricting physical activity and not, in addition, feeding animals to excess. Since the design of this study aimed to measure the effects of perinatal interventions on adiposity and its related diseases and as I postulate that these effects would be rather small, a too severe method of inducing obesity might have overpowered the effects I primarily wanted to investigate. For example, if excess obesity led to substantial ectopic fat accumulation this may have induced insulin resistance in all groups and the mechanism underlying the IUGR-induced insulin resistance would have presented less clearly.

Total fat masses, both as measured by DXA scan and as mass of dissected visceral fat tissues, were higher in 'obese' animals as compared to 'lean' animals. However, relative fat and lean mass were very similar between all groups, independent of the environment in which they were maintained. Relative dissected visceral fat was still considerably higher in 'obese' groups than in NSL offspring. This is important as visceral adiposity is

thought to specifically increase risk of developing metabolic diseases in humans and makes up one of the components of the metabolic syndrome [40, 432].

3.4.3. Metabolites and glucose tolerance in post weaning offspring are influenced by maternal nutrient restriction

At 3 months, none of the metabolites and hormones measured differed between groups but since the growth rates maintained before this measurement could all be considered as physiological, a difference in metabolites and hormones would not have been expected. This is also true for a majority of plasma measurements carried out at 7 months of age.

During the GTT at 7 months of age, plasma glucose concentrations in NSL offspring were lower at most time points as was the overall area under the curve. Since body weights at 7 months of age of NSL offspring were not lower than those of NSO offspring, this is most likely an effect of the higher degree of physical activity in NSL offspring, due to the lower number of animals housed in a given area. Physical activity is, in humans, both used as a preventive [433] and as a therapeutic intervention [434] against insulin resistance.

NAO offspring had the highest plasma insulin after glucose injection, and the AUC reached statistical significance compared to NSO offspring. Increased pancreatic excretion of insulin has been described as an early stage of developing impaired glucose tolerance [435], which characteristically has a slow onset before ß-cell function and insulin secretion decreases and postprandial and fasted plasma glucose concentrations increase in susceptible individuals [435].

As the 16 month old offspring body weight and body composition were not different between the obese groups, the difference seen during the GTT in insulin secretion between RAO and NAO animals is not due to differences in fat mass by itself. This is consistent with the findings from the Dutch famine follow-up studies. NSL offspring show a much lower insulin response, as they already had at 7 months of age, now due to both a higher physical activity and a lower body weight as compared to the obese offspring.

Interestingly, in the obese adult sheep, both NEFA and TG were not significantly higher than in lean animals and differed only slightly between perinatal intervention groups with a higher TG concentration in RAO as compared to NAO animals. Higher circulating NEFA and TG concentrations are thought to be major factors linking obesity with insulin resistance in humans [231, 436]. In humans and rodents, visceral fat is associated with increased circulating lipid concentrations [437, 438], which can lead to higher hepatic and pancreatic TG content and a subsequent decrease in insulin sensitivity [59, 439, 440]. As NEFA and TG were not strikingly different between any groups irrespective of current body weight, it is unlikely that insulin resistance is mediated through ectopic fat accumulation. Indeed, in a human study, individuals born small for gestational age (SGA) had reduced insulin sensitivity in liver and muscle but this could not be attributed to increases in fat deposition in these organs [59, 334], supporting the conclusion that ectopic fat accumulation is not the mechanism mediating insulin resistance after late gestational IUGR.

Not only is the insulin AUC in response to glucose highest in NAO and NSO but also the baseline measure, HOMA-IR, calculated from fasted plasma glucose and fasted insulin concentrations, is higher in NAO than in both

RAO and NSO, although fasted plasma glucose and insulin concentrations by themselves did not differ significantly. This suggests that NAO animals of this age show a dysfunction of the glucose regulatory system that is not only affected in the postprandial state but which also expands to a dysfunction during the fasted state [441]. The glucose tolerance of RAO offspring may, therefore, have further deteriorated compared to that at 7 months of age. Keeping in mind that the animals are still at a young adult age at the time of the later GTT, this is a striking finding.

Since in both the NAO and NSO animals neither the amount of white adipose tissue (WAT) nor the circulating lipid concentrations were altered, insulin sensitivity and/or insulin secretion must have been influenced in other ways. It is likely that the change in maternal plasma glucose concentrations accompanying nutrient restriction causes a long term change of glucose regulation. Both maternal hyperglycaemia and hypoglycaemia lead to disturbed offspring glucose tolerance but through different mechanistic pathways [442]. Since most of the mechanistic studies were conducted in rodents it is still unclear which mechanisms exactly lead to adult insulin resistance in, for example, humans and sheep. One of the mechanisms suggested in rodents is a permanent loss of pancreatic tissue. Unlike a study inducing hypoglycemia in late gestation through more severe nutrient restriction [325], the maternal nutrient restriction in this study did not lead to lower offspring pancreatic weight at dissection at 17 months of age. However, since we saw an increase of insulin secretion during the GTT, this does not suggest a dysfunction of insulin secretion but of peripheral glucose tolerance.

Disturbance of peripheral glucose tolerance can result from both a permanent increase in gluconeogenesis and from a decrease in glucose uptake by skeletal muscle, adipose tissue and liver. Fetuses of

normoglycemic mothers do not induce hepatic gluconeogenesis [273], but since fetuses subjected to hypoglycemia have been shown to express key enzymes of gluconeogenesis [330-332], it is possible that this pathway is permanently altered in these animals. To assess this component of glucose tolerance, a hyperinsulinemic, euglycemic clamp test carried out in the adult animals would have been ideal. Even though HOMA-IR can be used as an approximation of the overall glucose tolerance, the analysis of a clamp study allows calculation of glucose production rates [41, 79].

Both skeletal muscle and white adipose tissue of these animals have been examined but not specifically with regards to insulin resistance. With expanding WAT, monocytes are increasingly attracted to the tissue, forming crown-like structures and excreting inflammatory cytokines which, in turn, further decrease insulin sensitivity of muscle and WAT itself [41, 443]. Distinguishing between hyperplastic and hypertrophic adipocytes and identifying the number of crown-like structures could indicate a cause of insulin resistance. In histological analysis, adipocyte size could not be shown to be different between obese groups and an analysis of crown-like structures is now being undertaken. Seeing that the circulating leptin concentrations were higher in RAO than in NAO despite similar body fat mass suggests that WAT of RAO animals has a different phenotype concerning endocrine secretion, which may be accompanied by, for example, a difference in inflammatory profile.

Whilst I attempted to measure proinflammatory cytokines interleukin (IL)-6 and tumour necrosis factor (TNF) in plasma samples, concentrations higher than the negative control were not detected, possibly due to the very short half-life of these cytokines [444, 445].

Changes in basal endocrine and immunological state could be indirectly represented in the metabolomic fingerprint measured in the adult obese offspring. Specifically RAO and NAO differ from each other, with 22 compounds differing in quantity, showing that the maternal nutrient restriction had a more general effect than the early postnatal growth rate with only 4 compounds significantly differing between NAO and NSO. Due to technical limitations, only one of the 22 compounds could be identified, citrulline, whilst none of the 4 compounds affected by postnatal growth rate could be identified.

Citrulline is an amino acid which is part of the urea cycle and also accrues from arginine and oxygen in biosynthesis of the signalling molecule nitrogen oxide (NO). Citrulline, secondary to arginine and NO, may be linked to diabetes [446, 447] but the amino acid concentrations in plasma of adult offspring would have to be investigated more closely to allow any conclusions with regards to this biological system.

The overall difference in the metabolomic fingerprint, approximated by the number of compounds differing in quantity between groups, is similar in extent to the difference in overall metabolic changes, e.g. plasma leptin concentrations and change in glucose tolerance. In order to have a better basis of interpreting the changes in single metabolites, it is necessary to have a higher rate of positive identification, as is achieved by comparing plasma LC-MS results with results for pure standards. In the current analysis, only about 10% of metabolites could be identified and this does not allow me to draw extensive conclusions about mechanisms involved in these metabolic changes.

3.5. Conclusions

As is consistent with other studies, nutrient restriction in late pregnancy leads to a decrease in maternal plasma glucose concentrations, a difference in birth weight and early postnatal growth rates of the offspring but not to a change in offspring adult body weight and composition. Nevertheless, glucose tolerance is decreased in these offspring, with an earlier onset in offspring subjected to an accelerated growth rate in early postnatal life as compared to offspring subjected to a standard growth rate. These changes may be mediated by long term adaptations in pancreas, liver, muscle and white adipose tissue to the hypoglycemia experienced in utero. Potential mechanisms are summarised in Figure 3.15.

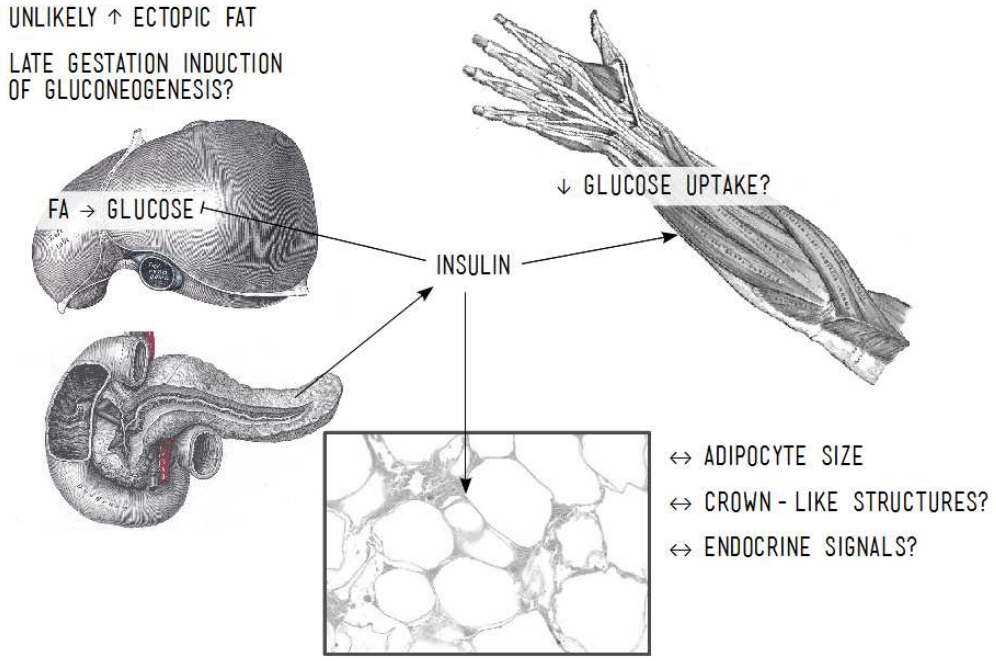

Figure 3.15: Insulin resistance develops in offspring after maternal nutrient restriction. Insulin secretion from the pancreas is increased, suggesting peripheral insulin resistance. This could be due to insufficient capacity to inhibit hepatic glucose production or due to reduced glucose uptake in muscle and white adipose tissue. Plasma leptin concentration is increased and this may indicate further changes in the endocrine secretion of white adipose tissue.

4. The influence of perinatal interventions on food intake and the appetite and cortisol regulatory systems in the hypothalamus

4.1. Introduction and hypotheses

This chapter further explores the study introduced in Chapter 3 with focus on the long term consequences of accelerated perinatal growth on the offsprings' appetite regulation. The studies around the Dutch famine cohort introduced earlier did not find an effect of famine in late pregnancy on offsprings' adult body weight or food intake preferences [234]. In contrast, individuals who were born with low birth weight but gained weight quickly in the early postnatal period are more likely to be overweight and diabetic as adults [243, 244]. Therefore, it appears that rapid early neonatal growth, not birth weight alone, has long term consequences for body weight regulation. Growth restriction during fetal life can lead to further changes, which are independent of alteration of body weight. Animal studies with larger species, i.e. not rodent studies, have not extensively investigated this possible mechanism. To assess this, offspring food intake and postprandial dynamics of hormones and metabolites were measured and hypothalamic gene expression analysed after perinatal interventions including maternal macronutrient restriction in late gestation and accelerated early postnatal growth.

1) As I have already found that there were no differences in adult body weight and physical activity between groups, I hypothesised that food intake would not be changed significantly.
2) The insulin resistance seen in the adult glucose tolerance test (GTT) of NAO offspring (offspring whose mothers were subjected to nutrient restriction in late pregnancy, followed by accelerated growth in early postnatal life and an obesogenic environment after weaning) was hypothesised to affect the hypothalamus, since peripheral and central insulin sensitivity are related [448]. Central insulin and leptin resistance may be mediated through an upregulation of suppressor of cytokine signalling 3 (SOCS3) and protein tyrosine phosphatase, non-receptor type 1 (PTP1B) and/or a down regulation of insulin receptor (IR) and leptin receptor (obRb).
3) However, since plasma insulin and leptin concentrations were higher in NAO animals, these may compensate for insulin resistance and overall there may be an anorexigenic effect in the primary and secondary neurons of the arcuate nucleus (ARC), i.e. an upregulation of pro-opio melanocortin (POMC) and melanocortin receptors 3 and 4 (MC3R/MC4R) and/or a down regulation of neuropeptide Y (NPY).
4) Lastly, since plasma cortisol concentrations were not significantly changed, I hypothesised that there would be no change in hypothalamic expression of genes involved in the regulation of cortisol secretion from the adrenal glands.

4.2. Methods

The study design in this part of the study was as described in Chapter 2. In brief, 4 groups were created from combinations of the 3 intervention periods: mothers were fed to requirements (R) or nutrient restriction in late pregnancy (N), offspring were subjected to accelerated (A) or standard (S) early postnatal growth rate and, after weaning, offspring were maintained in either an environment designed to induce obesity(O) or one to maintain lean animals (L). Within these groups, comparisons were drawn between animals if their interventions differed in only one factor, i.e. RAO vs NAO, NAO vs NSO and NSO vs NSL, allowing separate assessments of the impacts of maternal nutrient restriction, early postnatal growth rate and adult obesity, respectively.

At 16 months of age, offspring were separated into single housing and ad libitum intake of their standard feed, i.e. straw nuts and high-energy pellets for obese animals but only straw nuts for lean animals, was recorded over 10 days. The difference of feed used in this test was due to the difference in environment they were in as lean animals only had access to grass and a mineral stone after weaning. Adding high-energy pellets to their diet for this test would have misrepresented their normal food intake. However, due to this difference, food intake between lean and obese animals is not directly comparable.

Additionally, after an overnight fast of 18h, ad libitum food intake was observed for 24h in short intervals of 0-2h, 2-4h, 4-8h and 8-24h after start of feeding (9 a.m.) and blood samples were taken at each of these time points. Feed was presented in the morning and remained available throughout the 24h period. This shorter feeding test was also confounded

by the use of different feeds for lean and obese animals as described above. Blood samples were analysed for concentrations of glucose, non-esterified fatty acids (NEFA), insulin, leptin and cortisol. The blood sample taken at time point 0h is a fasted morning sample, before feeding starts, and the sample taken at 24h is a morning plasma sample taken in the fed state. The 10 day feeding test was performed to more closely represent the offsprings' normal feeding behaviour as it was averaged over a longer time period.

Finally, offspring were humanely euthanased and dissected at 17 months and expression of genes involved in hypothalamic appetite regulation, insulin and leptin resistance and cortisol regulation was assessed.

4.3. Results

4.3.1. Feeding test

At 16 months of age, food intake was measured over 10 days and averaged to food intake per day. Food intake of NSL offspring was statistically lower than NSO (Figure 4.1).

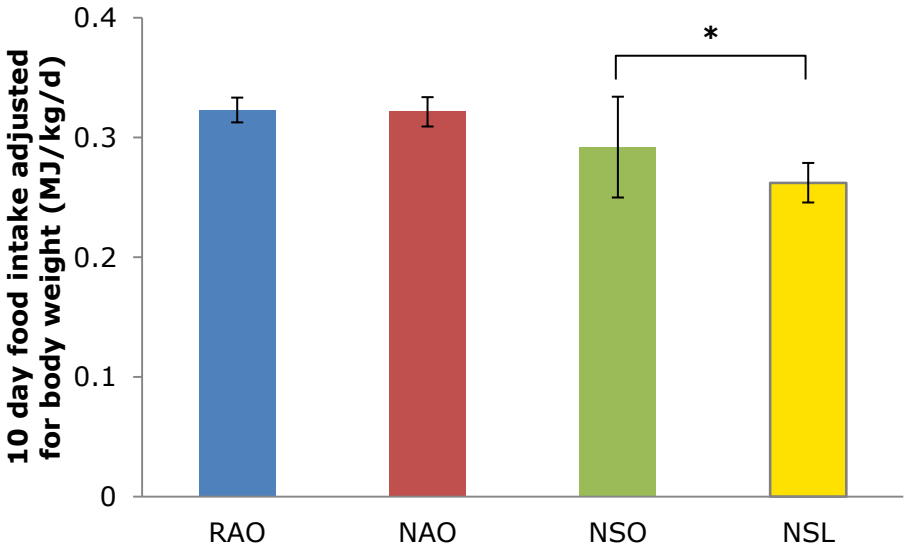

Figure 4.1: Food intake adjusted for body weight in adult offspring averaged to intake per day from measurements over a 10 day period at 16 months of age.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=9) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=8) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=9).

*** p<0.05. Data are means and error bars represent SEM.**

For the analysis of postprandial dynamics of plasma hormones and metabolites, food intake was measured over a 24h period after an overnight fast accompanied by blood sampling. In this 24h measurement, food intake was lower in NAO as compared to both RAO and NSO and lower in NSL than NSO (Figure 4.2).

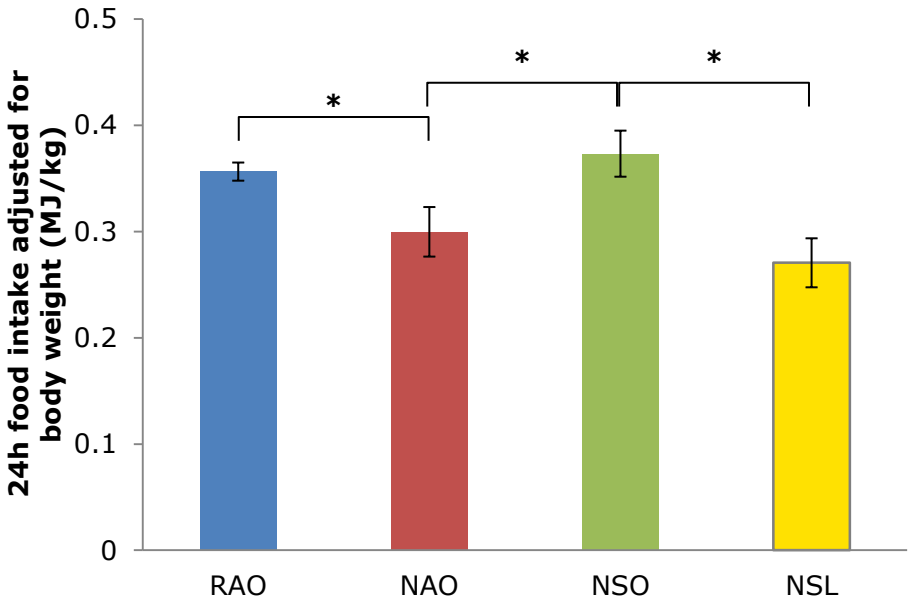

Figure 4.2: Offspring total food intake at 16 months of age over 24h after an overnight fast, adjusted for body weight.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7).

*** $p<0.05$. Data are means and error bars represent SEM.**

Most food intake occurred in the first 2h after feeding with a lower food intake in NSL compared to NSO and a slightly higher intake in NSO as compared to NAO at 4 to 8h after feeding (Table 4.1).

Table 4.1: Offspring food intake at 16 months of age over the course of 24h after an overnight fast and adjusted for body weight.

Food intake (MJ/kg)	RAO	NAO	NSO	NSL
0-2h	0.17 ± 0.01	0.16 ± 0.01	0.19 ± 0.02 [a]	0.10 ± 0.01 [b]
2-4h	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
4-8h	0.08 ± 0.01	0.06 ± 0.01 [c]	0.09 ± 0.01 [d]	0.07 ± 0.01
8-24h	0.10 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, n=7).

Comparisons a versus b, p=0.001; c versus d, p=0.044. Data are displayed as means ± SEM.

Fasting plasma leptin was higher in NAO as compared to NSO, as described in Chapter 3. NSL had reduced plasma leptin concentrations throughout the interventions and these were significantly lower than NSO at 24h (Figure 4.3). Both NAO and NSO leptin concentrations were higher at 24h (fed) than at baseline (fasted, $p<0.05$).

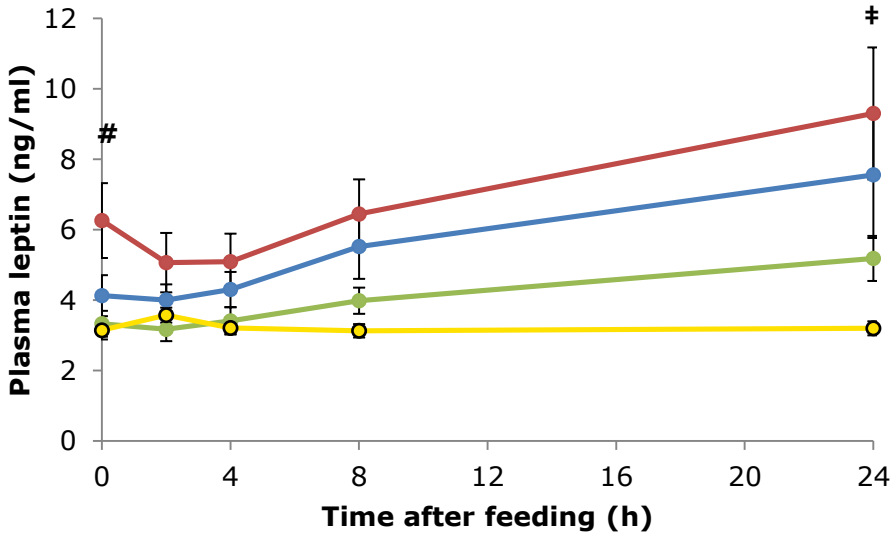

Figure 4.3: Plasma leptin concentrations over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period just before and after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7).

 # $p<0.05$ for NAO versus NSO; ‡ $p<0.05$ for NSO versus NSL. Data are means and error bars represent SEM.

When expressing leptin concentrations relative to the initial, fasted measurement to emphasise the postprandial dynamics, NAO had a fall in leptin concentrations at 2h and 4h after feeding as compared to NSO and RAO (Figure 4.4).

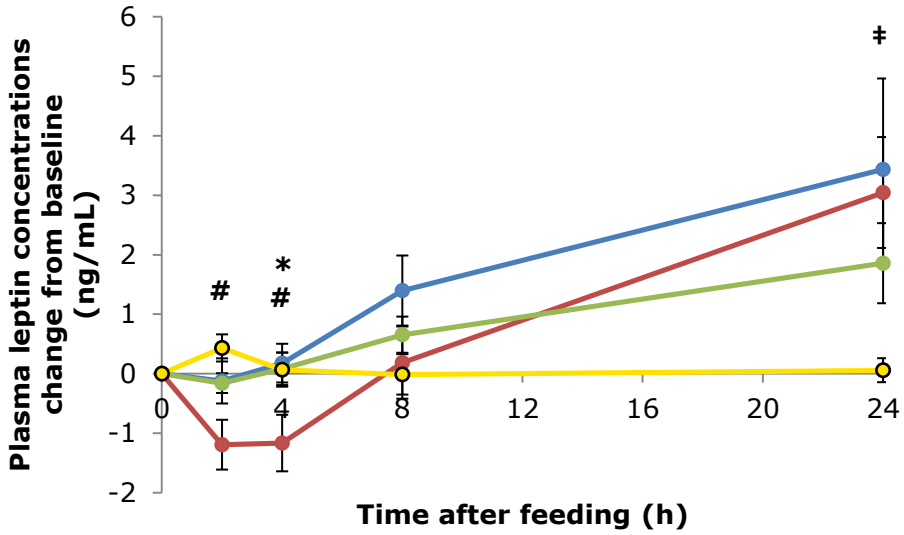

Figure 4.4: Plasma leptin concentrations relative to fasted concentration over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7).

*** p<0.05 for RAO versus NAO; # p<0.05 for NAO versus NSO; ‡ p<0.05 for NSO versus NSL. Data are means and error bars represent SEM.**

Plasma glucose concentrations were lower in NSL animals throughout and were significantly lower compared to NSO at 4h and 8h (Figure 4.5).

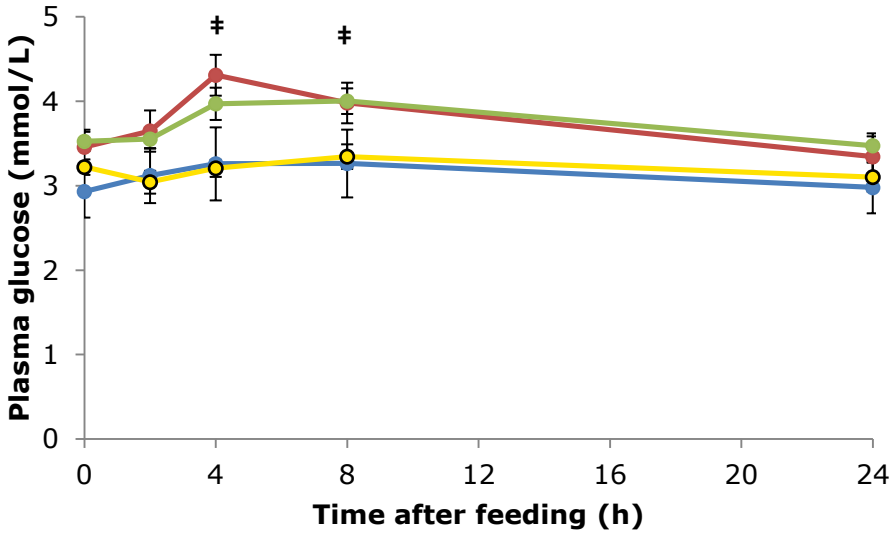

Figure 4.5: Plasma glucose concentrations over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=7), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7).

‡ $p<0.05$ for NSO versus NSL comparison. Glucose concentrations did not differ between 24h and baseline. Data are means and error bars represent SEM.

On considering postprandial dynamics relative to fasted concentrations, plasma glucose was higher in NAO as compared to RAO at 4h after feeding (Figure 4.6).

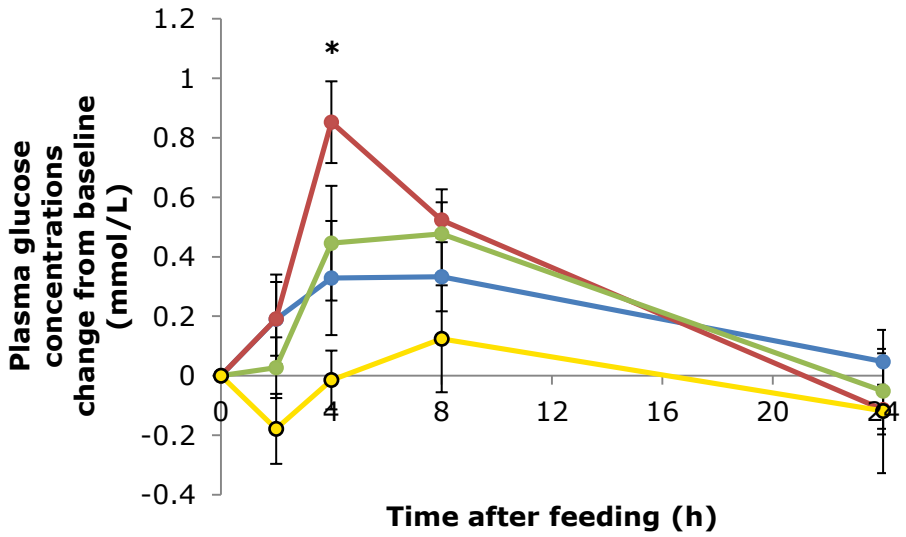

Figure 4.6: Plasma glucose concentrations relative to fasted concentration over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=7) or born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7).

*** $p<0.05$ for RAO versus NAO comparison. Data are means and error bars represent SEM.**

Similarly to the plasma glucose concentrations, NSL insulin concentrations were lower than in NSO, with significant differences at 0, 4, 8 and 24h. NSO insulin concentrations were higher at 24h (fed) than at baseline (fasted, p=0.023; Figure 4.7). Since the fasted concentrations of insulin between groups were very close together, insulin dynamics, in contrast to fasted concentrations, did not show any further differences (Figure 4.8).

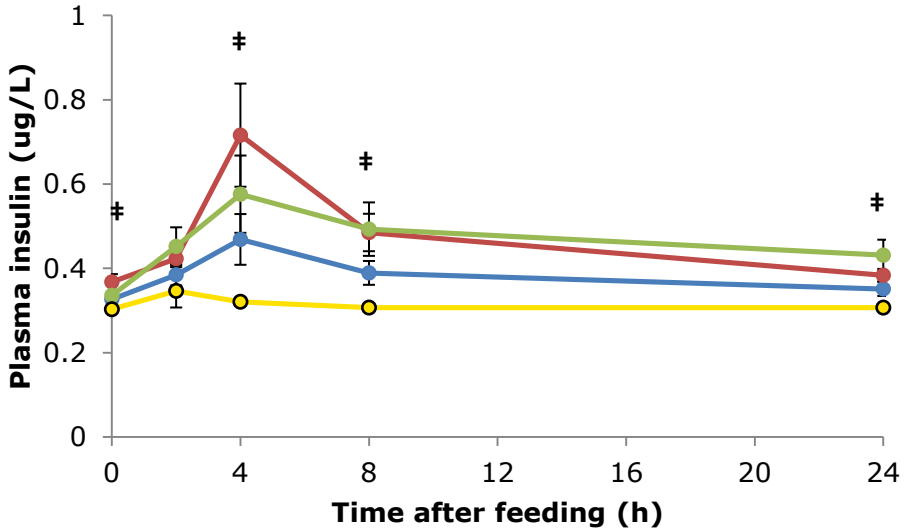

Figure 4.7: Plasma insulin concentrations over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=5).

‡ $p<0.05$ for NSO versus NSL. Data are means and error bars represent SEM.

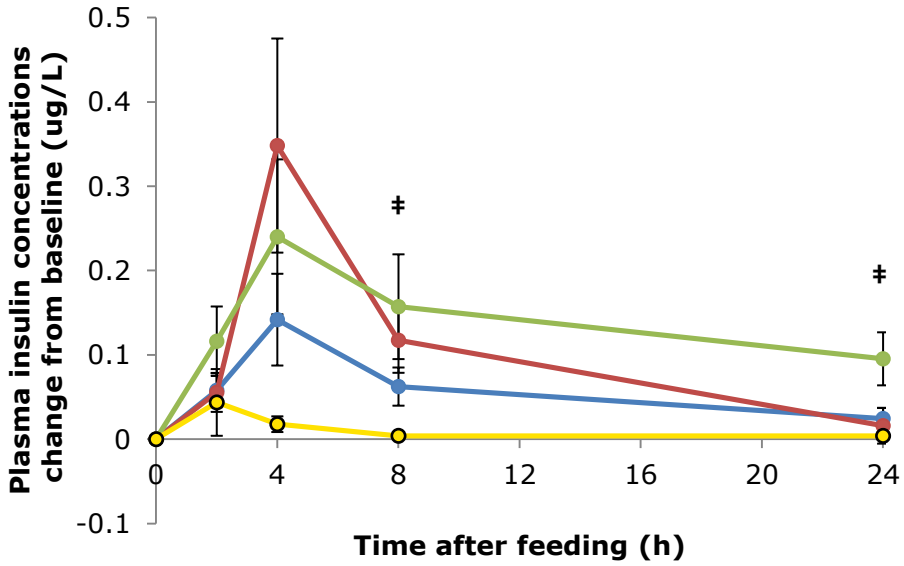

Figure 4.8: Plasma insulin concentrations relative to fasted concentration over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=5).

‡ $p<0.05$ for NSO versus NSL. Data are means and error bars represent SEM.

Plasma NEFA concentrations did not differ throughout the feeding test, both when expressed as absolute values (Figure 4.9) and when expressed relative to the baseline measurement (Figure 4.10). However, NEFA concentrations were higher at 0h before feeding (fasted) than at 24h (fed) in all groups ($p<0.05$).

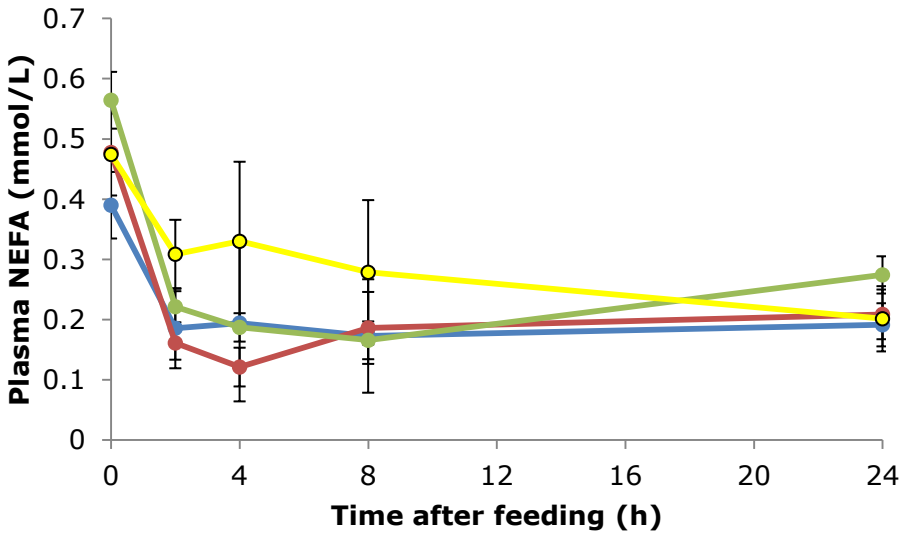

Figure 4.9: Plasma NEFA concentrations over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=7), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7). Data are means and error bars represent SEM.

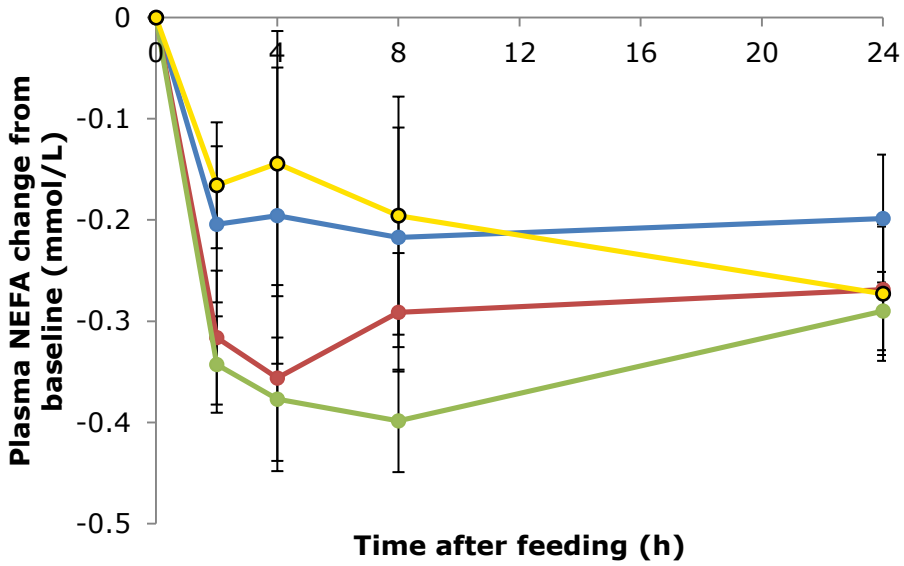

Figure 4.10: Plasma NEFA concentrations relative to fasted concentration over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=7), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7). Data are means and error bars represent SEM.

Cortisol concentrations were higher in NAO than in RAO throughout the feeding test but were only significantly different at 4h (Figure 4.11). The area under the curve was higher in NAO than in RAO, with RAO, 46 ± 42 nmol/L over 24h; NAO, 347 ± 181 nmol/L over 24h; NSO, 112 ± 37; NSL, 73 ± 22 nmol/L over 24h; RAO versus NAO, p=0.046.

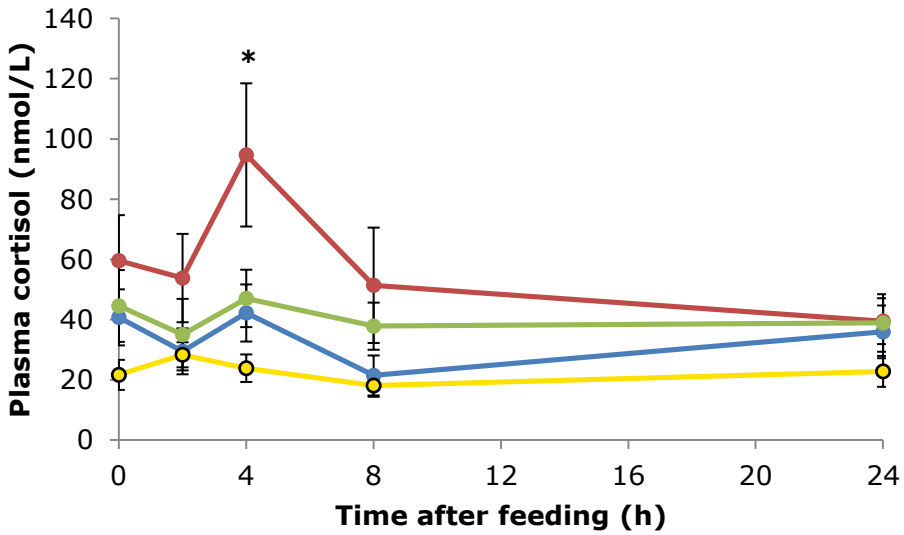

Figure 4.11: Plasma cortisol concentrations over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7).

*** p<0.05 for RAO versus NAO. Data are means and error bars represent SEM.**

When expressing plasma cortisol concentrations relative to the baseline value the difference in cortisol dynamic was diminished (Figure 4.12).

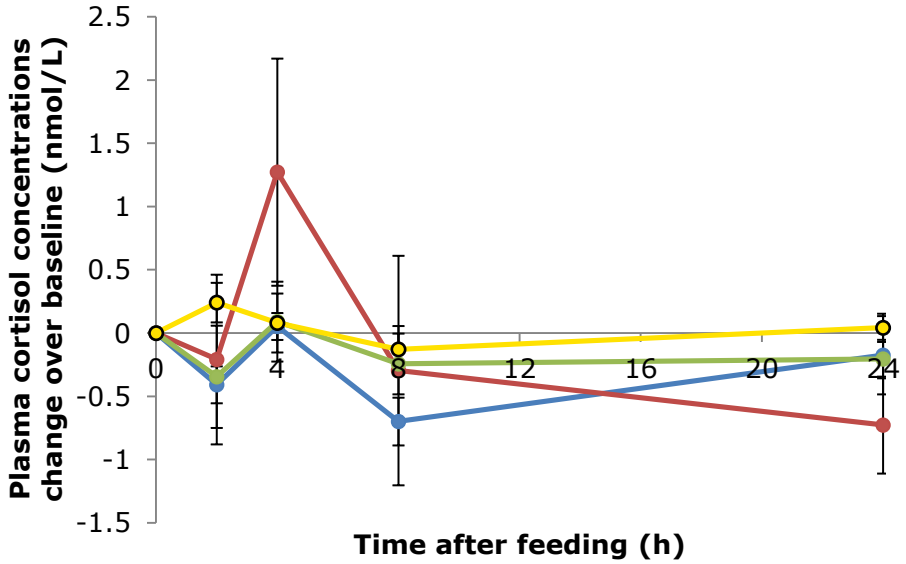

Figure 4.12: Plasma cortisol concentrations relative to fasted concentration over the course of the feeding test after an overnight fast. Blood samples were taken over a 24h period after feeding the animals.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=8), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=7). Data are means and error bars represent SEM.

4.3.2. Hypothalamic gene expression

As described in Chapter 2, the expression of genes of interest was normalised with expression of housekeeping genes. The expression of 18S was stable within and between groups and was used as the housekeeping gene for normalisation throughout. Other housekeeping genes considered were YWHAZ and RPO, which also showed a very low variation (Table 4.2) but were expressed at a lower concentration (higher Ct).

Table 4.2: Cycle threshold of housekeeping genes used. Expression of 18S was measured in all 3 dilutions of complementary DNA used for gene expression analysis. YWHAZ and RPO were only measured once. Measurements of 18S were correlated between dilutions: a versus b, R^2=0.55; b versus c, R^2=0.46; a versus c, R^2=0.44.

Ct	RAO	NAO	NSO	NSL
18S (1st dil.) [a]	13.4 ± 0.1	13.6 ± 0.2	13.3 ± 0.1	13.4 ± 0.1
18S (2nd dil.) [b]	11.8 ± 0.2	12.0 ± 0.2	11.8 ± 0.1	11.7 ± 0.2
18S (3rd dil.) [c]	11.1 ± 0.3	11.6 ± 0.1	11.3 ± 0.1	11.4 ± 0.2
YWHAZ	18.2 ± 0.1	17.6 ± 0.2	17.8 ± 0.2	18.1 ± 0.2
RPO	18.9 ± 0.2	18.9 ± 0.1	18.9 ± 0.2	19.2 ± 0.2

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, n=5), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RAO and NAO (NSO, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, n=8). Data is displayed as means ± SEM.

18S, ribosomal 18S; YWHAZ, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; RPO, RNA polymerase I polypeptide A.

4.3.2.1. Hypothalamic insulin and leptin sensitivity

The gene expression of PTP1B was higher in NSO offspring as compared to NSL (Figure 4.13 A) but expression of IR, obR and SOCS3 were not changed between groups (Figure 4.13 B-D).

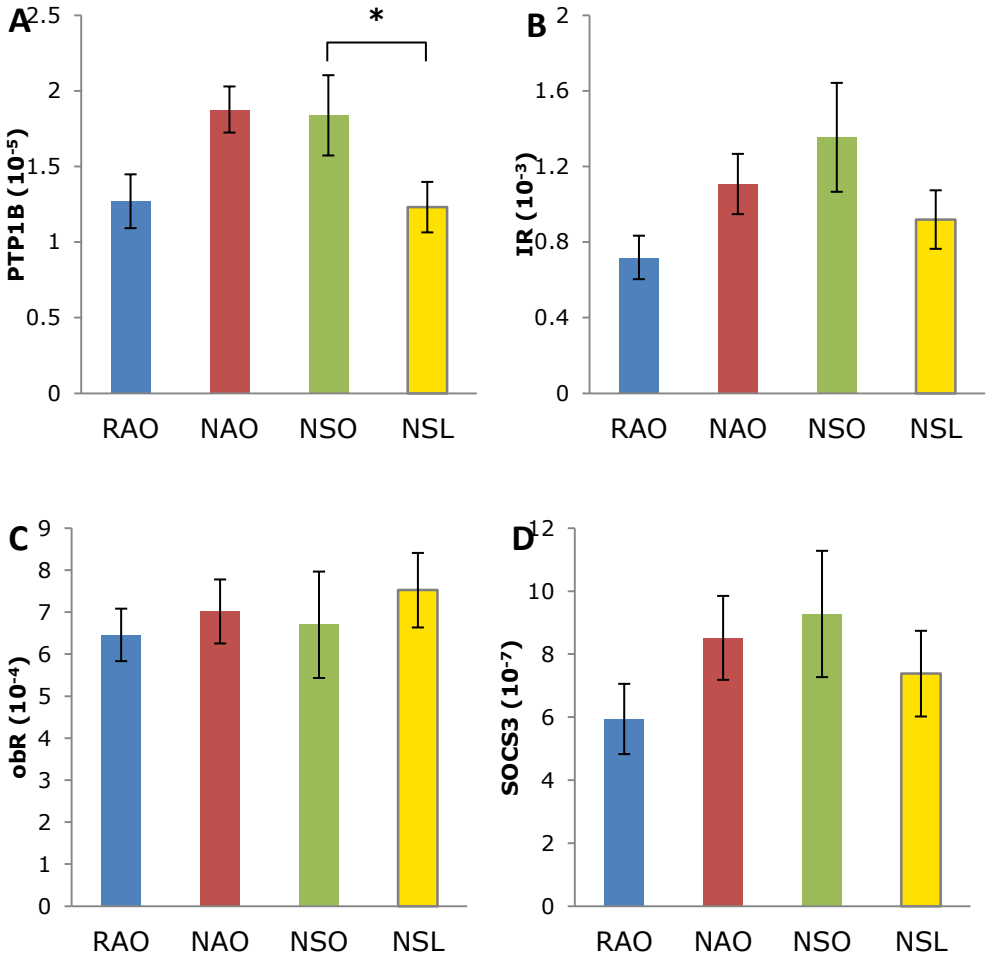

Figure 4.13: Gene expression of hypothalamic genes normalised for 18S, displayed in arbitrary units.

A) PTP1B, protein tyrosine phosphatase, non-receptor type 1; B) IR, insulin receptor; C) obR, leptin receptor; D) SOCS3, suppressor of cytokine signalling 3.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=5), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=8).

*** $p<0.05$. Data are means and error bars represent SEM.**

4.3.2.2. Hypothalamic appetite regulation

Gene expression of both the orexigenic NPY and the anorexigenic MC3R were higher in NSL as compared to NSO. The abundance of messenger ribonucleic acid (mRNA) of both receptors of adiponectin were lower in NSL as compared to NSO and higher in NAO as compared to RAO (Figure 4.14 A, B, D, E). The expression of growth hormone stimulating receptor (GhSR), agouti-related peptide (AgRP) and MC4R were not altered between groups (Figure 4.14 C, F, G).

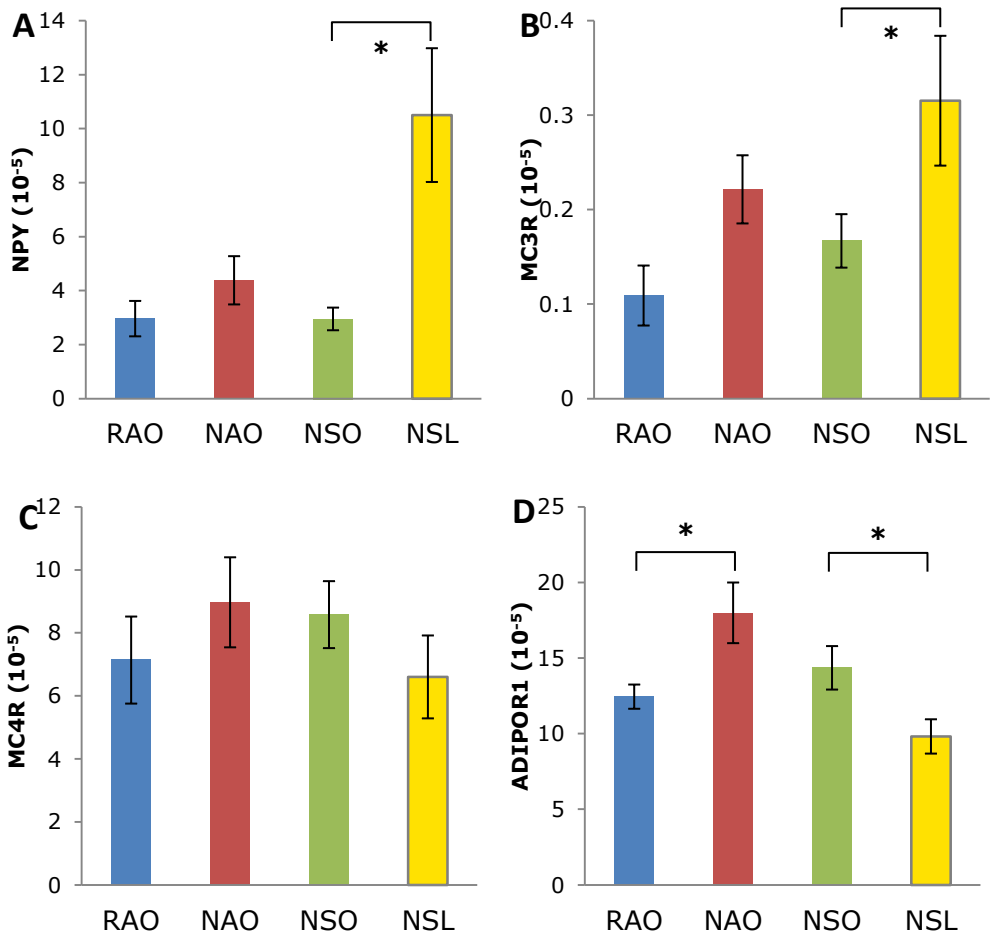

Figure 4.14, continued on the next page.

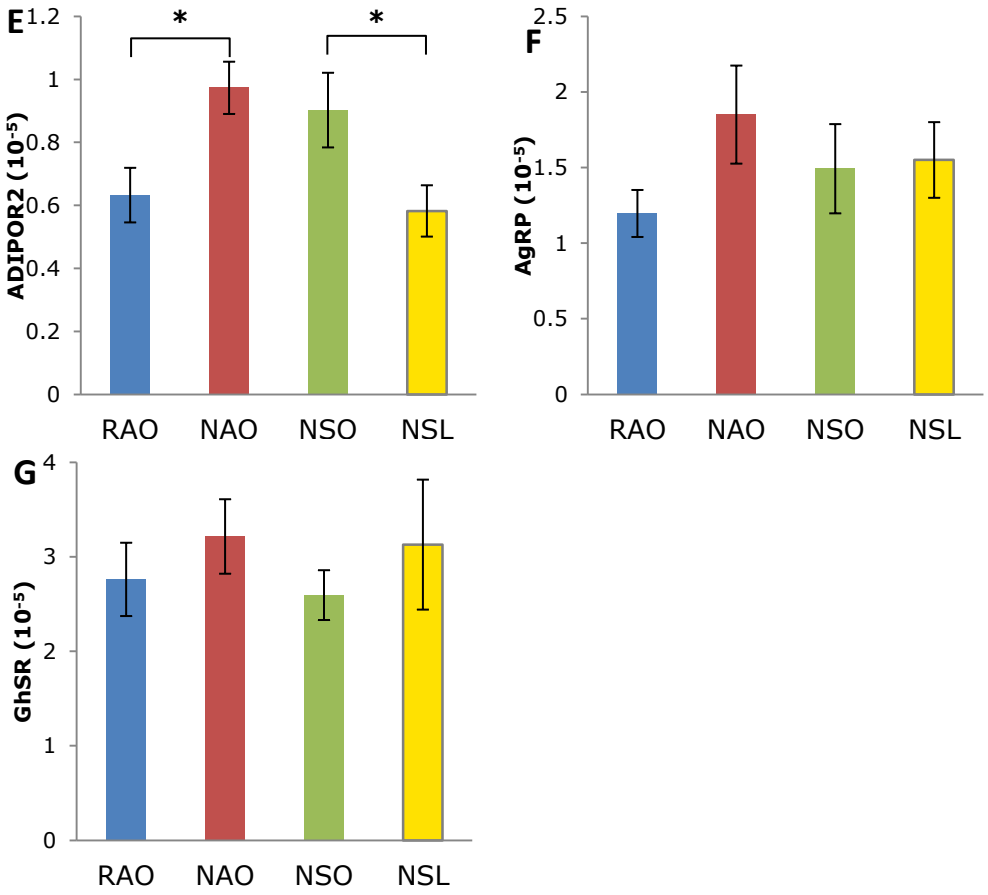

Figure 4.14: Gene expression of hypothalamic genes normalised for 18S, displayed in arbitrary units.

A) NPY, neuropeptide Y; B) MC3R, melanocortin 3 receptor; C) MC4R, melanocortin 4 receptor; D) ADIPOR1, adiponectin receptor 1; E) ADIPOR2, adiponectin receptor 2; F) AgRP, agouti-related protein; G) GhSR, growth hormone stimulating receptor (ghrelin receptor).

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=5), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=8).

*** $p<0.05$. Data are means and error bars represent SEM.**

4.3.2.3. Hypothalamic cell signalling

Gene expression of cell signalling molecules which play a role in both insulin and leptin sensitivity and appetite regulation, mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and FTO, were upregulated in NAO as compared to RAO and mTOR and AMPK were also

higher in NSO as compared to NSL (Figure 4.15 A-C). Expression of the brain-derived neurotrophic factor (BDNF) gene was not altered (Figure 4.15 D).

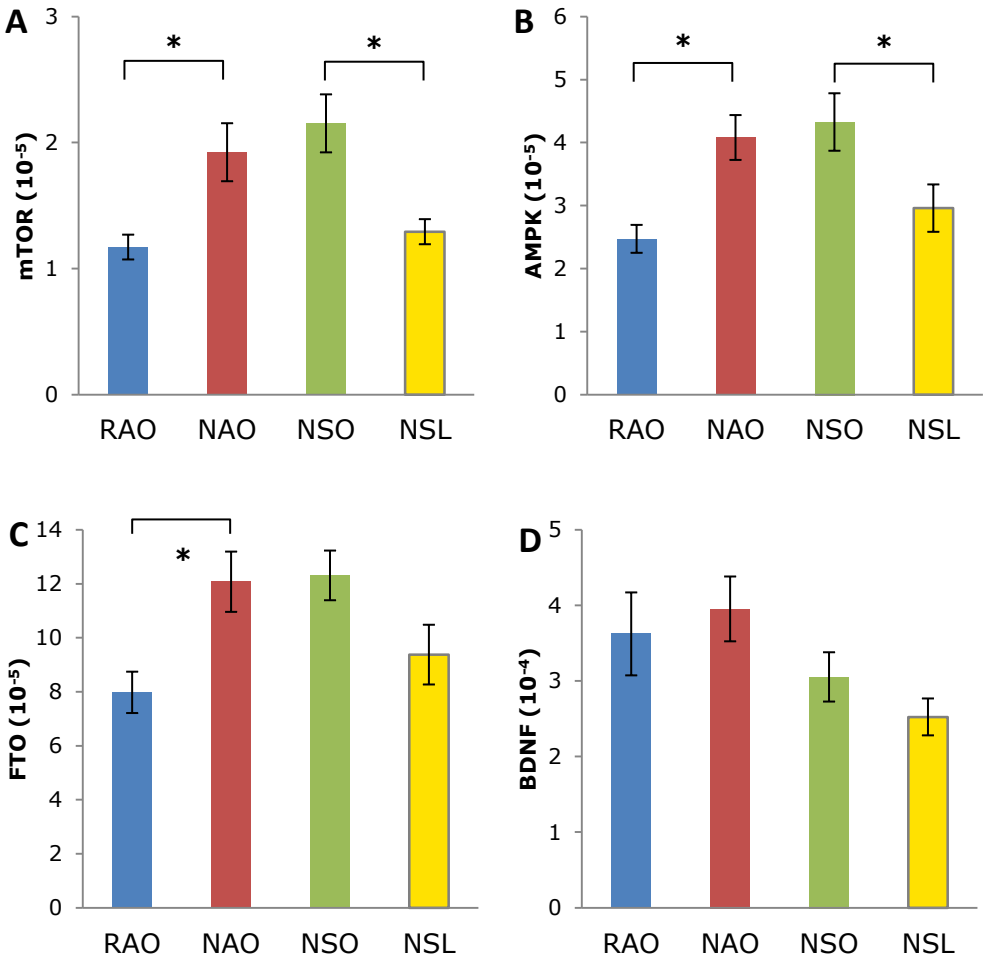

Figure 4.15: Gene expression of hypothalamic genes normalised for 18S, displayed in arbitrary units.

A) mTOR, mammalian target of rapamycin; B) AMPK, adenosine mono phosphate-activated kinase; C) FTO, fat mass and obesity associated gene; D) BDNF, brain-derived neurotrophic factor.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=5), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=8).

*** $p<0.05$. Data are means and error bars represent SEM.**

4.3.2.4. Hypothalamic control of the cortisol system

Expression of GCR was higher in NSO than in NSL and expression of corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) higher in NAO as compared to RAO (Figure 4.16).

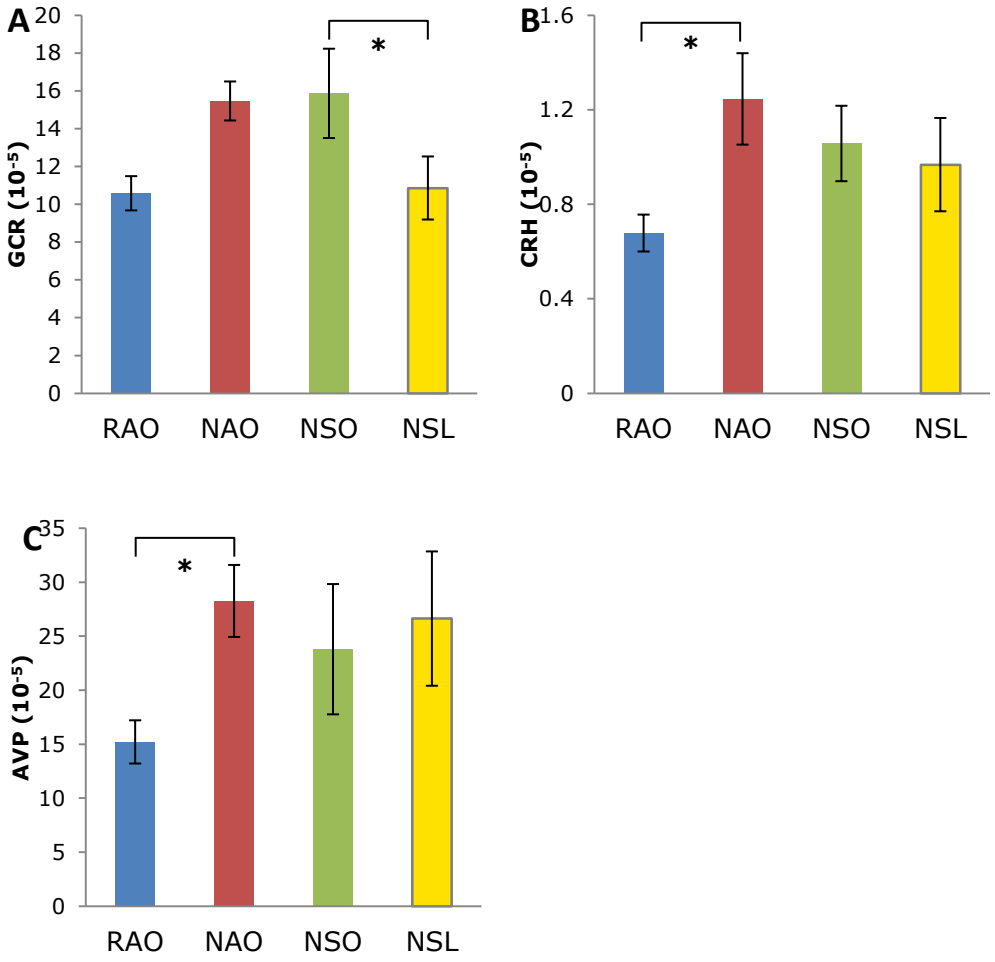

Figure 4.16: Gene expression of hypothalamic genes normalised for 18S, displayed in arbitrary units.

A) GCR, glucocorticoid receptor; B) CRH, corticotropin releasing hormone; C) AVP, arginine vasopressin.

Offspring were either born to mothers fed to requirements followed by accelerated early postnatal growth rate (RAO, blue, n=5), born to mothers nutrient restricted in late gestation followed by accelerated early postnatal growth rate (NAO, red, n=8) or born to mothers nutrient restricted in late gestation followed by standard early postnatal growth rate (NS). After weaning, a subset of NS offspring were maintained in the same restricted environment as RA and NA (NSO, green, n=7) whilst others were maintained in a less restricted environment, inducing a lean body weight (NSL, yellow, n=8).

*** $p<0.05$. Data are means and error bars represent SEM.**

4.4. Discussion

In adult offspring, maternal nutrient restriction, but not early postnatal growth rate, was accompanied with changes in gene expression which are consistent with a lack of clear upregulation of an anorexigenic response and insulin resistance. However, observed food intake did not differ after either intervention when it was averaged from a 10 day long observation of food intake. In a separate measurement of food intake over 24h following an overnight fast, food intake was lower in NAO as compared to RAO and NSO, plasma glucose, leptin and cortisol were higher. Both food intake and plasma metabolites during the 24h period show the most marked differences between lean and obese animals.

The food intake as measured and averaged over 10 days is likely the more representative overall determination of food intake, which means that, at 17 months of age, all animals maintained in the obesogenic environment had the same body weight, fat mass and food intake, regardless of their perinatal intervention. Dietary habits of the adult offspring have also been shown to be unaffected by famine during their late fetal development [234, 449], which is consistent with the absence of an effect on adult body weight [229, 233]. Part of the difference between the 24h and the 10 day measurements of food intake may be explained by the fact that for the single 24h measurement all animals were subjected to an overnight fast before the food intake test and blood sampling. Plasma leptin, insulin and NEFA concentrations differed significantly between baseline and 24h, both measurements representing morning samples in fasted and fed state, respectively. This indicates that endocrine and metabolic status and, possibly, food intake behaviour are changed by the overnight fast. However, NAO offspring did not show a markedly different pattern from the

other groups in their metabolic response to an overnight fast which could have explained the lower food intake observed after overnight fasting in this group. As it is, there is little evidence for the difference in food intake to be much more than by chance.

In addition, as animals were euthanased in the fasted state, the gene expression observed in the hypothalamus is representative of the fasted state at the beginning of the single 24h food intake measurement.

4.4.1. Maternal nutrient restriction followed by obesity changes the hypothalamic appetite regulation of adult offspring and their central insulin and leptin sensitivity in the fasted state

Whilst the plasma leptin concentration was higher in NAO in the fasted state, which would induce an anorexigenic response, genes displaying a higher expression in NAO as compared to RAO have been characterised both as anorexigenic, i.e. mTOR [203], and orexigenic, AMPK [191], but the effects of adiponectin receptors [22] and FTO on food intake are not entirely clear. A lack of clear anorexigenic response to increased plasma leptin concentrations may be at least partially explained by central leptin resistance, i.e. a combination of a decrease of leptin transport across the blood brain barrier and a decrease in signalling following the binding of leptin to its receptor [450].

Maternal nutrient restriction in late pregnancy did not lead to a direct difference in hypothalamic gene expression involved in insulin and leptin resistance as both receptors, IR and obRb, were not changed in expression and whilst there was a trend to higher expression of direct inhibitors PTP1B and SOCS3 in NAO as compared to RAO (>1.4-fold, p=0.051 for PTP1B),

this did not reach statistical significance. NSL hypothalami did not show a change in gene expression of IR, obRb and SOCS3 but PTP1B was reduced, suggesting a higher hypothalamic insulin and leptin sensitivity in NSL as compared to NSO.

The primary neurotransmitters involved in hypothalamic appetite regulation are NPY, AgRP and α-melanocyte stimulating hormone (α-MSH), which binds to receptors MC3R and MC4R. Whilst I could not measure gene expression of POMC, the gene coding for α-MSH, due to circumstances and time restrictions, none of the other genes were differentially regulated between obese groups. However, NSL animals had substantially higher expression of NPY and MC3R as compared to NSO. Since NPY and MC3R stimulation have opposing effects with regards to appetite regulation, a direct effect to increase or decrease of food intake could not be extrapolated from these findings.

Although food intake of NSO and NSL animals cannot be compared with regards to physiological changes in appetite regulation because the feed given differed significantly, it does give further insight into the causes of the lower body weight shown by the lean group, i.e. both the higher physical activity and a lower food intake relative to body weight contributed to this difference. This difference in food intake was not intended to be part of the interventional design as feed, i.e. grass, was present ad libitum. The higher food intake in obese animals may, therefore, be representative of the higher palatability of the high-energy pellets and/or the assumed (relative) central insulin and leptin resistance.

The associated lack of responses in plasma leptin, insulin and glucose in these lean animals as compared to obese animals are consistent with

common observations of postprandial metabolic differences between obese, insulin-resistant and lean, insulin-sensitive individuals [451-453].

Expression of genes downstream of insulin was potentially altered as hypothalamic AMPK, which is inhibited by insulin and leptin [454, 455], was higher in the NAO group than in RAO. AMPK is activated by phosphorylation in the insulin signalling cascade, so an increase in gene expression does not allow any conclusions on protein activity, especially since there is, to my knowledge, no clear data available on the regulation of AMPK gene expression or of the association of AMPK expression and activity. I, therefore, commenced an analysis of AMPK protein abundance in both its phosphorylated form and of total AMPK hypothalamic protein by Western Blotting but this was unsuccessful due to technical and time limitations. Whilst the higher gene expression of AMPK in the NAO group does not allow any direct conclusions on insulin and leptin action in these hypothalami, it is striking that the AMPK expression in the NSL animal was altered in the opposite direction, i.e. there was a lower expression in NSL as compared to NSO animals. Therefore, although what influences AMPK expression is unknown, it is tempting to see a relationship with the animals' overall insulin sensitivity. In the hypothalamus [456] and other tissues [193, 457], AMPK has been reported as an inhibitor of mTOR, so it is curious to see here that AMPK and mTOR expression display similar patterns between groups. It would, therefore, be of interest to investigate this further at the level of protein activation.

Another hormone thought to activate AMPK by phosphorylation is adiponectin. Receptors for adiponectin are expressed on the primary neurons of the ARC [458]. Plasma adiponectin concentrations are negatively associated with body weight [91] and their association with food intake is not entirely resolved, with studies indicating both an increase and

a decrease of food intake with administration of adiponectin [22, 94]. Plasma adiponectin would be expected to be reduced in 'obese' animals, but as a result of its multiple active isoforms it is very challenging to measure and, therefore, we have no information on offsprings' adiponectin concentrations. Since expression of adiponectin receptors 1 and 2 (ADIPOR1/2) was lower in lean offspring, i.e. in animals with potentially higher plasma adiponectin concentrations, it is feasible that these receptors are more highly expressed in obese animals to compensate for lower plasma adiponectin concentrations. However, direct conclusions cannot be drawn without the information on plasma adiponectin concentrations.

PTP1B is thought to be one of the main mechanisms mediating insulin and leptin resistance as it dephosphorylates the activated form of the insulin receptor [459] and dephosphorylates JAK2 in the leptin signalling pathway [460], hence attenuating the signal despite insulin or leptin binding to their receptors. PTP1B is thought to be upregulated both in peripheral organs and in the hypothalamus by inflammatory mechanisms including tumour necrosis factor (TNF) [461] but higher PTP1B protein levels have also been observed in muscle and white adipose tissue (WAT) of obese or diabetic subjects [462], decreasing with weight loss [463]. To my knowledge, PTP1B expression has not previously been explored in studies with manipulation of the fetal environment, but it is a likely candidate for mediating at least the central part of proposed insulin and leptin resistance in this study, following nutrient restriction in late pregnancy and accelerated early postnatal growth.

Whilst it has been clearly established in humans that a polymorphism in the FTO gene is associated with obesity [464] and a knockout model results in a lean phenotype [465], the relationship of mRNA expression in

adipose tissue and obesity in individuals not carrying that polymorphism is less clear and has been reported as both a positive and a negative relationship [466, 467]. In rodents, general overexpression of FTO including the hypothalamus as a result of polymorphisms has been associated with hyperphagia, high body weight and fat mass [468]. Long term nutrient restriction leads to a decrease in hypothalamic, but not adipose tissue, FTO expression suggesting that FTO gene expression is inhibited downstream of the leptin-induced signal transducer and activator of transcription 3 (STAT3) pathway [469]. Acute fasting, however, does not change hypothalamic FTO expression [470]. The higher expression of FTO in NAO offspring may, therefore, be indicative of a more orexigenic regulation, possibly secondary to hypothalamic leptin resistance.

The changes we can see after maternal nutrient restriction in late pregnancy are consistent with the reported timing of hypothalamic maturation with regards to the appetite regulating system. Both in humans and in sheep, appetite-related neuropeptides are expressed from mid-gestation i.e. from 21 weeks in humans [275] and 81d in sheep [276, 277], after which the number of neurons expressing these peptides increases, the hypothalamus differentiates further morphologically and, at least in the non-human primate, projections are formed in late pregnancy [80]. Therefore, it would seem likely that characteristics of the appetite-regulatory parts of the hypothalamus can be influenced with long term consequences during this period of plasticity in mid- to late gestation. Besides rodent models, there are few studies which investigate the effect of maternal nutrient restriction during pregnancy on the young or adult offspring. In a previous study from our group, when applying nutrient restriction in sheep in mid pregnancy, offspring at one year of age had a lower food intake but the gene expression of main ARC neuropeptides were

not altered [471]. Nutrient restriction of sheep in late pregnancy did not lead to changes in body weight, body composition or food intake but markers of appetite regulation were not assessed [429]. On the other hand, overnourishing sheep in late pregnancy leads to a change in initial neonatal intake of mother's milk and expression of hypothalamic neuropeptides but these offspring were not maintained until adulthood [343].

Since food intake regulation in the brain is very complex and involves homeostatic circuits including the ARC and hedonic influences, both of which are not yet fully understood, it would be very challenging to conduct a study that would give a clear picture of changes in the appetite-regulatory system. Food intake can be regulated very finely within this complex system, so that changes in one part of the system, i.e. hypothalamic gene expression in NAO offspring, might be compensated for in other parts of the system and hence not lead to an overt change in phenotype. Adult NAO offspring had both higher plasma leptin and insulin concentrations and, as Sebert et al. reported in our sheep study, a higher expression of ghrelin in the abomasum, potentially increasing both anorexigenic and orexigenic peripheral signalling, respectively [472]. Therefore, the changes in hypothalamic gene expression might result from these changes in offspring endocrine and metabolomic phenotype rather via long term programming of the hypothalamus directly. However, the majority of the peripheral signalling compounds that were increased in NAO as compared to RAO animals, i.e. leptin, insulin and glucose would result in anorexigenic signalling, not the partially orexigenic response observed. It is reasonable to assume that anorexigenic response to insulin and leptin may be blunted in these animals if the trend to increase in PTP1B reached biological significance but glucose would still have a similar

effect. Because plasma ghrelin concentrations are very difficult to determine in ruminants [473], the concentration of mRNA measured in the abomasum is the only indication that NAO offspring may express peripheral orexigenic signals.

Finally, whilst other research suggests that accelerated early postnatal growth may be positively associated with adult overweight in humans [243], this was not demonstrated in this study, as NAO and NSO offspring had very similar body weight, composition, appetite regulatory gene expression and only differed in peripheral insulin sensitivity and plasma leptin concentrations.

4.4.2. Maternal nutrient restriction and early postnatal growth rate influence the postprandial dynamics of plasma leptin and glucose

As most food intake occurs during the first 2 hours after the overnight fast, the main postprandial response would be expected between 2 and 4 hours after feeding. Leptin shows a different dynamic relative to its higher fasted baseline measurement in NAO as compared with RAO and NSO animals, i.e. a drop in leptin concentrations at 2 and 4 hours after feeding. A similar drop in postprandial leptin was observed when comparing obese, non-diabetic men with lean men [452]. At the same time, plasma glucose concentrations have a stronger postprandial peak at 4 hours after feeding in NAO as compared to RAO groups, again pointing to a reduced glucose tolerance in these animals.

This alteration in postprandial endocrine and metabolic dynamics is part of the phenotype for which offspring of mothers who were nutrient restricted in late pregnancy appear to be programmed. That is, despite having the

same body weight and body composition as the other obese offspring, the obesity developed by NAO offspring has metabolic effects that are more adverse than can be seen in both RAO and NSO animals with respects to leptin signalling and peripheral insulin sensitivity.

4.4.3. Maternal nutrient restriction affects cortisol-related gene expression in adult offspring

Plasma cortisol concentrations were not significantly altered between groups in any of the fasted measurements in mothers or offspring (see Chapter 3) but was higher in a postprandial measurement during the food intake test in NAO as compared to RAO groups. Since ovine plasma cortisol follows a diurnal rhythm of about 10 peaks per day, starting with a peak about 60 minutes after initial feeding [474], the sampling frequency of our study was too low to distinguish between those peaks or to cover a clear postprandial response. Therefore, the reported plasma cortisol concentrations can only give a general overview of the cortisol system.

Since crucial parts of cortisol regulation take place in the hypothalamus, I measured gene expression of some key genes and they showed significant differences. The expression of the glucocorticoid receptor (GCR) and, with that, probably the sensitivity of the hypothalamus to negative feedback with regards to AVP and CRH expression and secretion showed a trend to higher values (>1.4 fold, $p=0.064$) in NAO as compared to RAO without reaching statistical significance and was significantly lower in NSL as compared to NSO animals. Regardless of this assumed higher sensitivity to the negative feedback in NAO, expression of AVP and CRH were significantly higher in NAO as compared to RAO animals whilst neither were changed in NSL as compared to NSO groups. From the expression of

AVP and CRH alone, one would expect a higher ACTH secretion from the pituitary and, subsequently, higher cortisol secretion from the adrenal gland but this was not demonstrated in these animals. Therefore, despite a lack of clear phenotypic consequence, there are changes on the regulatory level after maternal nutrient restriction, which are not further influenced by early postnatal growth of the offspring. Whether this also occurs at the protein level of these genes should be confirmed and, if so, downstream consequences these changes have without increasing plasma cortisol should be explored.

Whilst periconceptional nutrient restriction causes an earlier fetal increase in prepartum cortisol, earlier delivery [475] and changes in related hypothalamic gene regulation in the fetus at late gestation [476], little is known on long term programming of the HPA axis by late gestational nutrient restriction. It was reported that human intra-uterine growth restricted (IUGR) fetuses have higher plasma cortisol concentrations than normal weight fetuses [326, 477]. However, in a study of a similar maternal nutrient restriction of 50% from day 118 of gestation in sheep conducted by Burrage et al., there was no significant effect on fetal plasma cortisol, ACTH and glucose at 140 days gestation [294]. In both our and Burrage's studies, cortisol concentrations at the different age points and both in mothers and in offspring were consistently higher after maternal nutrient restriction but this did not reach statistical significance. It is possible that the mechanism in sheep is similar to the one in human IUGR but is less pronounced, so that the sample numbers in these animal studies are not sufficient to show a clear effect. Similarly, at 16 months of age, cortisol measurements in NSL offspring were consistently lower compared with NSO animals without reaching statistical significance. In humans, fasted plasma cortisol concentrations are not correlated with body

mass index (BMI) but some regulatory parts of the cortisol system may be altered in obese subjects as gene expression and protein activity of 11ß-hydroxysteroid dehydrogenase type 1, the enzyme converting inactive cortisone to active cortisol, is increased in adipose tissue of obese individuals [478, 479]. Similarly, in sheep, obesity does not influence cortisol or other stress signalling molecules unless there was a stressor added, which induced a stronger response [480] whilst our data might suggest a trend to higher plasma cortisol concentration in obesity. This is of importance as glucocorticoids positively regulate hypothalamic expression of the anorexigenic neurotransmitter POMC [481] and may, therefore, change the perception of appetite.

Since CRH and GCR have been shown to be susceptible to long term regulation of gene expression during the periconceptional period, which may be an effect of epigenetic programming [476, 482], it would be of interest to investigate the hypothalamic samples of this study with regards to histone acetylation and DNA methylation of these genes to see if a similar effect can be reached in late pregnancy. Secondarily, it would be interesting to measure POMC gene expression to see if it would be correlated to cortisol concentration or GCR expression.

To my knowledge, alteration of hypothalamic GCR expression has, to date, only been observed in offspring subjected to maternal nutrient restriction during the periconceptional phase [476]. Although it has been reported that maternal nutrient restriction in early to mid pregnancy in sheep has an effect on offsprings' pituitary and adrenal responses, this study did not further investigate the hypothalamic component of cortisol regulation [483]. So even though NAO offspring are phenotypically unremarkable as their plasma cortisol concentrations are similar to that of RAO, changes in hypothalamic gene expression can be observed in adult offspring that

might be adaptive to the intervention in late gestation. The change in offspring hypothalamic GCR, CRH and AVP expression is unlikely to be caused by changes in maternal (and fetal) cortisol concentrations since these were subtle. Edwards et al. showed a relationship of very low maternal plasma glucose concentrations with fetal HPA axis responsiveness, associated with maternal nutrient restriction in late pregnancy [484]. However, this difference only manifested in acute hypoglycaemia and alteration of plasma ACTH or cortisol during the chronic hypoglycemia caused by the nutrient restriction was not observed. This suggests that chronic hypoglycemia causes changes in the regulatory mechanisms which do not show in the plasma phenotype and that measuring plasma cortisol concentrations is not sufficient to detect all changes in this system.

It would, therefore, be necessary to assess whether maternal nutrient restriction in late pregnancy consistently leads to the changes in gene expression we observed and whether these changes would lead to phenotypical differences in a stress situation in the adult offspring.

Especially AVP expression would be of interest for further research because of its implication with hypertension [485]. AVP binds to 2 receptors, V1R, which increases systemic vascular resistance, and V2R in the kidneys, which increases blood volume, so that AVP leads to increased arterial pressure [486]. If the increased gene expression in NAO offspring translates to a higher hypothalamic and pituitary AVP protein expression and higher plasma AVP then this may be seen as a higher blood pressure in these animals. Although blood pressure was measured in the adult offspring in our study, these measurements were deemed unreliable as the animals were anaesthetised at the time. In the human studies following the Dutch famine cohort, an earlier onset of coronary heart disease was

seen in offspring of mothers affected by the famine in early pregnancy but no difference was seen for offspring of mothers affected in late pregnancy as compared to individuals unaffected by the famine [487-489]. However, independently of the time of famine, the individuals who developed coronary heart disease tended to have lower birth weight [487], but neither effect was accompanied by a significant change in blood pressure. The association of cardiovascular disease, as well as higher blood pressure, with low birth weight was also seen in the studies presented by Barker et al. [223]. This combination of the timing of famine and birth weight is surprising since offspring of mothers affected in late pregnancy are far more likely to be growth restricted and thus small at birth than offspring affected in early pregnancy. It has been postulated that the rapid postnatal growth associated with small babies is the crucial factor leading to later coronary heart disease [487]. Since NAO offspring were both small at birth and then experienced accelerated early postnatal growth they do fit the risk pattern of cardiovascular morbidity.

4.4.4. General strengths and limitations of this study

Even though the comparisons available to me in this study, i.e. RAO vs NAO, NAO vs NSO and NSO vs NSL, are sufficient to differentiate the effects of the interventions applied at each developmental period, it would have been desirable to include additional groups. Since maternal nutrient restriction in late pregnancy appears to be the most prominent influence on later metabolic health of the offspring, additional groups, e.g. RSO (no maternal intervention, standard early postnatal growth rate followed by obesity, to compare with NSO) and RSL (no maternal intervention,

standard early postnatal growth rate followed by a lean body weight, to compare with NSL).

Secondly, for a better comparability between obese and lean offspring it should be considered whether lean animals could be fed the highly energy-dense pellets as well as grass. This would enable better interpretation of feeding test and of all postprandial metabolites and hormones as described in this chapter. Presumably, the difference in body weight between obese and lean offspring would be less pronounced if the only difference in treatment is indeed the higher physical activity in lean animals. I would hypothesise that other changes between NSO and NSL would persist since activity is of very high importance in the regulation of insulin sensitivity and, possibly, leptin sensitivity.

4.5. Conclusions

Maternal nutrient restriction, but not additional accelerated early postnatal growth, leads to changes in adult offspring hypothalamic gene expression that are consistent with decreased insulin and leptin sensitivity (Figure 4.17, Figure 4.18) and modification of the cortisol system. The opposite effects were seen if NS offspring were not subjected to obesity in adulthood and maintained lean. However, these changes were not associated with higher food intake or body weight, likely due to the highly complex nature of appetite regulatory circuits. Changes in gene expression can be partially explained by insulin and leptin resistance whilst expression of other genes may be under long term regulation by perinatal changes in epigenetic markers. Genes known to be susceptible to epigenetic regulation in rodents include POMC [490], BDNF [308], CRH [482], GCR [317, 491] and AVP [492], which would be of interest to examine more

closely with regards to DNA methylation and histone acetylation in this study to see if it matches the gene expression patterns observed. Besides the metabolic changes in NAO offspring discussed in Chapter 3, i.e. increased insulin resistance and plasma leptin concentrations, these animals also showed changes in postprandial dynamics of plasma leptin and glucose. Alteration of hypothalamic cortisol regulation was observed but plasma cortisol itself was unaffected. Overall, NAO animals display changes which are consistent with stronger adverse consequences of obesity, despite not being more obese in adulthood than RAO offspring. Figures 4.17 and 4.18 sum up the changes in NAO as compared to RAO offspring in hypothalamic appetite regulation both on an inter- and intra-cellular level.

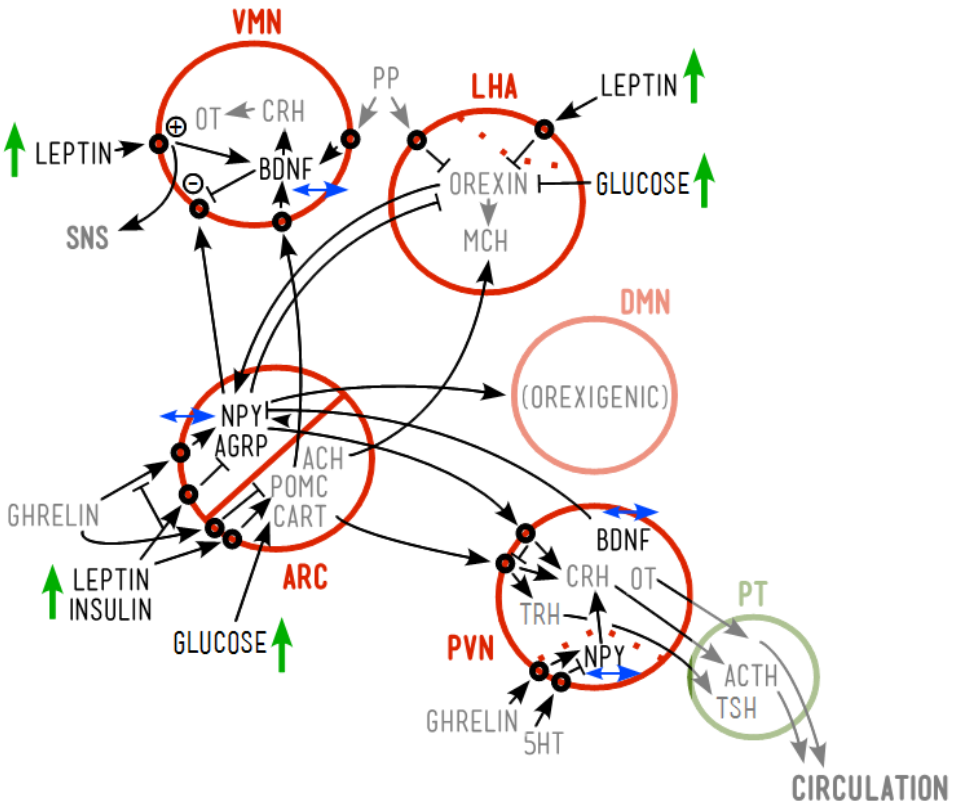

Figure 4.17: Overview of changes in plasma hormones and metabolites and hypothalamic gene expression in NAO offspring as compared to RAO offspring at 17 months of age. Plasma leptin, insulin and glucose were all shown to be higher at fasting, during the GTT or in the 24h metabolite measurements. Nevertheless, appetite regulatory proteins NPY, AgRP and BDNF were not changed in gene expression.

Nuclei are: arcuate nucleus (ARC), ventromedial nucleus (VMN), lateral hypothalamus (LHA), dorsomedial nucleus (DMN), paraventricular nucleus (PVN).

NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opio melanocortin; CART, cocaine and amphetamine related transcript; OT, oxytocin; CRH, corticotropin releasing hormone; BDNF, brain-derived neurotrophic factor; PP, pancreatic polypeptide; MCH, melanin concentrating hormone; ACTH, adrenocorticotropic hormone; TRH, thyroid releasing hormone; TSH, thyroid stimulating hormone; 5HT, serotonin; SNS, sympathetic nervous system. Adapted from figures in Chapter 1 and [95-99].

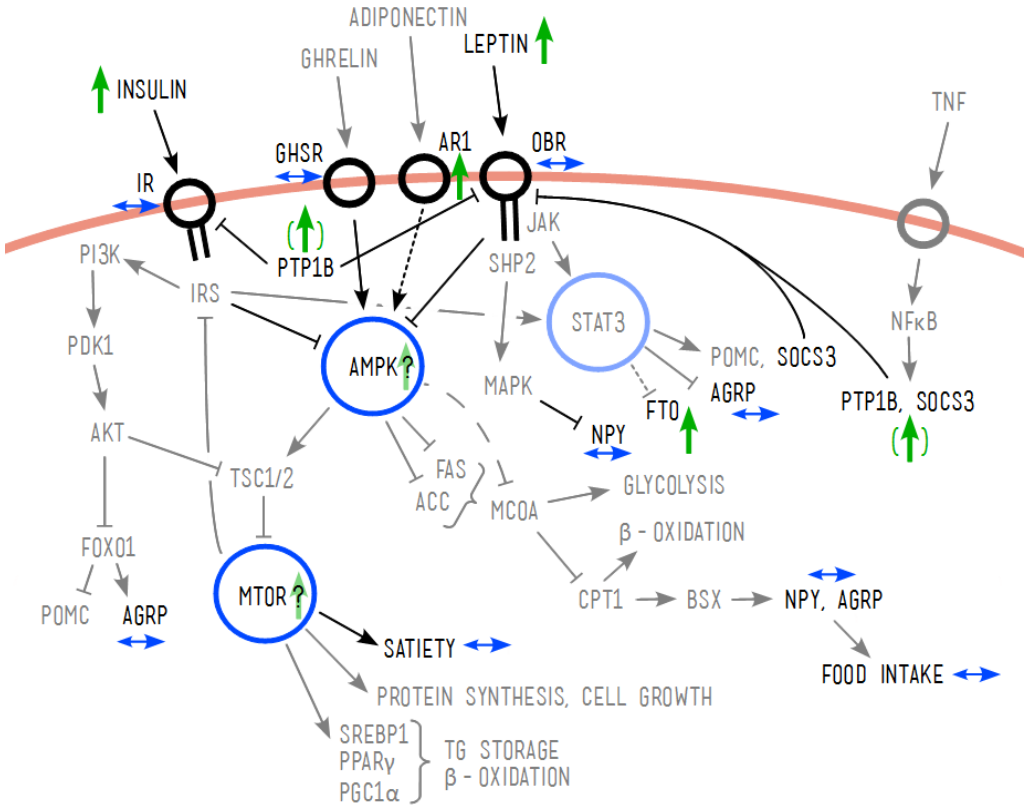

Figure 4.18: Overview of changes in hypothalamic gene expression with focus on intracellular signalling in NAO offspring as compared to RAO at 17 months of age.

Plasma insulin and leptin concentrations were higher in fasted state or after the GTT. Signalling of insulin and leptin receptors were potentially inhibited by higher expression of SOCS3 and PTP1B although this didn't reach significance. FTO, which is thought to be inhibited in expression by leptin through STAT3, was increased. Primary orexigenic neurotransmitters NPY and AgRP are not changed in gene expression. Whilst AMPK and mTOR are increased in gene expression it is not clear what this means on a protein activity level. Overall food intake is not changed in these animals.

Adenosin monophosphate-activated protein kinase; BSX, brain-specific homeobox transcription factor; CPT1, carnitine palmitoyl-transferase 1; FAS, fatty acid synthase; FOXO1, forkhead box O1; GHSR, growth-hormone secretagogue receptor; IR, insulin receptor; IRS, insulin receptor substrate; JAK, janus kinase; MAPK, MCOA, malonyl-CoA; MTOR, mammalian target of rapamycin; NFκB, nuclear factor κB; NPY, neuropeptide Y; OBR, leptin receptor; PDK1, pyruvate dehydrogenase kinase 1; PGC1α, PPAR γ coactivator 1α; PI3K, phosphoinositide-3-kinase; POMC, pro-opiomelanocortin; phosphoinositide-3-kinase; PPAR, peroxisome proliferator-activated receptor γ; PTP1B, protein tyrosine phosphatase non-receptor type 1; SHP2, protein tyrosine phosphatase non-receptor type 11; SOCS3, suppressor of cytokine signalling 3; SREBP1, sterol regulatory element-binding protein 1; STAT3, signal transducer and activator of transcription 3; TG, triglyceride; TNF, tumour necrosis factor; TSC1/2, tuberous sclerosis 1 and 2.

This figure is based on the papers as referenced throughout the corresponding section of the introduction and especially reviews [182, 184, 185, 192].

5. The acute and long term influences of dietary choline on metabolic health of lactating mothers and their offspring

5.1. Introduction

As I have described in more detail in Section 1.9.2.3 of the introduction, most experimental work modulating choline intake during development concentrated on describing the role of choline during pregnancy. The lactation period, however, has not been extensively investigated.

Previous studies have shown that a choline-deficient diet during pregnancy leads to increased gene expression of an inhibitor of cell cycle progression, CDKN3, of the fetal hippocampus [372, 373]. These changes had long term effects, as the adult offspring demonstrated altered hippocampal function [374], decreased memory performance [375, 376] and attention to stimuli [368] compared to offspring whose mothers had been fed sufficient choline in the form of a salt. A postnatal intervention of choline supplementation as intubation or subcutaneous injection of choline chloride, affected spatial memory, as measured in a maze test, and increased the number of hippocampal dendritic spines and memory performance was positively correlated with hippocampal capacity to release acetylcholine (Ach) [386, 493]. In rats, the period of 16-30 days of age has been suggested as a developmental phase during which spatial memory can be positively influenced in a long term manner [386, 493] and age-related memory loss ameliorated by administering additional choline. Studies examining

postnatal choline supplementation are few and, to my knowledge, only examined the consequences on spatial memory in the offspring. Our study was, therefore, novel by looking at other outcome measures and by providing a model closer to the human situation, in which breastfed infants rely mainly on choline content of mother's milk which would most likely be influenced by maternal choline intake [494].

Within these studies, differences in availability and routes of uptake of choline as salt as opposed to phosphatidylcholine (PC) have not been taken into account and there are not many studies comparing choline salt with PC. Free choline is taken up through a low-affinity and low-specificity transport system by organic cation transporters (OCT), through a high-affinity system by choline transporter 1 (CHT1) or intermediate-affinity systems by choline transporter-like proteins [495]. PC and SM, on the other hand, are taken up into tissues through transporters CD36 and scavenger receptor class B type 1 (SR-B1) and, possibly, through non-specific internalisation of lipid particles, e.g. uptake of micelles into intestinal epithelium [496]. In fact, PC and its derivative carrying only one fatty acid, lyso-PC, are facilitating micelle uptake and lipoprotein secretion and are thus instrumental for lipid digestion [497]. As not all tissues display the same receptors, choline salt and PC may differ in distribution but this has not been well described. Upon digestion, free choline enters the circulation in the hydrophilic phase of plasma whilst PC and SM are circulating as part of lipoproteins and both pool in the liver, which has enzymes to carry out the CDP-choline pathway and the phosphatidylethanolamine methyltransferase (PEMT) pathway to produce PC from free choline and PE, respectively. Thus it might be hypothesized that metabolically these two forms of choline are unlikely to be the same.

Choline and its metabolites are strongly involved in cell replication and tissue growth as it is essential for cell survival, membrane integrity and signalling, protein and DNA methylation and in neuronal signalling (as reviewed in [365]). During the lactation period rat pups increase their body weight about 6 times (approximately from 8g at birth to 50g at 21d), and is, therefore, a period of rapid growth. Compared to humans or other larger mammals, rats are born relatively immature and most of the rapid growth occurs during rat lactation, making this period comparable to the third trimester of fetal development in larger mammals and humans. In the early postnatal period of the rat pancreas [498], the immune system [499] and brain [383, 384] including the appetite regulating system [500], continue to develop and are sensitive to environmental stimuli, which may result in lasting effects on organ development and function.

Rat milk choline concentrations are about 20-fold higher than in maternal serum [501], which suggests that the pup has a high requirement for choline in early postnatal life. Although mammary glands synthesise PC from both the PEMT [502] and the CDP pathway [503], phosphocholine concentration, one of the main choline metabolites in milk, is reduced in rat milk after feeding choline-devoid diet for 21d [494]. This suggests that endogenous synthesis cannot meet the needs of the mammary gland. Choline is additionally transported from plasma into milk [501] and feeding rats a choline-devoid diet during late pregnancy and lactation led to depletion of choline metabolites from the liver [504], suggesting that, during lactation, maternal choline stores are preferentially utilised for milk production.

5.1.1. Aims and Hypotheses

It has been previously observed, both by our group and others [397] but not considered in the majority of published studies [369, 379], that, at least during lactation period, rapid choline depletion leads to a decrease in food intake and body weight and, if not reversed, death.

1) I hypothesised that there is an acute effect on both the mothers' and offsprings' food intake and this is due to singalling changes in appetite regulation.
2) Due to the important structural and functional role of choline in the brain, I hypothesised that these early changes, affected by both the availability and the form of choline in the diet, are mediated through structural changes in the brain.
3) These changes would be evident both in early life and extending into adulthood.
4) Since choline has such a crucial role in cell membrane structure and growth, I hypothesised that the morphology of the small intestine, an organ dependent on rapid cell turnover, would be compromised by choline deficiency.

As with a majority of organs, the pancreas is still developing before weaning in rats. Recently, it has been shown that drastically reduced methyl donor availability, i.e. methionine, folate and choline, in early postnatal life in rats alters pancreatic characteristics and improved glucose tolerance permanently [505]. Also, as described in the introduction, early postnatal growth rate may have effects on long term glucose tolerance in rodents. Since previous studies conducted by our group in 2009/2010 have shown that rat offspring whose mothers were fed a choline-devoid diet

weighed less at weaning, this could potentially alter, or be associated with, glucose tolerance, independently of the effects of choline availability.

5) I, therefore, hypothesised that this intervention may influence glucose tolerance in adult offspring.

To address these objectives and hypotheses, I fed diets differing in the content and form of choline to lactating rats. Brain phospholipids and brain fatty acid composition on PC and PE molecules were analysed to determine the effect of choline on brain structure, membrane fluidity and signalling. I measured established effects of choline deficiency, including liver fat accumulation [506] and estimated the concentrations of choline metabolites in mother's milk, by analysing the suckling offsprings' stomach content at weaning (21 days of age). Abundance of the predominant appetite regulatory proteins neuropeptide Y (NPY) and α-melanocyte stimulating hormone (α-MSH) was determined in the hypothalamic arcuate nucleus (ARC) to first gain insight into choline's role in appetite regulation. Glucose tolerance was assessed in the offspring at a young adult age of 9 weeks.

5.2. Methods

The design and all methods for this study are described in sections 2.10-2.21. In short, my study was designed to assess the long term effects of low choline availability and the difference between choline salt and PC during the lactation period. As in previous studies carried out by this group (2009/2010), pregnant rats of 10-12 weeks of age at mating were entered into the experiment. After giving birth, they were fed a diet in which the choline content and source (form) was manipulated but all other nutrients

were kept the same. The diets were isocaloric and isonitrogenous and the micronutrients, with the exception of choline, were identical and met the requirements of lactating and growing rats. Two female offspring from each mother were weaned to a semi-purified diet containing 1g/kg choline in the form of free choline until 11 weeks of age. This study extended earlier work by the group with the added step of following some of the offspring fed a diet containing a standard amount of choline until young adult age. Since designs were very similar some of the results presented in this chapter were derived from the 2009/2010 studies and will be marked accordingly and differences between the studies discussed as necessary. The study was designed by myself together with Prof. Catherine Field, Dr. Rene Jacobs and Ms Sue Goruk. In the daily animal work I was assisted by Ms Nicole Coursen and staff of the animal facility. I performed and/or supervised the analytical studies. I have compiled, analyzed and interpreted the results of the experiments described in this chapter.

5.3. Results

5.3.1. Maternal body weight and food intake

The food intake of mothers fed a choline-devoid diet was lower than that of the two choline groups at 2 weeks post partum and both D mothers (Figure 5.1) and D offspring (Figure 5.7) had a lower body weight at postnatal day 14 as compared to C and PC. This was more than predicted from earlier studies and the research team rescued these mothers by feeding the choline sufficient (salt) diet for the remaining five days until weaning (days 16-21). In doing this to the study design, the study represented insufficient maternal dietary choline deprivation during the last part of lactation. After weaning, these D mothers were kept on a diet containing

0.1 g/kg choline added as a bitartrate salt until they were killed at week 4 post-partum. When feeding the choline-restored diet, D mothers gained weight, but body weight remained significantly lower than C but not PC mothers 21 days after giving birth. After weaning, maternal weights further aligned and showed no difference in body weight at euthanisation as compared to C and PC mothers. At euthanasia at 4 weeks after birth, PC mothers showed a lower body weight compared to C mothers (Figure 5.1).

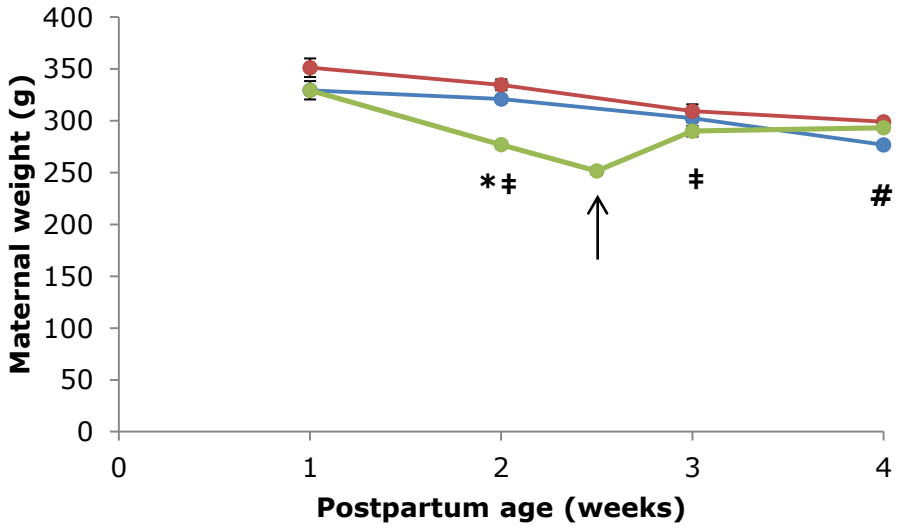

Figure 5.1: Maternal weight development during intervention over 4 weeks postpartum. The arrow represents the time point of re-introducing choline into the diet of D mothers.

PC (blue, n=4): mothers fed a diet containing 1 g choline as PC per kg diet; C (red, n=4): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green, n=3): mothers fed a choline-devoid diet; all diets were fed from giving birth to euthanisation at 4 weeks postpartum except for D as described.

*** p<0.05 for PC versus D comparison; ‡ p<0.05 for C versus D comparison; # p<0.05 for PC versus C comparison. Data are means and error bars represent SEM.**

As is consistent with their weight development, D mothers showed a trend for a lower food intake as measured in week 2 postpartum and as compared to C and PC mothers (p=0.057 for both comparisons, Figure 5.2). Food intake did not differ between C and PC mothers.

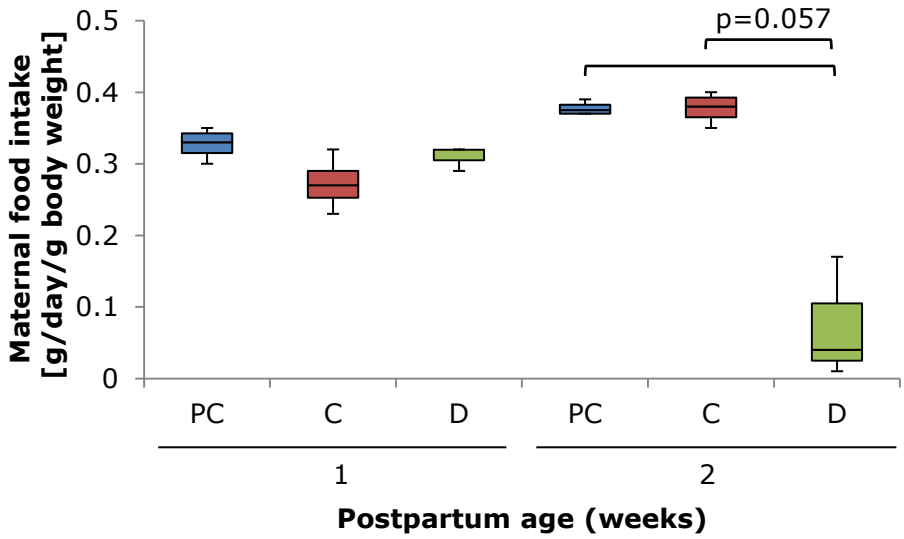

Figure 5.2: Maternal food intake adjusted for body weight as measured at weeks 1 and 2 postpartum.

PC (blue, n=4): mothers fed a diet containing 1 g choline as PC per kg diet; C (red, n=4): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green, n=3): mothers fed a choline-devoid diet; all diets were fed from giving birth to euthanisation at 4 weeks postpartum except for D as described. Data are means and error bars represent SEM.

5.3.2. Effects on maternal organs and fat mass

At 4 weeks postpartum mothers were euthanised and dissected. There was no effect on tissue weight when adjusted for maternal body weight between groups, nor was intestinal length different between groups (Table 5.1).

Table 5.1: Maternal tissue weights at 4 weeks postpartum.

Tissue	PC	C	D
BAT (mg/g)	1.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.1
Gonadal fat (mg/g)	11 ± 2	14 ± 1	11 ± 2
Spleen (mg/g)	2.7 ± 0.2	2.4 ± 0.2	3.1 ± 0.2
Liver (mg/g)	37 ± 1	41 ± 2	43 ± 4
Intestine (cm)	140 ± 1	136 ± 3	137 ± 4

PC: mothers fed a diet containing 1 g choline as PC per kg diet, n=4; C: mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=4; D: mothers fed a choline-devoid diet, n=3; all diets were fed from giving birth to euthanisation at 4 weeks postpartum except for D as described. Data is displayed as means ± SEM. BAT, brown adipose tissue.

Examination of maternal small intestinal morphology demonstrated significant differences in length of villi in all sections (duodenum, jejunum, ileum), with the longest villi in PC mothers and the shortest in D mothers (Figure 5.3).

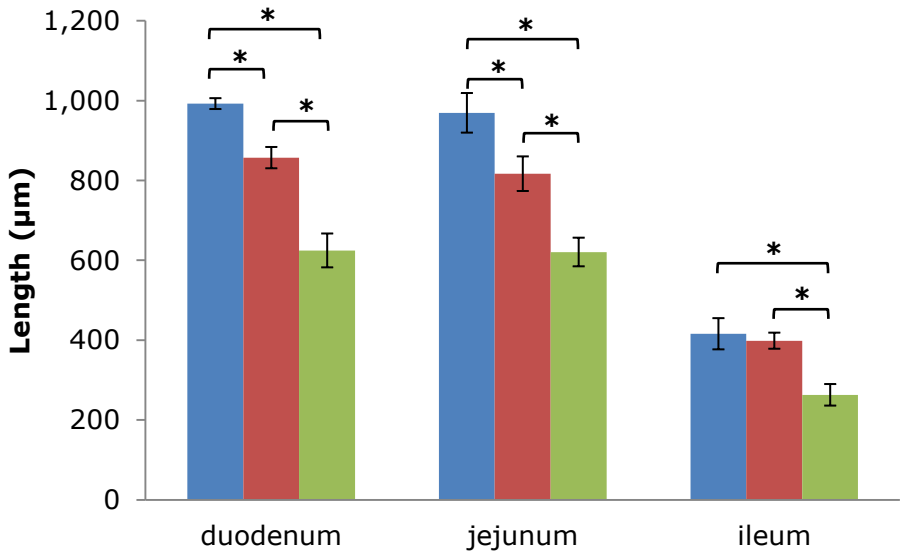

Figure 5.3: Villus length in mothers divided by intestinal section and intervention groups, in samples obtained from the 2009/2010 studies.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, n=4; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=4; D (green): mothers fed a choline-devoid diet, n=4; all diets were fed from giving birth to euthanisation at 4 weeks postpartum except for D as described.

*** $p<0.05$. Data are means and error bars represent SEM.**

Maternal crypt was deeper in D mothers in the duodenum as compared to C and PC and shallower in the jejunum of D mothers as compared to C (Figure 5.4).

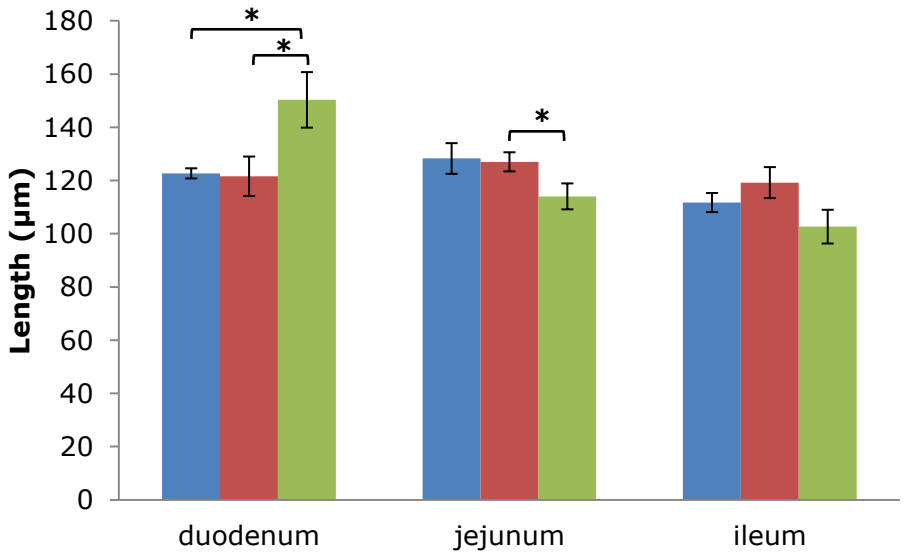

Figure 5.4: Crypt length in mothers divided by intestinal section and intervention groups, in samples obtained from the 2009/2010 studies.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, n=4; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=4; D (green): mothers fed a choline-devoid diet, n=4; all diets were fed from giving birth to euthanisation at 4 weeks postpartum except for D as described.

*** $p<0.05$. Data are means and error bars represent SEM.**

5.3.3. Offsprings' choline intake at the end of lactation

In offsprings' stomach content the highest free choline concentration was found in C offspring and the lowest in D. PC concentration was higher in the PC group than in C or D. Sphingomyelin was lower in the PC group as compared to C and glycerophosphocholine was lower in D as compared to C (Figure 5.5).

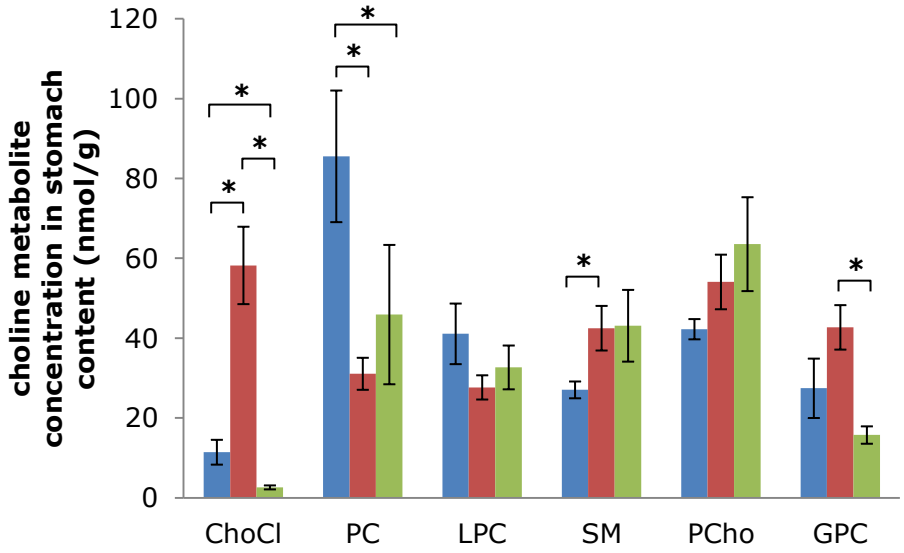

Figure 5.5: Offspring stomach content concentrations of choline chloride (ChoCl), phosphatidylcholine (PC), lyso-phosphatidylcholine (LPC), sphingomyelin (SM), phosphocholine (PCho) and glycerophosphocholine (GPC) in nmol/g, divided by intervention group, at 3 weeks of age, in samples obtained from the 2009/2010 studies.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet (n=8); C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet (n=11); D (green): mothers fed a choline-devoid diet (n=7); all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum.

*** p<0.05. Data are means and error bars represent SEM.**

Despite differences in the forms of choline there was no significant difference in the overall choline content of the stomach contents. Total phospholipid concentration was substantially higher in PC as compared to C and D offspring (Figure 5.6).

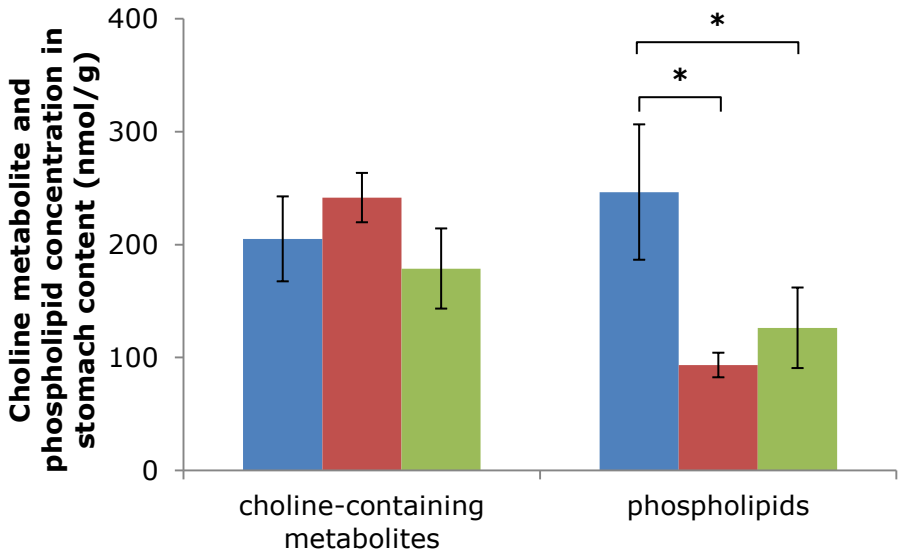

Figure 5.6: Offspring stomach content concentrations of a sum of choline-containing metabolites (choline chloride, lyso-PC, PC, sphingomyelin, phosphocholine and glycerophosphocholine) and of the sum of phospholipids (PC, lyso-PC, PE, lyso-PE, phosphatidylserine, phosphatidylinositol) in nmol/g, divided by intervention group at 3 weeks of age, in samples obtained from the 2009/2010 studies.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet (n=8); C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet (n=11); D (green): mothers fed a choline-devoid diet (n=7); all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum.

*** $p<0.05$. Data are means and error bars represent SEM.**

5.3.4. Offspring growth and food intake after weaning

Offspring weight and length did not differ at 1 week postnatal age but D offspring weighed less than C offspring from 2-5 weeks of age. After weaning at 3 weeks the remaining two offspring per mother were weaned to a diet with 1 g choline as bitartrate per kg diet. D offspring restored their weight by 6 weeks of age. Weights did then not differ significantly after that up to euthanisation at 11 weeks of age (Figure 5.7).

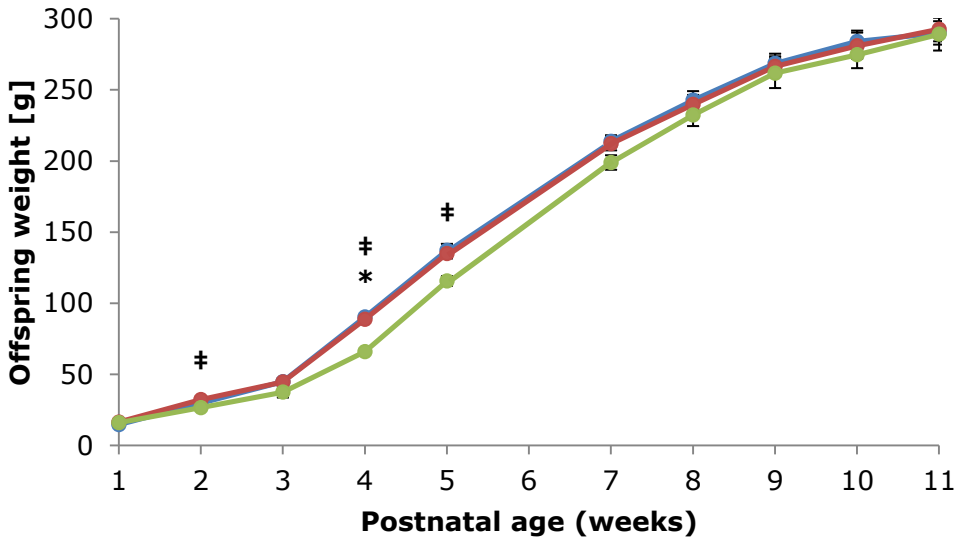

Figure 5.7: Offspring postnatal weight development.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green): mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Offspring data is averaged within litters at 1 and 2 weeks of age (PC, 4 litters; C, 4 litters; D, 3 litters) and numbers are n=8 (PC) and n=7 (C and D) for 3-11 weeks of age.

*** p<0.05 for PC versus D; ‡ p<0.05 for C versus D. Data are means and error bars represent SEM.**

D offspring had lower weight gain during suckling without reaching statistical significance (p=0.057 as compared to PC and C for 1-2 weeks and p=0.057 as compared to PC for 1-3 weeks of age, Figure 5.8).

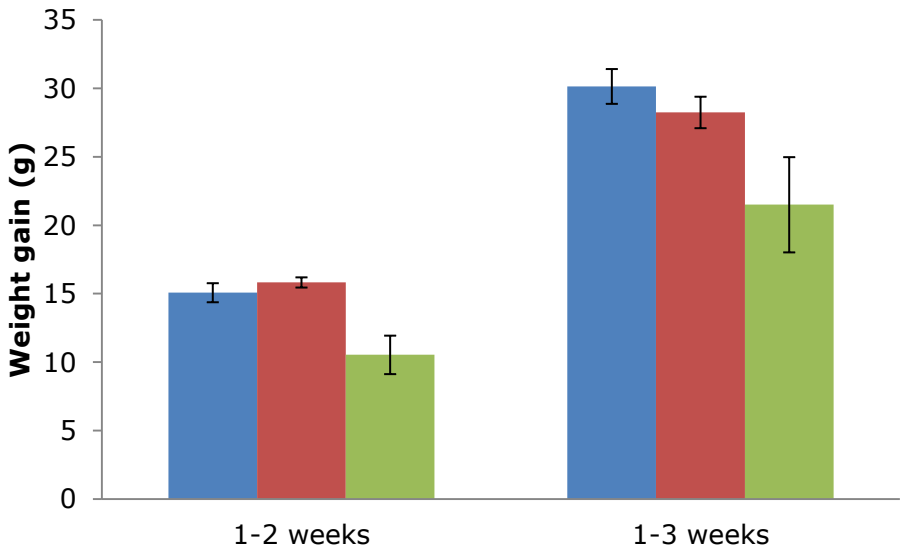

Figure 5.8: Weight gain of offspring between age 1 week and 2 weeks (left) and 3 weeks (right).

PC (blue, 4 litters): mothers fed a diet containing 1 g choline as PC per kg diet; C (red, 4 litters): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green, 3 litters): mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described. Data is averaged within litters. Data are means and error bars represent SEM.

Whilst body weight of the offspring did not significantly differ between groups at weaning at 3 weeks of age, D offspring had less gonadal adipose tissue, but similar brown adipose tissue (BAT) mass, as compared to C and PC offspring (Table 5.2).

Table 5.2: Offspring tissue weights at weaning.

Tissue	**PC**	**C**	**D**
BAT (mg/g)	3.8 ± 0.1	4.0 ± 0.3	3.5 ± 0.5
Gonadal fat (mg/g)	1.7± 0.2 [a]	1.5 ± 0.2 [a]	0.8 ± 0.4 [b]

PC: mothers fed a diet containing 1 g choline as PC per kg diet, 4 litters; C: mothers fed a diet containing 1 g choline as bitartrate per kg diet, 4 litters; D: mothers fed a choline-devoid diet, 3 litters; all diets were fed from giving birth to euthanisation at 3 weeks postpartum except for D as described.

Comparison a vs b (groups PC versus C), p=0.022; a vs b (groups C versus D), p=0.048. Data are displayed as means ± SEM.

After weaning, offspring food intake was measured regularly and when adjusted for body weight, food intake was higher in D and PC as compared to C offspring only at 4 weeks of age (Figure 5.9).

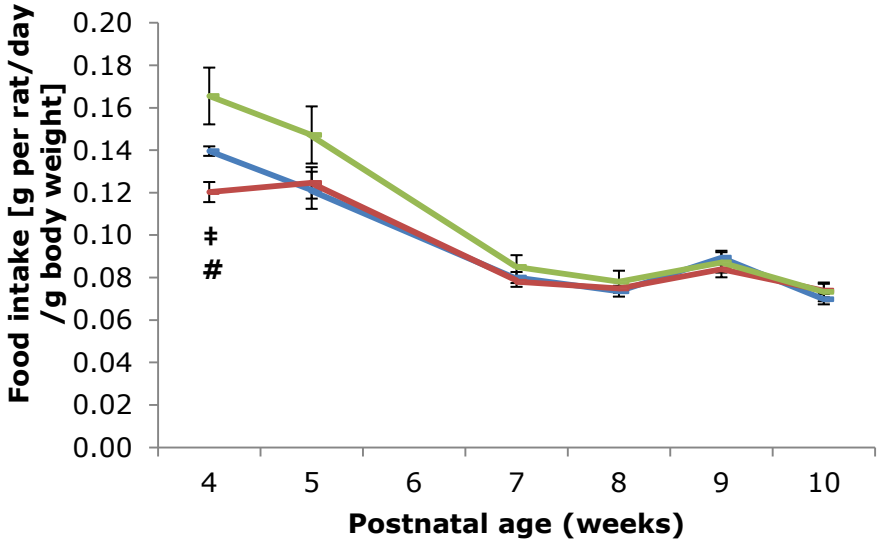

Figure 5.9: Offspring food intake adjusted for body weight after weaning.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, n=8; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=7; D (green): mothers fed a choline-devoid diet, n=7; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet.

‡ $p<0.05$ for C versus D; # $p<0.05$ for PC versus C. Data are means and error bars represent SEM.

5.3.5. Intraperitoneal glucose tolerance test (GTT)

Over the 120 minute course of the GTT at 9 weeks of age neither glucose (Figure 5.10) nor insulin (Figure 5.11) concentrations nor either the area under the curve (AUC) or the homeostatic model assessment for insulin resistance (HOMA-IR,Table 5.3) differed between groups.

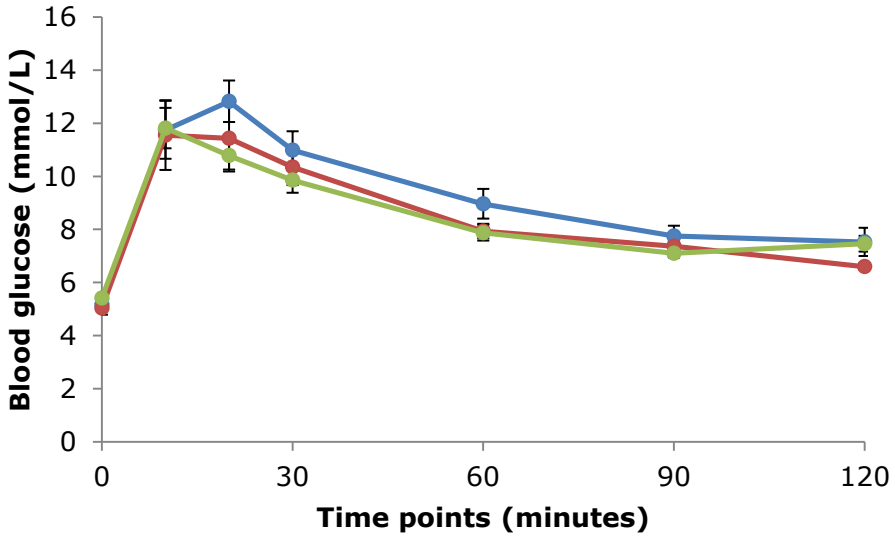

Figure 5.10: Blood glucose concentration measured during the GTT.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, n=8; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=6; D (green): mothers fed a choline-devoid diet, n=7; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Data are means and error bars represent SEM.

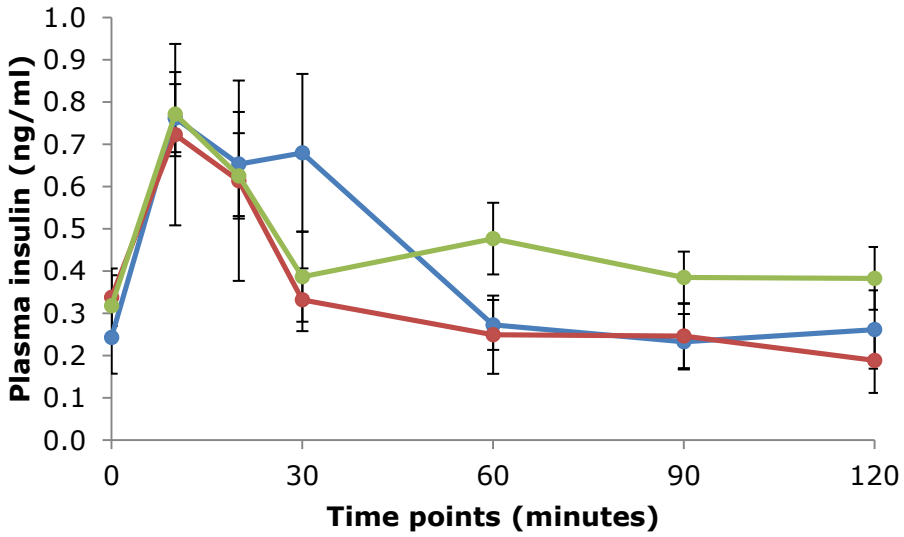

Figure 5.11: Plasma insulin concentration measured during the GTT.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, n=8; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=6; D (green): mothers fed a choline-devoid diet, n=7; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Data are means and error bars represent SEM.

Table 5.3: Characteristics of the GTT: glucose and insulin AUC and HOMA-IR. Glucose was measured in mmol/L and insulin in ng/ml and the AUC determined over 120 minutes.

Measurement	PC	C	D
AUC glucose	486 ± 48	416 ± 42	362 ± 39
AUC insulin	23 ± 5	13 ± 5	22 ± 8
HOMA-IR	1.9 ± 0.6	1.8 ± 0.3	1.5 ± 0.1

HOMA-IR was calculated from baseline glucose and insulin measurements.

PC: mothers fed a diet containing 1 g choline as PC per kg diet, n=8; C: mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=6; D: mothers fed a choline-devoid diet, n=7; all diets were fed to mothers from giving birth to the end of lactation at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Data is displayed as means ± SEM.

5.3.6. Long term effects on offspring organs and body composition

At 11 weeks of age, offspring showed no differences in fat mass or organ weight. The small intestine was slightly shorter in PC as compared to C offspring (Table 5.4).

Table 5.4: Offspring tissue measures at 11 weeks of age.

Tissue	PC	C	D
PAT (mg/g)	22.8 ± 4	28.2 ± 2	23.7 ± 3
BAT (mg/g)	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Gonadal fat (mg/g)	19.7 ± 2	21.9 ± 1	22.6 ± 3
Spleen (mg/g)	2.6 ± 0.1	2.2 ± 0.1	2.6 ± 0.1
Liver (mg/g)	32.2 ± 1	32.8 ± 1	33.3 ± 1
Intestine (cm)	111 ± 1 [a]	119 ± 2	116 ± 1 [b]

PC: mothers fed a diet containing 1 g choline as PC per kg diet, n=8; C: mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=7; D: mothers fed a choline-devoid diet, n=7; all diets were fed from giving birth to euthanisation at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet.

Comparison a vs b, p=0.018. Data is displayed as means ± SEM.

5.3.7. Brain phospholipids

Maternal brain concentrations of phospholipid classes lyso-phosphatidylethanolamine (LPE) and lyso-phosphatidylcholine (LPC) were lower in PC than in C. D adult offspring had significantly lower concentrations of PC and SM as compared to PC offspring. In weanling offspring, C animals had generally lower phospholipid concentrations as compared to PC and D, with significant differences between PC and C for phosphatidylinositol (PI), PE, phosphatidylserine (PS), SM and LPC and

significant differences between C and D for PE, LPE, PC, SM and LPC (Figure 5.12).

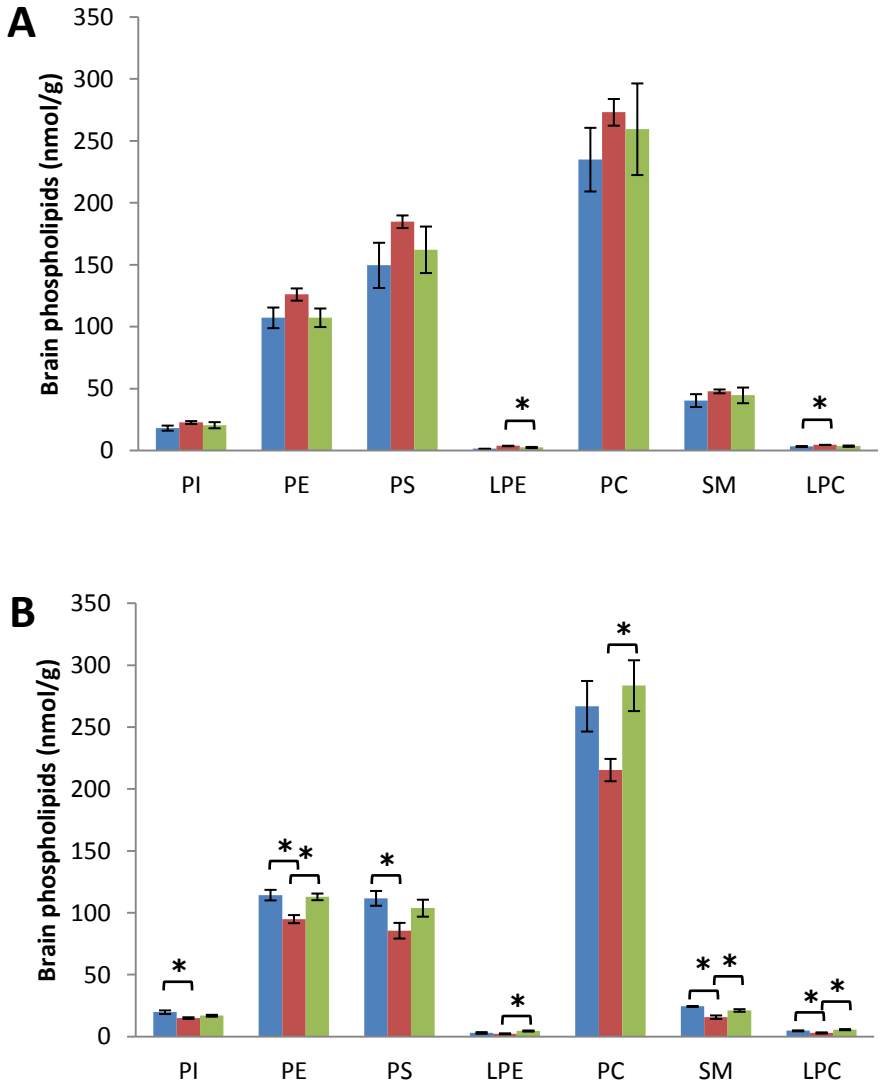

Figure 5.12, continued on the next page.

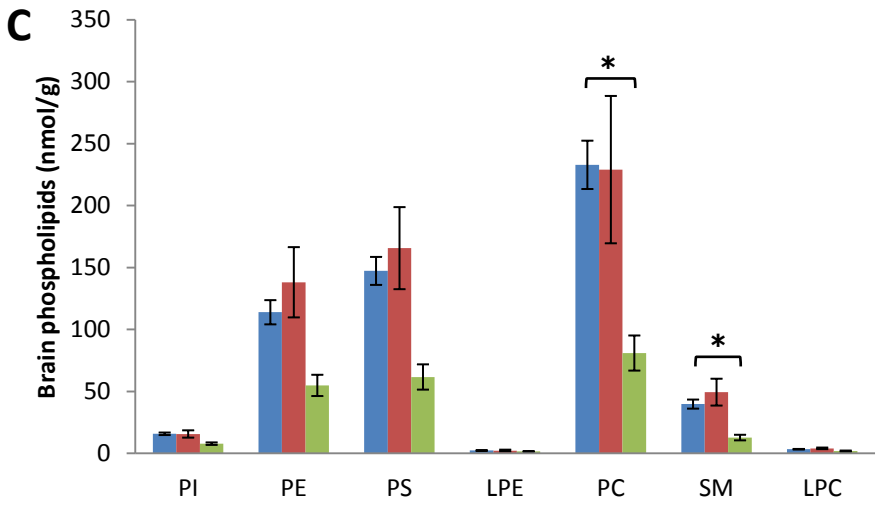

Figure 5.12: Phospholipid class concentrations divided by age groups and intervention groups.

Panel A, mothers;

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, n=4; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=4; D (green): mothers fed a choline-devoid diet, n=3; all diets were fed to mothers from giving birth to euthanisation at 4 weeks postpartum except for D as described.

Panel B, weanling offspring;

PC (blue): offspring raised by PC mothers, 4 litters; C (red): offspring raised by C mothers, 4 litters; D (green): offspring raised by D mothers, 3 litters; offspring were euthanased at 3 weeks of age.

Panel C, adult offspring;

PC (blue): offspring raised by PC mothers, n=7; C (red): offspring raised by C mothers, n=6; D (green): offspring raised by D mothers, n=4; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet.

PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; LPE, lyso-phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lyso-phosphatidylcholine.

*** $p<0.05$. Data are means and error bars represent SEM.**

Total phospholipid classes were not changed in mothers but were lower in D adult offspring as compared to C and lower in C weanling offspring as compared to D and PC (Table 5.5).

Table 5.5: Total phospholipid concentrations in the brain, divided by age groups and intervention groups. Samples from weanlings are taken from offspring at 3 weeks of age, from adult offspring at 11 weeks of age and mother samples are taken 4 weeks postpartum.

Phospholipids (nmol/g)	PC	C	D
Mothers	556 ± 59	663 ± 10	601 ± 72
Weanling	545 ± 31 [a]	432 ± 21 [b]	549 ± 31 [c]
Adult offspring	556 ± 43	712 ± 95 [d]	222 ± 36 [e]

PC: mothers fed a diet containing 1 g choline as PC per kg diet; C: mothers fed a diet containing 1 g choline as bitartrate per kg diet; D: mothers fed a choline-devoid diet; all diets were fed from giving birth to 4 weeks postpartum except for D as described; after weaning at 3 weeks all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Numbers (n) as in Figure 5.12.

Comparison a vs b, p=0.017; b vs c, p=0.021; d vs e, p=0.001. Data is displayed as means ± SEM.

5.3.8. Brain fatty acids attached to PC

When comparing fatty acid classes attached to brain PC, mothers and adult offspring did not show any differences whilst PC weanling offspring had a slightly higher percentage of saturated fatty acid (FA) as compared to C weanlings (Table 5.6 A). Percentages of functional fatty acids arachidonic acid and docosahexaenoic acid (DHA) were not changed in any of the groups (Table 5.6 B).

Table 5.6: Fatty acids attached to PC in the brain as percentage of total fatty acids, divided by age groups and intervention groups;

A, fatty acids as divided into main classes; B, functional fatty acids docosahexaenoic acid (DHA) and arachidonic acid.

A

FA class (%)	PC	C	D
SFA			
Mothers	61 ± 0.3	60 ± 0.7	61 ± 1.2
Weanling	68 ± 0.9 [a]	65 ± 1.0 [b]	66 ± 0.4
Adult offspring	62 ± 0.8	63 ± 0.4	61 ± 0.5
MUFA			
Mothers	26 ± 2.7	27 ± 2.7	26 ± 3.7
Weanling	21 ± 1.2	23 ± 0.6	23 ± 0.2
Adult offspring	26 ± 1.3	25 ± 1.4	27 ± 0.5
ω-3 PUFA			
Mothers	2.5 ± 0.2	2.5 ± 0.1	2.6 ± 0.1
Weanling	1.6 ± 0.1	2.2 ± 0.3	2.0 ± 0.1
Adult offspring	2.2 ± 0.2	2.4 ± 0.1	2.4 ± 0.2
ω-6 PUFA			
Mothers	3.8 ± 0.1	4.2 ± 0.5	3.8 ± 0.4
Weanling	5.2 ± 0.2	5.9 ± 0.4	5.4 ± 0.3
Adult offspring	4.2 ± 0.4	4.2 ± 0.2	5.3 ± 0.7

B

FA (%)	PC	C	D
Arachidonic acid			
Mothers	2.8 ± 0.2	3.1 ± 0.5	2.7 ± 0.2
Weanling	4.4 ± 0.2	4.8 ± 0.4	4.2 ± 0.3
Adult offspring	3.2 ± 0.3	3.3 ± 0.1	3.9 ± 0.3
DHA			
Mothers	1.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.1
Weanling	1.3 ± 0.1	1.8 ± 0.3	1.6 ± 0.1
Adult offspring	1.5 ± 0.1	1.6 ± 0.1	1.7 ± 0.1

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green): mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Numbers (n) as in Figure 5.12.

SFA: saturated FA; MUFA: mono-unsaturated FA; ω-3 PUFA: omega 3 poly-unsaturated FA; ω-6 PUFA: omega 6 poly-unsaturated FA. Comparison a vs b, p=0.043. Data is presented as means ± SEM.

5.3.9. Brain fatty acids attached to PE

None of the fatty acid classes attached to PE in mother's brains, expressed as a percentage of total fatty acids, differed between groups. However, DHA concentrations were lower in PC mothers as compared to D mothers. Weanling brain PE had a higher percentage of mono-unsaturated fatty acids (MUFA) attached in PC offspring as compared to D due to a difference in oleic acid (18:1; PC, 18 ± 1% w/w; C, 14 ± 1% w/w; D, 12 ± 2% w/w; PC versus D, p=0.017). C adult offspring brain PE had a significantly higher percentage of docosahexaenoic acid and overall ω-3 FAs attached to it as compared to PC (Table 5.7), whilst α-linolenic acid (18:3n3; PC, 5.2 ±

0.6% w/w; C, 4.0 ± 0.3% w/w; D, 4.8 ± 0.2% w/w; PC versus C, p=0.040) was higher in PC than in C adult offspring.

Table 5.7: Fatty acids attached to PE in the brain as percentage of total fatty acids, divided by age groups and intervention groups; A, fatty acids as divided into main classes; B, functional fatty acids docosahexaenoic acid (DHA) and arachidonic acid.

A

FA class (%)	PC	C	D
SFA			
Mothers	65 ± 17	42 ± 4	38 ± 2
Weanling	51 ± 2	50 ± 4	48 ± 2
Adult offspring	44 ± 3	40 ± 3	42 ± 4
MUFA			
Mothers	23 ± 13	35 ± 1	31 ± 1
Weanling	18 ± 1 [a]	14 ± 1	13 ± 2 [b]
Adult offspring	31 ± 2	26 ± 1	28 ± 1
ω-3 PUFA			
Mothers	5 ± 5	13 ± 2	17 ± 1
Weanling	13 ± 1	17 ± 3	18 ± 1
Adult offspring	13 ± 2 [c]	18 ± 1 [d]	16 ± 3
ω-6 PUFA			
Mothers	7 ± 1	10 ± 3	13 ± 1
Weanling	17 ± 2	18 ± 1	20 ± 1
Adult offspring	11 ± 1	15 ± 1	14 ± 2

B

FA (%)	PC	C	D
Arachidonic acid			
Mothers	2.0 ± 2.0	6.3 ± 1.8	8.4 ± 0.5
Weanling	12 ± 1	13 ± 1	14 ± 1
Adult offspring	7 ± 1	9 ± 1	9 ± 2
DHA			
Mothers	2 ± 2 [e]	7 ± 3	12 ± 1 [f]
Weanling	12 ± 1	15 ± 3	16 ± 1
Adult offspring	7 ± 2 [g]	14 ± 1 [h]	11 ± 3

PC: mothers fed a diet containing 1 g choline as PC per kg diet; C: mothers fed a diet containing 1 g choline as bitartrate per kg diet; D: mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Numbers (n) as in Figure 5.12.

SFA: saturated FA; MUFA: mono-unsaturated FA; ω-3 PUFA: omega 3 poly-unsaturated FA; ω-6 PUFA: omega 6 poly-unsaturated FA. Comparison a vs b, p=0.040; c vs d, p=0.014; e vs f, p=0.022; g vs h, p=0.025. Data is presented as means ± SEM.

5.3.10. Accumulation of fat in the liver

The concentration of total fat in maternal livers was higher in PC mothers as compared to C mothers. In PC weanling offspring livers had a lower fat content as compared with D weanling offspring in that study (Figure 5.13).

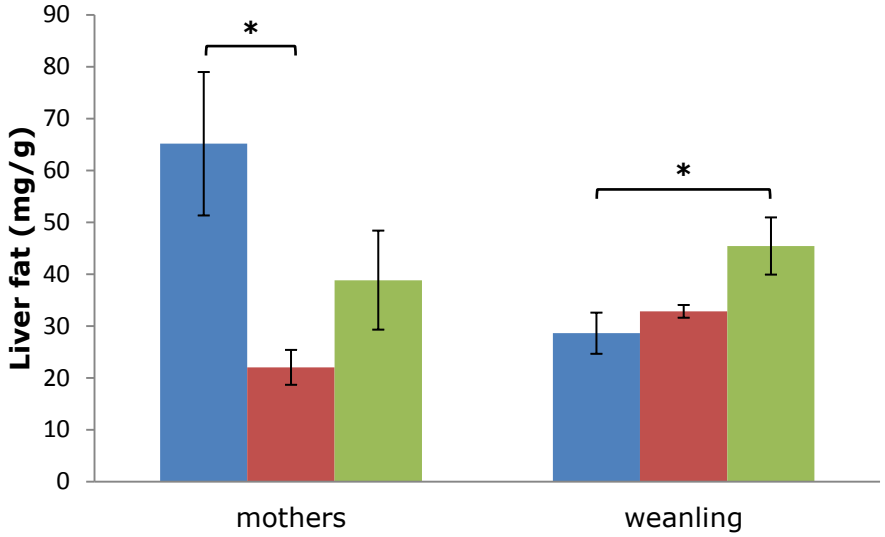

Figure 5.13: Hepatic fat content of mothers and weanling offspring as assessed in the 2009/2010 study.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet, mothers n=8, offspring n=6; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet, mothers n=8, offspring n=7; D (green): mothers fed a choline-devoid diet, mothers n=6, offspring n=7; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum.

*** $p<0.05$. Data are means and error bars represent SEM.**

Choline-containing phospholipids and PE were measured in C and D but, as a PC diet group was not included in the 2009 study these livers were taken from, not PC mothers. Expressed as metabolite content for the entire liver, D mothers had lower stores of PC and betaine (Table 5.8). In the 2009 study, livers of D mothers were lighter than of C mothers (8.8 ± 0.7 g and 11.4 ± 0.4 g, respectively, p=0.005).

Table 5.8: Estimation of choline metabolite content for the whole liver of C and D mothers, i.e. mg/liver, as assessed in the 2009 study.

metabolite (mg)	PC	free choline	betaine	PE	SM
C	113 ± 6 [a]	0.4 ± 0.1	1.6 ± 0.1 [c]	39 ± 4	9.3 ± 0.4
D	76 ± 7 [b]	0.5 ± 0.2	1.2 ± 0.1 [d]	38 ± 5	8.3 ± 0.5

C: mothers fed a diet containing 1 g choline as bitartrate per kg diet, n=8; D: mothers fed a choline-devoid diet, n=6; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum.

Comparisons a versus b, p=0.002; comparison c versus d, p=0.032. Data is presented as means ± SEM.

5.3.11. Effects on appetite regulation in the ARC: NPY and α-MSH

The number of NPY-positive cells counted in the ARC and standardised for area was higher in PC mothers as compared to D mothers. There was no difference observed between any of the adult or weanling offspring groups (Figure 5.14).

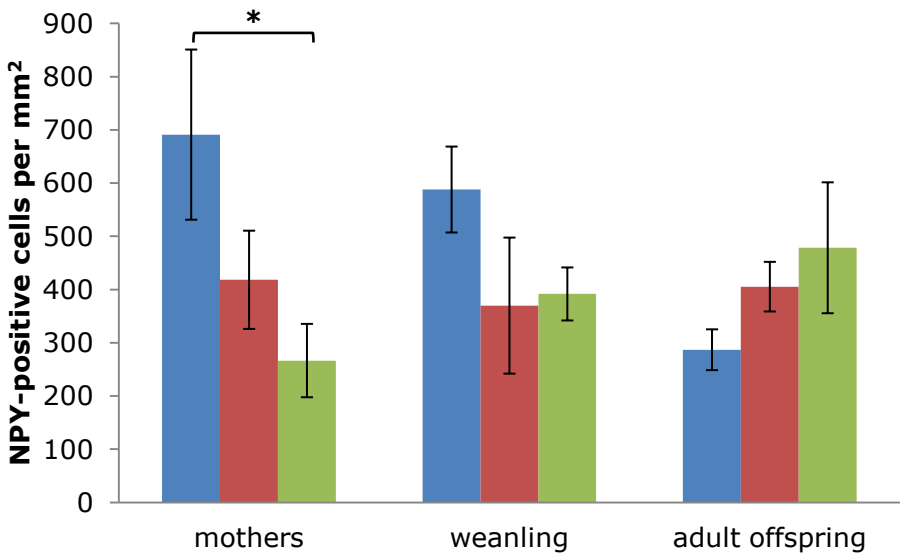

Figure 5.14: Number of NPY-positive cells counted in the ARC and standardised for area, divided by age groups and intervention groups.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green): mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to the end of lactation at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Numbers (n): mothers, PC n=2, C n=4, D n=2; weanling offspring, PC n=7, C n=6, D n=5; adult offspring, PC n=6, C n=5, D n=7.

*** $p<0.05$. Data are means and error bars represent SEM.**

Number of α-MSH-positive cells counted in the ARC and standardised for area did not differ between any of the groups but was highest in D weanling offspring (Figure 5.15).

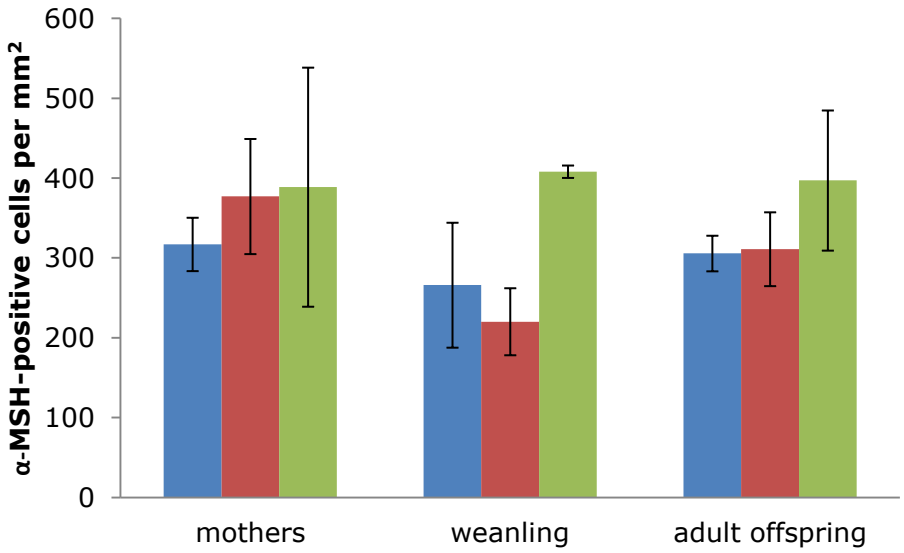

Figure 5.15: Number of α-MSH-positive cells counted in the ARC and standardised for area, divided by age groups and intervention groups.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green): mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Numbers (n): mothers, PC n=2, C n=4, D n=2; weanling offspring, PC n=7, C n=6, D n=5; adult offspring, PC n=6, C n=5, D n=7. Data are means and error bars represent SEM.

The ratio of NPY- to α-MSH-positive cells within the ARC and standardised for area did not differ between any of the groups (Figure 5.16).

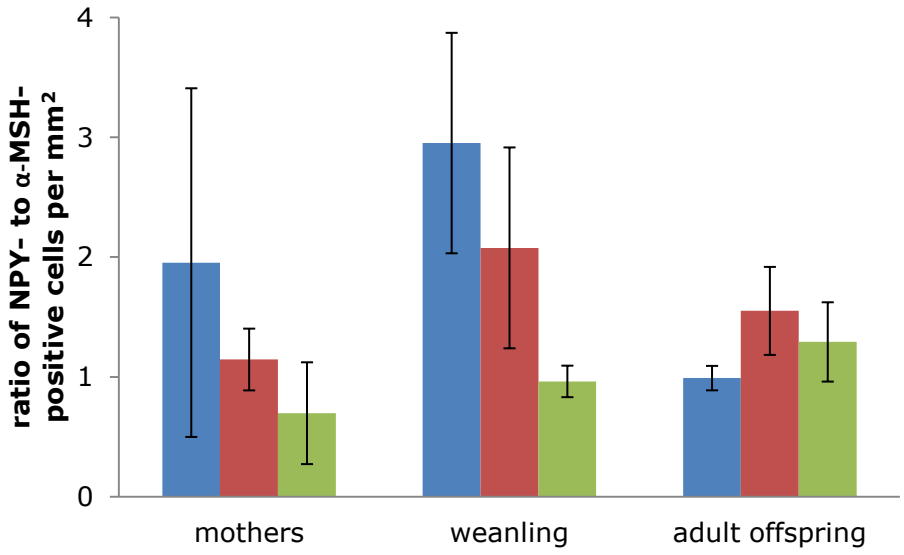

Figure 5.16: Ratio of NPY- to α-MSH-positive cells counted in the ARC and standardised for area, divided by age groups and intervention groups.

PC (blue): mothers fed a diet containing 1 g choline as PC per kg diet; C (red): mothers fed a diet containing 1 g choline as bitartrate per kg diet; D (green): mothers fed a choline-devoid diet; all diets were fed to mothers from giving birth to weaning at 3 weeks postpartum except for D as described; after weaning all offspring were fed a diet containing 1 g choline as bitartrate per kg diet. Numbers (n): mothers, PC n=2, C n=4, D n=2; weanling offspring, PC n=7, C n=6, D n=5; adult offspring, PC n=6, C n=5, D n=7. Data are means and error bars represent SEM.

Due to the experimental difference between the 2011 and 2009/2010 studies explained at the beginning of this result section, interpretation has to take into account the length of feeding a choline-devoid diet to mothers. For an easier overview, information about which study specific data is taken from is listed below (Table 5.9).

Table 5.9: Overview of data origin.

Results taken from the 2011 study (D, choline-devoid diet day 1-16)	**Results taken from the 2009/2010 studies** (D, choline-devoid diet day 1-21)
all growth data	intestinal morphology
all food intake data	stomach content data
all tissue weights at dissection	maternal liver lipids and choline contents
glucose tolerance test	offspring liver lipids
all brain data	

5.4. Discussion

5.4.1. Effects of feeding a choline-devoid diet during lactation

Diminishing mother's choline intake during lactation led to a trend to lower maternal food intake and changes in intestinal morphology (shorter villi in the jejunum, ileum and duodenum without a change in intestinal length) that may result in disturbed nutrient uptake. Consistent with reduced nutrient availability through either a potential decrease in intake and/or a reduction in nutrient absorption, offspring of mothers fed the choline-devoid diet during early lactation grew more slowly and had a smaller gonadal fat pad at 3 weeks of age. Although the total choline metabolite concentrations in the offsprings' consumption of mother's milk were similar at the end of lactation, there were differences in the form of choline due to diet. At this point D weanling offspring had higher liver lipid concentrations and showed higher concentrations of brain phospholipids. Offspring reached the same weight as offspring whose mothers were fed choline-containing diets by adulthood and also showed no other differences in organ and fat masses, except that phospholipid concentrations were now much lower in the brain. This suggests that growth differences can be compensated for by providing sufficient choline after early lactation but there may be lasting effects on the lipid composition of the brain.

5.4.1.1. Acute effects on maternal outcomes

With regards to acute effects in D mothers as measured at 4 weeks post partum it must be considered what influence the re-introduction of choline into their diet had (1 g/kg for days 16-21 postnatal age and 0.1 g/kg from day 21 to euthanisation on day 28). The immediate effects on maternal

body weight were rapid and considerable. The effects we observed in the hypothalamic neurons at week 4 are, therefore, unlikely to be a direct effect of choline depletion as appetite regulatory signalling including neurotransmitter expression in neurons of the ARC is a rapidly responding system. Measurement of hypothalamic NPY and α-MSH would therefore, at week 4, most likely reflect the effect of a low-choline diet rather than the earlier choline-devoid diet and, therefore, a milder form of depletion. D mothers re-gained weight quickly after choline was re-introduced into their diet and although food intake was not recorded during this time it is very likely that this weight gain was accompanied by higher food intake. Overfeeding has been associated with a voluntary reduction of food intake, associated with a slight reduction in NPY [507] and an increase in pro-opio melanocortin (POMC) gene expression in the ARC [508]. Rapid weight gain in choline-depleted D mothers could, therefore, indeed be associated with a decreased ratio of NPY- to POMC-positive neurons in the ARC once normal weight is re-established, i.e. the same weight as C and PC mothers. Therefore, the results of hypothalamic immunohistochemistry at 4 weeks post partum do not allow to draw conclusions about the effect of choline depletion on the mothers' appetite regulatory system. From the data shown it can be assumed that the effects of feeding a choline-devoid diet seen on maternal food intake is not a mere effect of palatability as food intake after the first week on this diet is not changed compared to C and PC. A lower food intake is only seen once choline depletion has been established. The reduced food intake during choline depletion could be a dysfunction in feedback mechanisms through an unknown mechanism or it could indeed be a direct effect of choline deficiency in the form of a lack of acetylcholine signalling to the muscarinic acetylcholine receptor 3 (M3R) in secondary neurons.

A knockout model of the M3R shows a lean, hypophagic phenotype [509]. Consistent with that, blocking M3R with the antipsychotic drug olanzapine increases M3R binding capacity, food intake and body weight in a dose-dependent manner [510]. In the ARC, POMC-positive neurons also secrete acetylcholine and in the fasted state, i.e. when α-MSH concentrations are low, acetylcholine has an orexigenic effect by increasing melanin-concentrating hormone (MCH) expression in the lateral hypothalamic area (LHA) [511]. A lack of acetylcholine in the maternal brain could, therefore, affect appetite regulation directly. This is a hypothesis that would need to be explored further in following studies.

In all 3 studies we conducted with a design of feeding lactating mothers a choline-deficient diet (2009-2011) we saw the same decrease in food intake of these mothers after feeding this diet for a week, although this change did not reach significance in the 2011 data presented above. However, most other studies conducting experiments with choline-devoid diets in adult or pregnant rodents do not report this striking change in food intake. The reasons for this difference are unknown and, to my knowledge, have not been discussed previously.

A study conducted by Copeland et al. feeding choline-devoid diets to adolescent rats showed that 20% of rats lost body weight rapidly and died within the first 6 weeks on the choline-devoid diet. Pregnant females, lactating mothers and weanling offspring have increased requirements for choline relative (for secretion with milk and utilised in growth processes, respectively) to their body weight as compared to non-pregnant, non-lactating adult rats and might, therefore, show signs of rapid choline depletion which might differ from physiological signs of a slower depletion. However, in other studies with 3 week old rats a normal weight gain despite feeding a choline-devoid diet for 12 weeks was reported [512],

suggesting that weanling animals are not necessarily more vulnerable to choline depletion.

Deprivation of young male rats of zinc, another micronutrient essential for tissue growth [513], induced a decrease in their food intake by about 30% and rats stopped growing [514]. Besides a lower food intake, the growth rate relative to energy intake is inefficient in these animals, likely due to structural and catalytic effects of zinc [515, 516]. The mechanism by which food intake is decreased remains unclear [517]. Deficiency of other developmentally important micronutrients, including vitamin B6 [518], inhibit food intake as well whilst deficiency of most micronutrients, including selenium [519], vitamin D [520] and iron [521], do not result in changed food intake or growth rate in young rodents.

Even though a choline-devoid diet does not show an effect on appetite regulation of adult animals [522, 523], a diet devoid of both choline and methionine, a common intervention to induce hepatic steatosis, decreases food intake and body weight substantially [523-525]. This effect cannot be due to methionine alone as reducing diet content of methionine in presence of folate does not substantially decrease food intake [526, 527]. The mechanism through which methionine-choline-deficient diet decreases food intake is not known but, since both are interrelated in the one-methyl pathway, methylation processes or homocysteine may be involved in this mechanism in an as of yet unexplored manner. However, a diet devoid of both choline and folate again has no effects on body weight [528], making it less likely that methyl donor availability is the mechanism involved here. The diet used in our study had approximately 7.3 g/kg methionine (based on the assumption that casein has about 3% methionine [529]) which is considerably higher than the 3 g/kg that is used as a control diet next to MCD diet (cat no. 0296044010, MP Biomedicals, Illkirch, France) and,

therefore, D mothers were unlikely to have a methionine-and choline-deficient diet.

Most studies applying a choline-devoid diet during pregnancy only subject mothers to a short period of this diet, typically gestational day 11-17 [368, 377], which is shorter than the period necessary in our study to induce hypophagia in D mothers (2 weeks). A lack of dietary choline during this time frame of pregnancy resulted in long term changes in the hippocampus of the offspring but has not been reported to produce changes in food intake or weight of mother and offspring [368].

Lactation period is a period of an even higher requirement for choline than pregnancy, as the main storage forms of choline in the liver have been shown to be decreased whilst liver triglyceride concentrations are higher in lactating rats as compared to pregnant or non-mated rats when all 3 groups received the same choline concentration in their diets [504]. When feeding a choline-devoid diet to lactating rats, hepatic choline metabolite concentrations are further reduced [504]. Whilst this study did not mention an impact on maternal and offspring weight development or food intake, Liapi showed that feeding a choline-devoid diet only during pregnancy does not change offspring weight but if this diet is continued throughout lactation, offspring are 46 % smaller at weaning as compared to control offspring [530]. This supports our findings that there is a difference in response to feeding a choline-devoid diet during lactation. However, this finding was not further explored and maternal weight development or food intake were not reported in this study.

Because PC is closely involved in lipid digestion [497] and transport [358] it could be reasoned that choline-deprived animals develop an aversion for dietary fat as a dysfunction in lipid absorption has unpleasant side effects

[531]. Since our maternal intervention diets were relatively high in fat, compared to other rodent diets, a lipid aversion could have explained the sudden drop in food intake of D mothers. Raubenheimer et al. showed that a choline-devoid diet does not have an effect on food intake of adult male mice both in low- and high-fat diets [522] but this is a concept that could be further explored in lactating animals due to their apparent vulnerability to the effect of choline depletion on appetite.

D mothers' brain phospholipid concentrations and fatty acid profile of brain PC were not changed but brain PE had a higher percentage of DHA attached as compared to PC mothers. This may suggest a decrease in PC biosynthesis through the PEMT pathway as PE with long-chain poly-unsaturated fatty acids (PUFA) attached are more likely to be converted to PC than PE with shorter, saturated fatty acids attached [532]. However, in an acute need for PC, PEMT activity is unlikely to be reduced. It is more likely that brain phospholipid turnover is slow, as the general lack of change suggests, leaving no explanation for the change in DHA percentage.

Taken from the 2009/2010 studies and, therefore, without choline re-introduction in D mothers, finding that villi are shorter suggests that nutrient absorption may be disturbed in these animals as villi increase the absorptive area of the intestine [533]. If shorter villi have an effect on nutrient uptake into the blood stream, e.g. lower plasma glucose concentrations, this could increase food intake [180] but cannot explain the decrease of food intake observed in these mothers. The shorter villi could be caused either by the lack of choline, i.e. during the rapid turnover of villi cells there was not sufficient choline available for cell replication, or it is feasible that the shorter villi are actually a result of the decreased food intake or a combination of both. Since nutrients from the gut lumen are

crucial for cell proliferation, food deprivation is associated with lower rates of cell replication and this process is reversible by refeeding [534, 535]. Furthermore, the dysfunction of lipid absorption that can be assumed to take place in D mothers [536] may contribute to changes in intestinal morphology since orlistat, a pharmacological lipase inhibitor which decreases lipid absorption, has the side effect of damaging the brush-border membrane and connective tissue of villi [537]. Therefore, it is unclear whether the changes in intestinal morphology are directly due to choline depletion or are secondary to reduced food intake or disturbed lipid uptake and more research would be necessary to ascertain the mechanism behind this. Intestinal crypts are primarily made up of epithelial cell and stem cells, with proliferation rates regulated in reaction to normal cell turnover and injury [538]. If the shortened villi in the duodenum are indeed disadvantageous for nutrient uptake this may induce the response of crypt cell proliferation, which we showed to be longer in D mothers. However, this response was not seen in jejunum or ileum.

5.4.1.2. Effects on maternal choline metabolism and milk production

Mothers fed a choline-devoid diet are partially able to compensate for lack of dietary choline in the milk they express, as overall choline content is not different and milk only differs in concentration of specific choline-containing metabolites as compared to milk from C and PC mothers. This lack of difference in overall choline content is not due to the re-introduction of choline into the diet of D mothers which occurred in the 2011 study, as the stomach contents collected were from 2009/2010 offspring after feeding a choline-devoid diet to mothers throughout the lactation period. Measurements for mother's milk were carried out in the offsprings' stomach content, which at 21 days of age represents mother's milk but

may also contain an amount of the solid diet provided for mothers since rat offspring start consuming solid foods around 17 days postnatal age [539]. Our results show that offspring are primarily protected from short term maternal choline deficiency with regards to total choline concentrations. However, offspring are smaller and have less central body fat mass at weaning and show differences in adult brain phospholipid concentrations, suggesting that there are changes in mother's milk that have acute and long term effects on the offspring.

In lactating mothers, choline and choline-containing phospholipids secreted in milk are likely to be derived from both biosynthesis and storage forms in the liver and other tissues. Rate of biosynthesis and the extent of utilisation of stores may depend on dietary choline availability. In the 2009/2010 studies livers were lighter in D as compared to C animals, likely due to glycogen mobilisation in response to reduced food intake to maintain stabile plasma glucose concentrations. When choline metabolites were expressed as total metabolite content per liver rather than per gram liver to compensate for the change in size, the amounts of PC and, to a smaller extent, betaine were lower in D livers. This suggests that liver-stored PC was used in milk production in these animals. The reduction of PC in the liver did not lead to steatosis whilst in other studies feeding choline-devoid diet liver PC is not necessarily further reduced to induce steatosis [540]. However, hepatic steatosis has only been shown to be induced by feeding a choline-devoid diet in male rodents, not females, probably due to the higher PEMT activity in females caused by oestrogen [541]. This protection may persist despite depletion of liver PC stores in D mothers.

The reduction in food intake may indicate the distinctiveness of choline metabolism during the lactation period, which needs to be explored in further studies.

5.4.1.3. Acute effects on offspring

It is, so far, not entirely clear how feeding choline in different forms affects the availability and the tissue distribution of choline. It has to be assumed that D offspring had access to feed, i.e. mother's milk, containing similar overall choline concentrations as compared to C and PC but the amount of milk ingested and, with that, total choline intake rather than choline concentrations could not be assessed.

In rodents, macronutrient restriction during lactation decreases offsprings' body weight at the time point of weaning [542, 543]. This suggests that if rodent mothers are undernourished to a high degree during lactation, as was the case for D mothers after the first week of lactation in our study, this will indeed have direct impact on offsprings' development. This effect may be mediated by a lower milk output or by a lower energy density of the milk. In humans, general malnutrition reduces milk output and fat content of the milk [544, 545]. If milk consumption in D offspring was indeed lower than in C and PC offspring then this would mean that D offspring ingested less choline during lactation. The lower growth rate seen in D suckling offspring could, therefore, be caused by a lower availability of nutrients, including choline, secondary to mother's loss of body weight. An overall decrease in choline intake secondary to lower milk intake in D offspring could help explain the increase in liver lipid concentrations at weaning as production of very low-density lipoproteins (VLDL) may be impaired in these animals. However, it is not clear why brain phospholipid

concentrations would be higher in D as compared to C offspring if their overall availability of phospholipids was limited, compared to C and PC offspring. However, in the 2011 study we had to provide choline (as free choline) to the mothers during the last week of lactation as weight loss was very high and this may have affected the availability of choline to the developing brain in the offspring of D mothers. This might suggest that there is preferential choline deposition in the brain. Further tests may show whether this change is phenotypically relevant by changing behaviour of these animals.

There are some experimental designs described [546] that measure offsprings' food intake during suckling and these could be considered for future studies. It would be of interest to express mother's milk directly [547] instead of drawing conclusions about maternal metabolism from offsprings' stomach content. To closer monitor offsprings' choline availability throughout lactation it may, additionally, be desirable to euthanise one offspring per mother per week and collect their stomach contents. This would help determine if choline content of milk changes during lactation and if there is a critical period for later effects on the offspring.

5.4.1.4. Long term effects on offspring

Outcome measures in adult offspring that were significantly affected by a lack of choline in the maternal nutrition during lactation were not a mere continuation of the effects of this treatment at weaning. Early postnatal growth is commonly manipulated through two different study designs in rodents, with differing long term results. Similar to the findings of our study, directly restricting maternal food intake during lactation to 50%

showed an acute growth restriction of weanling offspring but no difference in body weight at an adult age of 23 weeks postnatal as compared to controls [542]. However, rats which were delayed in growth by being raised postnatally in a bigger litter than control animals, did not catch up in body weight after weaning [221, 548], which was associated with a lower food intake throughout lactation and adulthood. What exactly the difference between these two interventions might be is unclear. In order to determine whether any of the long term effects we saw in the offspring were indeed effects mediated by a difference in maternal choline intake or whether the delay in early growth was the main influencing factor it would be necessary to introduce a group pairfed to the D group.

The intervention did also not show an effect on offsprings' glucose homeostasis or appetite regulation. We had assessed brain phospholipids and fatty acid composition of PC and PE in these animals to gain some insights into structural changes, which might underlie behavioural changes connected to choline depletion during development. The changes in brain phospholipid concentrations were striking and it would be very interesting to see if these observations could be repeated in studies of animals with long term changes in spatial memory due to gestational choline depletion. Although phospholipid concentrations were drastically changed, fatty acids attached to PC and PE did not show any significant changes compared to C offspring.

Cholinergic signalling has recently been implied in myelination processes in the brain and also in the decline of myelination with age as is associated with Alzheimer's disease [549, 550]. Specifically, inhibiting breakdown of acetylcholine delays progression of Alzheimer's disease [550]. Since myelin is making up a major part of neuronal lipid mass, it is feasible that demyelination is associated with a lower phospholipid content seen in adult

D offspring. In order to get a better idea if this is indeed the case, brain acetylcholine content and neuronal degree of myelination should be determined in future studies. Since myelination starts just before birth and continues throughout lactation period until about 6 weeks of age in the rat [551], it would make sense that lower choline availability during early suckling influences myelination processes but in D offspring at weaning there is no indication of that as brain phospholipid concentrations are even higher than in C offspring. Again, a closer look at brain characteristics may give further ideas as to what mechanisms are involved and what consequences that might have for the animals' behaviour.

Figure 5.17 summarises findings and possible mechanisms of this comparison. D mothers are fed an isocaloric diet devoid of choline. Through an unknown mechanism mothers decrease their food intake drastically (although this did not reach statistical significance in this study), which is likely mediated through central appetite regulation, possibly through disturbance of acetylcholine signalling. Intestinal morphology is changed in these mothers with shorter villi in all 3 sections of the small intestine. This may be caused by decrease in choline availability or be secondary to the reduction in food intake. Hepatic PC stores are depleted but mother's milk choline concentrations are not altered. However, it is likely that the volume of mother's milk produced was reduced in D mothers, which would reduce offspring caloric and choline intake, leading to a reduced early postnatal growth rate. In the adult D offspring concentrations of phospholipids were drastically reduced, which may be associated with a loss of myelination and manifest in behavioural changes.

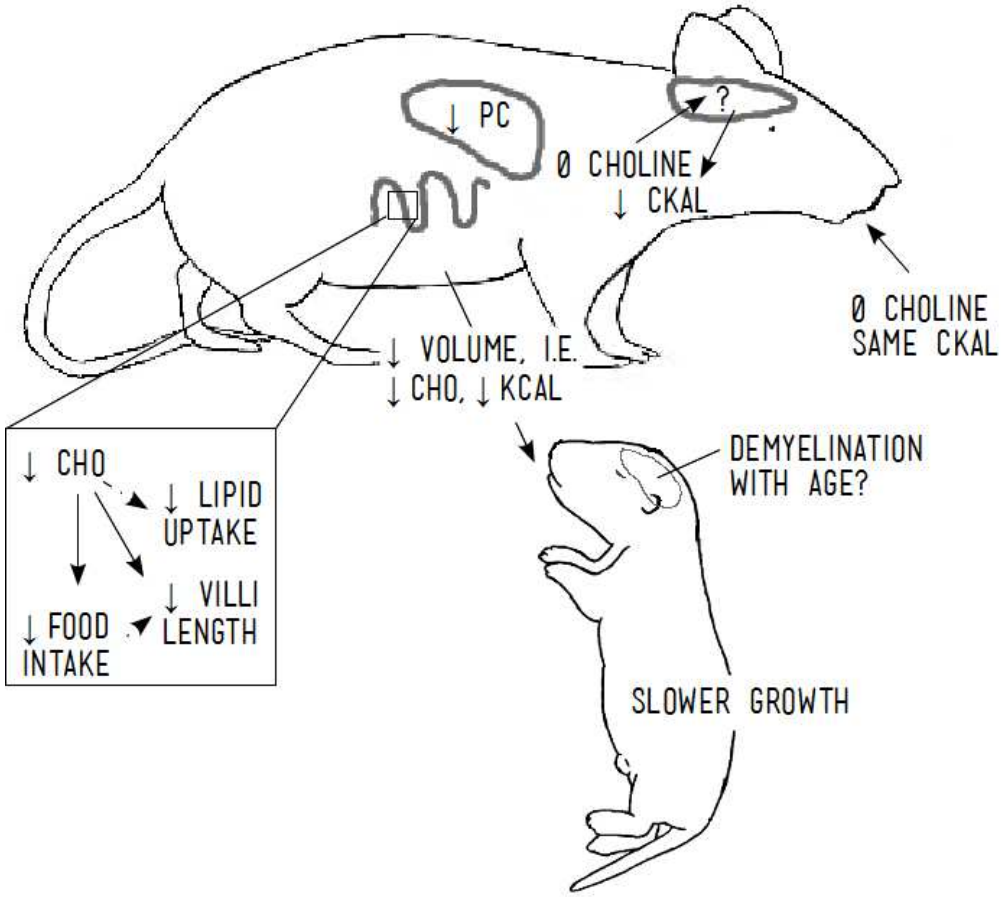

Figure 5.17: Overview of changes induced by feeding lactating mother a diet devoid of choline as compared to a standard amount of choline as bitartrate. Mothers have decreased food intake, intestinal villi are shorter and a disturbed lipid uptake can be assumed. Maternal liver PC is decreased and mother's milk is assumed to be decreased in volume although choline concentration itself is not changed, leading to a lower growth rate in offspring. In adult offspring, brains show a substantial decrease in phospholipid concentration, which may be associated with loss of myelin.

5.4.2. Effects of feeding choline in differing forms during lactation

Feeding mothers a different form of choline during lactation, i.e. PC as compared to choline chloride, had less pronounced effects than feeding a diet without choline but it is remarkable that there were differences between these mothers and offspring at all.

5.4.2.1. Acute effects in mothers and offspring

PC mothers showed longer intestinal villi and a 3 fold increase in liver lipids compared to mothers fed the C diet but there was no difference in maternal and offspring weight at any time. The increase in hepatic lipids in PC-fed mothers was unexpected since choline depletion, not choline supplementation is known to induce hepatic steatosis in males. However, there is no indication that 60 mg fat per g liver, i.e. 6% w/w fat, constitutes steatosis, which has, in other rat studies, been defined as 60% fat in hepatocytes as determined by histology [552] and over 25% in a magnetic resonance measurement of hepatic lipids [524]. A small part of the higher hepatic lipid content in PC mothers may be explained by the pooling of PC in liver due to its lipophilic nature. Dietary PC rather than free choline is stored primarily as PC (85%) in the liver, without further conversion, whilst free choline is primarily stored as betaine (85%) and only 15% as PC [553]. It has recently been shown that injecting mice with PC-rich HDL leads to accumulation of PC in the liver and that 30% of the PC is converted to triglycerides [554]. By feeding choline as PC rather than salt, PC-content of chylomicrons may be increased, which subsequently reach the liver and induce an increase in triglycerides there, even if the effect may not be large. To my knowledge, this has not been described before as choline supplementation studies commonly use choline salts, not PC, as the dietary supplement. In future studies, the choline phospholipid concentrations of the liver of mothers and offspring would have to be analysed, as was already carried out for C and D mothers, and compared with the other groups.

Milk of PC mothers had lower concentrations of free choline and sphingomyelin and a higher concentration of PC and overall phospholipids but no difference in overall choline concentration as compared to C and D

mothers. Choline concentrations in mother's milk are thought to be derived both from plasma and, with that, liver stores and from biosynthesis [502]. Our finding of higher PC concentration in milk of mothers fed PC is, therefore, consistent with studies by Cheng et al. as described on the previous page [553], which showed that dietary PC is predominantly stored as PC in liver, as these stores may then be utilised to reach the high choline concentrations usually found in mother's milk.

5.4.2.2. Long term effects in offspring

Brain phospholipid concentration of PC offspring was higher at weaning by 26% as compared to C offspring, which may reflect an acute effect of higher phospholipid intake and availability for brain deposition. In adulthood, PC offspring had a lower percentage of DHA and overall ω-3 FA but higher α-linolenic acid attached to brain PE, whilst total concentration of PE in the brain was not changed. The changes in ω-3 FAs attached to PE may have implications for membrane fluidity and anti-inflammatory signalling [354]. Brain concentrations of ω-3 FAs and specifically DHA have been negatively associated with anxiety-like behaviour in rats [555]. In animals with high choline availability in the form of PC rather than choline, it could be assumed that activity of PC biosynthesis through the PEMT and CDP-choline pathways was reduced. Changes in PC fatty acids may indicate PEMT activity and the contribution of this pathway to the PC present in a tissue. There were only few differences in PC fatty acids in brains of this study and certainly not of the magnitude described by DeLong et al. [556]. This paper showed that PC synthesised through the CDP-choline pathway has predominantly medium length, saturated fatty acids attached whilst PC synthesised from PE has more long chain, poly-unsaturated fatty acids.

This suggests that feeding choline as PC or free choline did not make a difference in adult offsprings' brain PC biosynthesis.

In the PEMT pathway, the molecular species of PE used has PUFA attached [532]. As PEMT activity is reduced when PC is available [557] it would follow that percentage of PUFA attached to PE is preserved in animals with higher PC availability. However, in adult PC offspring there is a lower percentage of ω-3 FAs attached to PE, suggesting that there may be another, unknown mechanism underlying this change.

In further studies, it will be of interest to test if differences in behaviour can be found between animals fed diets with different forms of choline. Small intestine of adult PC offspring was 7% shorter compared to C offspring. How these changes may be mediated by systemic PC availability is unknown.

Feeding the PC diet instead of the C diet led to a bigger difference in many of the outcomes measured as compared with D animals. This suggests either that the bioavailability of PC is better than that of choline bitartrate or that differences arise from limited capacity of the conversion from free choline to PC and vice versa through the CTP-choline pathway. Studies on bioavailability are few but they show that PC leads to higher and longer lasting elevation of blood choline concentrations [347, 349, 553] and are, therefore, consistent with my results.

Figure 5.18 summarises the findings and potential mechanisms for mothers fed a diet containing PC rather than free choline and their offspring. Mothers' villi are longer and liver triglyceride concentrations are higher. Mothers' milk contains higher concentrations of PC and phospholipids and their offspring show changes in brain composition as they have a higher phospholipid concentration at weaning age and a higher

percentage of MUFA but lower percentage of ω-3 PUFA attached to brain PE at a young adult age.

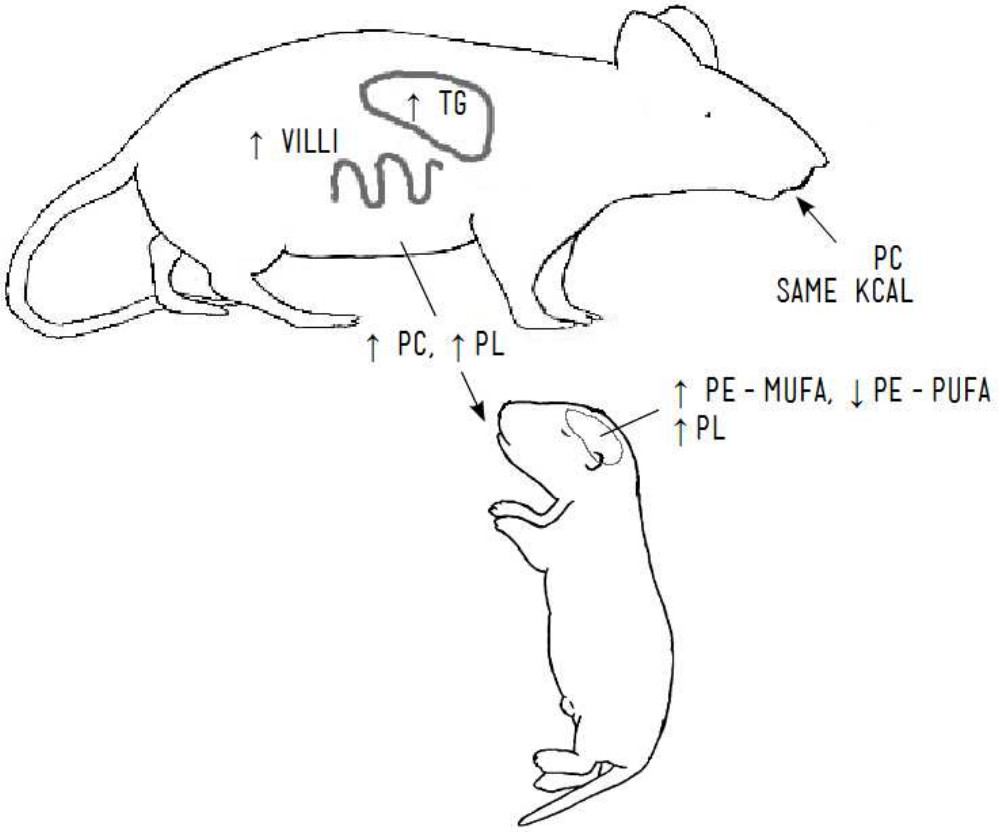

Figure 5.18: Overview of changes induced by feeding lactating mother a diet containing choline as PC as compared to choline bitartrate. Mothers have higher lipid concentrations in the liver and longer intestinal villi. Offspring receive a diet higher in PC and phospholipids (PL) and their brain lipids are changed to a higher overall phospholipid concentration and higher percentage of MUFA and lower percentage of ω-3 PUFA attached to brain PE.

5.4.3. General strengths and limitations of this study and future work

In this study some outcomes that had the appearance of being different between groups did not reach statistical significance were limited by lack of power due to a low n-numbers. The low number of animals used was due to this study being a combination of 1) a follow-up study to confirm some results in the immune system that had come out of the 2009/2010 studies

and of 2) a pilot study which was to elucidate if there may be any long term effects of this intervention, which had not been done before. In order to separate the effects of delayed postnatal growth and maternal choline deficiency during lactation it would be advisable to add a fourth intervention group. Mothers of this group would receive a sufficient amount of choline in the diet but would be pair-fed with the D mothers of the cohort. Furthermore, to follow up on the most prominent difference we observed in adult offspring, neurological assessment in connection with the suspected myelin deficit should be carried out, specifically testing for symptoms like fatigue and changed motor function, known to be associated with demyelination diseases like multiple sclerosis [558]. Brain structure would have to be analysed accordingly.

Since a complete lack of choline in mothers led to complications in the 2011 study to the point where animals had to be rescued with a standard choline diet it would be better to have a low choline group instead in future studies, i.e. mothers fed a diet containing 0.1 g/kg free choline during lactation. This may still reduce food intake and the effect of choline depletion and decreased food intake could be separated by having a group of mothers pair-fed to this group of low choline-fed mothers.

For comparability with human dietary intake of choline it may be desirable to include a group which receives a diet with the choline metabolites which are present in food at the highest concentrations, free choline, PC and glycerophosphocholine, in proportions similar to a human diet [350].

About 10% of bacteria have PC as part of their cell wall, with PC either being synthesised from exogenous choline sources or from methylation from PE or both [559]. Ability to produce PC from PE differs between bacterial species and, in a human study, it was suggested that the pre-

existing gastrointestinal bacterial composition is strongly predictive as to whether the subject develops fatty liver in response to choline depletion [560]. Gut microbiome might, therefore, explain some of the differences in response to choline-devoid diets reported in other studies. Similarly, since rats are coprophagic [561] hindgut bacteria might contribute to their choline intake quite substantially if they are not prevented from re-ingesting their faeces. However, in our study and many other studies effects of choline depletion could clearly be seen and bacterial PC production cannot be considered sufficient to prevent depletion.

5.5. Conclusions

Our findings clearly show that early lactation is a crucial period for choline intake as lactating mothers react to a choline-devoid diet differently from those fed sufficient choline and differently from non-lactating adult animals as the food intake of D mothers decreased substantially. Secondly, the amount and form of dietary choline consumed by the mother during lactation period is crucial for the offsprings' growth and brain development. This period needs to be studied more closely with regards to choline metabolism as the distinctiveness of this period and the mechanisms behind the changes we described are poorly understood.

In lactating mothers, choline availability and form do indeed change intestinal morphology and liver lipid and choline metabolite concentrations acutely. Offspring have a slower growth pattern in early postnatal life but glucose tolerance is not influenced in the offsprings' early adulthood whilst brain phospholipids, which may represent myelination, are substantially changed.

It is beyond the scope of this study to draw any definitive conclusions about mechanisms underlying the effects observed in this study. Further, carefully planned studies will give a better idea with regards to the mechanisms involved, as I pointed out throughout this discussion. As choline is involved in methyl group metabolism, a choline-devoid diet, especially fed during early development, could potentially change methylation of genes. This is a mechanism that could be examined in further studies, both for global DNA methylation and methylation of specific genes, but there is currently not sufficient information to indicate which genes could be mediating the changes described in this chapter. As for the structural characteristics of choline in membranes and structures like myelin, cell culture studies could be employed to gain further insights into the response of tissues to choline-depletion.

6. Conclusions

6.1. General aims

This thesis aimed to investigate the influence of perinatal nutritional interventions on the development of metabolic disease and appetite regulation in the adult offspring. This aim was considered in 2 different studies. The first investigated the influence of maternal macronutrient restriction in late pregnancy and the early postnatal growth rate in a sheep model. The second studied the influence of a diet devoid of choline, a vitamin with key roles in tissue growth and brain development, during the lactation period in a rat model.

In the sheep study, my aims were achieved by assessing glucose tolerance, food intake, the metabolic and endocrine responses to feeding, and appetite regulation. In the rat study, the main focus was on food intake of mothers and offspring, composition of mother's milk and changes in tissue growth and especially brain lipid composition.

6.2. Summary of findings

6.2.1. Macronutrient restriction in a sheep model

Restriction of maternal macronutrient intake in late pregnancy to 60% of maternal nutrient requirements caused maternal hypoglycemia and higher plasma non-esterified fatty acids (NEFA) concentrations and led to lower birth weights. Despite a higher growth rate relative to birth weight, N offspring (born to mothers nutrient restricted in late pregnancy) were still smaller than R offspring at weaning but were of similar weight by 6 months

of age, regardless of perinatal intervention. The insulin sensitivity of NAO offspring (N offspring subject to accelerated early postnatal growth and an obesogenic environment after weaning) was reduced compared to that of both RAO (R offspring with the same postnatal interventions as NAO) and NSO groups (N offspring subject to standard early postnatal growth and an obesogenic environment after weaning), supporting the concept that slow growth in late gestation, followed by accelerated growth in early postnatal life, is especially detrimental for adult glucose tolerance. Although it is likely that insulin resistance is programmed in one of the main organs involved in glucose metabolism, i.e. muscle, white adipose tissue or liver, preliminary research in these organs did not give further insights into the mechanisms behind the long term effects of perinatal interventions on offspring insulin resistance.

Despite higher fasted plasma leptin, postprandial insulin (glucose tolerance test) and 24h glucose concentrations in NAO offspring, food intake was not different in these offspring compared to RAO group. Hypothalamic gene expression suggested central insulin and leptin resistance as there was a non-statistically significant trend to higher gene expression of protein tyrosine phosphatase, non-receptor type 1 (PTP1B). Overall gene expression did not indicate an anorexigenic stimulus despite the increased plasma leptin, insulin and glucose. Although commonly associated with overweight and obesity, NAO offspring showed signs of leptin and insulin resistance at a similar body weight and composition as RAO offspring.

Whilst NAO glucose tolerance was affected earlier than that in NSO offspring, overall effects induced by maternal macronutrient restriction in late pregnancy did not differ significantly with respect to early postnatal growth rate. Contrary to my hypothesis, accelerated early postnatal growth following a low birth weight did not lead to greater obesity or fat mass in

this model. This might have been due to the use of sheep, as I am not aware of other sheep studies inducing increased adiposity by subjecting lambs to similar perinatal interventions. This may be a programming effect specific to humans.

NSL offspring (N offspring subject to a standard early postnatal growth rate and an environment inducing a lean body weight) did not show peripheral or central insulin and leptin resistance, suggesting that adverse effects of maternal macronutrient restriction in late pregnancy do not manifest, at least at a young adult age, if offspring obesity is prevented.

6.2.2. Choline intake during lactation in a rat model

In rats, feeding lactating mothers a choline-devoid diet decreased their food intake by an unknown mechanism, an effect that is not seen if male [512, 522, 523] or female non-pregnant or pregnant rodents [528, 562, 563] are subjected to a similar diet. This finding suggest that the demand for choline during lactation exceeds the ability to synthesise this nutrient. Although offsprings' intake of mother's milk did not differ in total choline and phospholipid concentrations, the volume of milk available may be decreased secondary to the reduction in maternal food intake. Offspring of mothers fed a choline-devoid diet for the first 2 weeks of lactation had a delay in growth but ultimately attained a normal mature body weight. In adult offspring, brain phospholipid concentrations were substantially decreased, suggesting that lactation is a sensitive period with regards to long term effects of maternal choline intake.

Dietary intake of choline as phosphatidylcholine (PC), rather than free choline, in lactating mothers increased PC and overall phospholipid concentration of mother's milk. Offspring growth was not affected but, at

weaning, brain phospholipid concentrations were higher. At a young adult age, their brain fatty acid profile was altered, with an increase in mono-unsaturated fatty acids and a decrease in ω-3 poly-unsaturated fatty acids attached to phosphatidylethanolamine, a prominent phospholipid in the brain. This may have implications for membrane fluidity and anti-inflammatory signalling.

6.3. Limitations of the models

6.3.1. Sheep as a model for human development

Sheep are a good model for human development as they both give birth to offspring at a similar stage of organ development as described in more detail in Chapter 1, with the highest increase in body weight and pancreas, adipose tissue and appetite regulatory pathway development occurring in the last third of pregnancy. Like humans, sheep predominantly give birth to single, twin or triplet offspring rather than large litters.

As ruminants, sheep have a distinctly different digestive system and their dietary requirements clearly differ from humans. The difference in digestive system has been accounted for in my experiments by having longer fasting periods before blood samples were taken, that is before glucose tolerance tests and the 24 hour feeding tests were performed, with an overnight fast of 18h as opposed to 9-12h, which is adopted in humans. Despite this difference, it has previously been shown that, consistent with human epidemiological studies, sheep develop obesity and/or insulin resistance after perinatal interventions [429, 471].

The animals in this study were subjected to a comparatively mild nutrient restriction, resulting in maternal hypoglycemia without distinct endocrine

changes. This may, therefore, not be a directly translatable model for intra-uterine growth restriction (IUGR) pregnancies in which the fetus experiences hypoinsulinemia, which could further influence intrauterine growth and later insulin resistance [231, 477]. However, the phenotype displayed by the offspring is consistent with findings in human IUGR cohorts with regards to adiposity and insulin metabolism.

6.3.2. Technical limitations during the sheep study

Unfortunately, the offspring of mothers allocated to be fed to requirement or nutrient restricted did not give birth to a balanced number of male and female offspring but, within the groups I analysed, there was no significant difference in outcomes between males and females, so that the imbalance was not likely to skew these data and conclusions. It would have been desirable to have a slightly greater number of animals in each group since several outcomes showed a trend to be different without reaching statistical significance. It can only be speculated that with a more strongly powered study these differences would have stood out more clearly.

Since sheep are not commonly used as an animal model besides for developmental research, commercial assays are not as readily available as they would be for human or rodent work. The genome of sheep is not entirely sequenced and some attempts to design primers had to use bovine or pig sequences and were not successful in the sheep samples. These included NFκB, which would have been desirable to measure in adipose tissue as a general marker of inflammation. Similarly, there are only few antibodies tested for measurement of sheep protein and, in the limited time frame I had to conduct the sheep and rodent studies in Nottingham and Edmonton respectively, it was not possible to spend more time

optimising Western blotting or immunohistochemistry, even though it would have been desirable to confirm gene expression findings on a protein level.

6.3.3. Rats as a model for human development

Rodents are a valuable model for human development as long as developmental differences are taken into account. As I was assessing the effects of early postnatal diet on development of brain and insulin resistance in rats, this may be used as a model for late pregnancy in humans as these organs develop in those periods in rats and humans, respectively.

This study is well-designed with regards to the distribution of sexes in the offspring examined. From a litter of 11 offspring per mother, one male and one female were selected for dissection at 3 weeks of age but no outcome differences could be detected between sexes except for gonadal fat mass, which is larger in males. For the extension of the study after weaning, two female offspring were selected per mother to reduce effect variance between animals due to sex differences. Oestrogen is known to increase messenger ribonucleic acid (mRNA) expression of phosphatidylethanolamine methyltransferase (PEMT) [564], therefore promoting choline biosynthesis from ethanolamine and potentially protecting females from choline deprivation as compared to males. If this study was to be repeated with male offspring, it is feasible that the outcomes seen after feeding mothers a choline-devoid diet would be stronger because males may be less able to compensate for choline depletion. However, at the time point of intervention there should not be a

significant sex difference as oestrogen concentrations and, with that, PEMT activity does not change before puberty [565].

6.3.4. Technical limitations during the rat study

For reasons to do with the timing of my work in Edmonton in relation to when the studies could be performed I had to combine data from several studies for the chapter included in this thesis. This is not ideal and in future studies all measurements would have to be repeated in a more consistent manner. However, for the purpose of my thesis comparing effects between groups within the studies was thought to be valid as effects on maternal decrease in food intake and weight loss relative to initial weight were comparable between studies.

6.4. Future work

6.4.1. Future directions for macronutrient restriction in sheep

As discussed in Chapter 2, the groups I investigated for my thesis were only half of the groups included in the original sheep study. I selected the groups I used based on hypotheses involving glucose tolerance and appetite regulation of the offspring. Future studies with a similar design could include fewer groups to more specifically investigate metabolic health and this would enable a larger number of animals in each group. For this, it would be desirable to have direct control groups for each of the nutrient restricted groups, i.e. RSO and RSL (i.e. a standard early postnatal growth rate with and without induction of obesity after weaning) additional to RAO. However, using the RAO group in the study discussed here as the only group not subjected to maternal nutrient restriction is legitimate since

the NAO, NSO and NSL groups could be compared to each other to differentiate the effects of the time periods affected by interventions. Secondarily, it is most representative of today's affluent societies to have offspring subjected to accelerated early postnatal growth and an obesogenic post-weaning environment as a control group.

Another tissue of interest to investigate would have been the pancreas, specifically the state of pancreatic ß-cells in the adult offspring, to further determine the cause and the likely continuation of insulin resistance with age in the maternally nutrient restricted animals. ß-cells have to be isolated immediately at post mortem and as glucose tolerance was not a crucial point of the original research question this was not done in this study. Additionally, it would be of interest whether the main contributor to impaired glucose metabolism is the liver, muscle or subcutaneous/visceral fat mass. This could be determined with gene expression analysis of insulin receptor substrates [566], PTP1B [462] and genes of lipogenesis, glycogenesis and gluconeogenesis. With regards to the changes seen in the hypothalamic cortisol regulatory system, further tests could be carried out, assessing markers of cardiovascular health and/or subjecting the offspring to a specific stress and recording the endocrine response to it.

Several of the hypothalamic genes with altered expression could be assessed for protein abundance and, especially if epigenetic modifications have been shown before for these genes, they could be analysed for DNA methylation and histone acetylation to assess the role of epigenetic changes in the long term mediation of metabolic health outcomes. At present this is difficult in sheep as the genome is not fully known and antibodies are not readily available.

6.4.2. Future directions for choline interventions during lactation in rats

As the long term choline study was performed as a pilot study, further studies may benefit by increasing the choline concentration of the D diet slightly, i.e. increasing it from 0 g/kg to 0.1 g/kg to avoid the drastic weight loss seen in D mothers. The effects of choline depletion and the weight loss secondary to it can be distinguished by adding a pair-fed group, i.e. a fourth group that is fed the same amount of diet with a standard choline concentration as D mothers are consuming.

It may also be of interest to have another group consuming a diet with all forms of choline at proportions similar to the human diet with a suboptimal total concentration of e.g. 0.5 g choline per kg diet, to closer represent human choline consumption.

With regards to the changes seen in the brain of offspring, which may lead to behavioural changes, further tests could be carried out for these animals, not only testing spatial memory as previously, but also tests investigating other behaviours such as anxiety. I discussed in Chapter 5 that the reduction in brain phospholipids of adult D offspring may be associated with a decrease in myelination. To assess this further, brain histology would have to be carried out.

6.5. Final remarks

The perinatal periods have been shown to be sensitive periods for long term development of offspring in the studies of my PhD, both in a restriction of overall macronutrient intake and in a restriction of a specific

micronutrient with a central role in brain development and tissue growth, choline. A crucial part of the effects seen may be mediated through changes in growth rates, i.e. slower growth in late pregnancy followed by accelerated growth in early postnatal life in the sheep study, and slower growth in early postnatal life in the rat study.

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Appendix A: Paper abstract

Endocrinology 152: 2816–2826, 2011

Maternal Nutrient Restriction During Late Gestation and Early Postnatal Growth in Sheep Differentially Reset the Control of Energy Metabolism in the Gastric Mucosa

Sebert SP, Dellschaft NS, Chan LLY, Street H, Henry M, Francois C, Sharma V, Fainberg HP, Patel N, Roda J, Keisler D, Budge H, and Symonds ME. Early Life Nutrition Research Unit (S.P.S., N.S.D., L.L.Y.C., H.S., M.H., C.F., V.S., H.P.F., N.P., J.R., H.B., M.E.S.), Academic Child Health, Division of Human Development and Nottingham Respiratory Medicine Biomedical Research Unit, School of Clinical Sciences, University Hospital, Nottingham NG7 2UH, United Kingdom; and Department of Animal Sciences (D.K.), University of Missouri, Columbia, Missouri 65201 USA.

Fetal growth restriction followed by accelerated postnatal growth contributes to impaired metabolic function in adulthood. The extent to which these outcomes may be mediated centrally within the hypothalamus, as opposed to in the periphery within the digestive tract, remains unknown. In a sheep model, we achieved intrauterine growth restriction experimentally by maternal nutrient restriction (R) that involved a 40% reduction in food intake through late gestation. R offspring were then either reared singly to accelerate postnatal growth (RA) or as twins and compared with controls also reared singly. From weaning, all offspring were maintained indoors until adulthood. A reduced litter size accelerated postnatal growth for only the first month of lactation. Independently from postnatal weight gain and later fat mass, R animals developed insulin resistance as adults. However, restricted accelerated offspring compared with both the control accelerated and restricted restricted offspring ate less and had higher fasting plasma leptin as adults, an adaptation which was accompanied by changes in energy sensing and cell proliferation within the abomasum. Additionally, although fetal restriction down-regulated gene expression of mammalian target of rapamycin and carnitine palmitoyltransferase 1-dependent pathways in the abomasum, RA offspring compensated for this by exhibiting greater activity of AMP-activated kinase-dependent pathways. This study demonstrates a role for perinatal nutrition in the peripheral control of food intake and in energy sensing in the gastric mucosal and emphasizes the importance of diet in early life in regulating energy metabolism during adulthood.

Appendix B: Conference abstracts

Canadian Nutrition Society, Vancouver, BC, Canada (poster presentation by Erin Lewis)

Maternal choline intake during suckling alters immune development in the offspring.

Lewis ED, Dellschaft N, Goruk S, Curtis JM, Jacobs R, Field CJ (2012). Agricultural, Food and Nutritional Sciences, University of Alberta, Alberta, Canada, T6G 2R3.

Choline is an essential nutrient for the normal function of all cells; however few studies have explored its role during immune system development. The most abundant forms of choline in the diet and breast milk are free choline and phosphatidylcholine (PC) which differ in their metabolism and may have different metabolic roles in the body. The objective of this research was to determine the essentiality of choline and the effect of feeding different metabolites of choline to dams on immune development in offspring. After giving birth, Sprague-Dawley dams were fed 1 of 3 high fat, isocaloric diets differing only in amount and form of choline; 0 g/kg choline (D, n=7), 1.0 g/kg free choline (FC, n=15) or 1.2 g/kg PC (PC, n=12). At 3 weeks, 2-3 of the suckled pups from each dam were killed and splenocytes isolated to measure the type of cells present (via flow cytometry) and the ability of cells to produce cytokines (by ELISA) ex vivo after stimulation. Total choline content of the dam's milk (pups' stomach content) did not differ significantly among groups, but the PC group had a higher PC content ($p<0.05$). Final body weight was lower in the D pups compared to PC and FC pups ($p<0.05$). The D group had a higher % of CD8+CD28+ T cells compared to choline groups and a higher % of NK cells (CD3+CD161+) compared to FC group ($p<0.05$), but the cytokine response after stimulation did not differ from FC. Cells from PC pups produced more (133%) IL-6 than the FC pups ($p<0.05$) when stimulated with ConA. Compared to FC, the PC splenocytes had a higher proportion (43%) of regulatory T cells (CD4+CD25+) and activated cytotoxic T cells (CD8+CD71) ($p<0.05$). The response to lipopolysaccharide (bacterial antigen) did not differ among groups. However, a higher proportion of IgM+ cells were found in PC group compared to FC group and a higher proportion of IgA+ cells compared to both FC and D groups ($p<0.05$). These findings suggest that the amount and form of choline in the maternal diet during suckling influences immune development in offspring.

World Congress on Developmental Origins of Health and Disease (DOHaD), Portland, OR, USA (poster presentation)

Dietary Choline Intake During Lactation Impacts the Growth of Offspring.

Dellschaft N, Jacobs R, Goruk S, Coursen N, Curtis J, Field C (2011). Agricultural, Food and Nutritional Sciences, University of Alberta, Alberta, Canada, T6G 2R3.

Aims: Although it has been established that choline is an important nutrient during pregnancy, the essentiality of choline (or the dietary forms of choline) in the maternal diet during the suckling period has not been established and is the purpose of the current study.
Methods: Sprague-Dawley dams (n=4/group) were fed a high-fat, isocaloric diet from delivery until the end of the suckling period (21d), containing phosphatidylcholine (PC), choline (C) or neither (D). Similar to our earlier studies, D mothers gain less weight during lactation. At 21d, stomach contents were collected (n=2/dam) and analysed by LC-MS for choline metabolites. 2 female pups per dam were weaned to a high-fat diet (20% w/w) containing 1g/kg choline as free choline and body weight and food intake were measured regularly until 11 wks of age.
Results: In offsprings' stomach content there was a lower concentration of free choline in D (0.3±0.1mg/g) compared to C (6.1±1.0) and a higher PC concentration in the PC group (66±13mg/g) compared to D (35±13) and C (24±3), $p<0.05$, suggesting that endogenous synthesis of PC is reduced in D mothers. Length and body weight for D pups was significantly lower than that of PC and C at 4 wks (weight: PC, 90±1; C, 89±2; D, 66±2g; $p<0.05$) and 5 wks of age (PC, 137±5; C, 135±4; D, 116±3g; $p<0.05$) but did not differ at 7 wks. This catch up growth was likely due to a higher food intake adjusted for body weight (wk 4, PC, 140±2; C, 120±5; D, 166±13 mg diet/g body weight*d; for PC vs. C, C vs. D $p<0.01$) by the D pups. At 9 wks of age, an intraperitoneal glucose tolerance test (IPGTT) was performed but insulin and glucose response (area under the curve) did not differ significantly among groups.
Conclusions: Our findings suggest that a source of choline is required in the maternal diet during suckling to provide choline for maternal milk and to induce normal growth in the offspring. The effects of maternal choline deficiency on pup body weight remained for several weeks, despite feeding a choline sufficient diet. Rapid catch-up growth occurred by 7-8 wks of age but glucose tolerance at 9 wks was not significantly affected. Currently, studies are underway in these animals to determine the effects of early choline deprivation on immune function and appetite regulatory pathways in the hypothalamus later in life. (Funded by ALMA, Alberta Egg Producers and NSERC.)

Experimental Biology, Washington, DC, USA (poster presentation)

Choline Intake During Lactation Alters Maternal and Infant Growth and the Ex Vivo Response of Splenocytes to Lipopolysaccharide (LPS).

Dellschaft N, Goruk S, Curtis J, Jacobs R, Field C (2011). Agricultural, Food and Nutritional Sciences, University of Alberta, Alberta, Canada, T6G 2R3.

To determine if choline is required in the diet during lactation, immediately after birth, the pups from 26 Sprague-Dawley dams were randomized to the dams and the dams fed one of 3 high fat (20% w/w) diets differing only in the amount and form of choline: a diet with the recommended 1g choline as bitartrate/kg diet (C, n=11) or phosphatidylcholine (PC, n=8), or a choline-devoid diet (D, n=7) Dams and pups were sacrificed at 21 d and splenocytes isolated from dams and stimulated in vitro with LPS. All results were compared to the choline salt diet, C. Dams and pups weighed less ($P<0.05$) when fed D. Spleens from D had a higher % of antigen presenting cells (IgM+, OX62+OX6+, CD68+11+) but cells produced less (30%) IFNγ after LPS. Dams, but not pups, weighed more and had a higher gonadal and perirenal fat mass ($P<0.05$) when fed PC. Spleens from PC had a higher % activated macrophages (CD284+) and Th2 (CD71+), and total and activated B (CD80+28+, IgG+) cells and a lower % T reg cells (CD25+Fox+) ($P<0.05$). PC cells produced more ($P<0.05$) IL-6 (188%) with LPS. These results suggest 1) that choline is needed in the maternal diet during lactation to ensure maternal and infant growth and to support the immune response to LPS and 2) that providing choline as PC instead of a salt may further promote maternal growth, alter the activation state of immune cells and the ability to produce IL-6 with LPS. (Supported by Alberta Innovates Biosolutions and NSERC)

Women, Child, Health Research Institute (WCHRI) Annual research day, Edmonton, AB, Canada (poster presentation by Peter Iglinski)

Dietary Choline Influences the Distribution of Immune Cells in the Spleens of Dams during Lactation.

Iglinski P, Dellschaft N, Goruk S, Jacobs R, Curtis J, Field C (2010). Agricultural, Food and Nutritional Sciences, University of Alberta, Alberta, Canada, T6G 2R3.

It is established that choline is required during pregnancy for fetal neural development. The objective of this summer project was to determine whether dietary choline is necessary for the maternal immune system

during lactation and to investigate whether supplementing different forms of choline affects immune function differently.

64 Sprague-Dawley pregnant females were fed standard rodent chow. Within 24h of delivery they were randomized to one of 6 nutritionally complete diets that differed only in the amount and form of choline. Choline bitartrate (CB) and lecithin (phosphatidylcholine (PC)) were used in the diets (added in a g/kg diet basis) as a source of choline. The diets were ZERO (0 g choline), CB (1g), 2xCB (2g), 4xCB (4g), PC (1g), and 2xPC (2g). After 21d the dams and pups were weighed and killed and the dams' spleens collected and splenocytes isolated. Cells were treated with monoclonal antibodies and the mononuclear cell population gated and analyzed by flow cytometry. Groups were compared using ANOVA and differences between groups ($P<0.05$) were identified by a Duncan's multiple range test.

Final body weight was lower in dams and pups fed the ZERO diet compared to CB ($P<0.05$). Additionally, the proportion of T cells (total CD3+, CD4+, CD8+) and the activated T cells (CD28+, CD27+) were significantly lower in dams fed the ZERO diet ($P<0.05$). Feeding 4xCB, but not 2xCB resulted in significantly lower body weights of both dams and pups compared to the 1xCB. Dams and pups fed the PC diet compared to the CB diet had higher body weights and a higher proportion of activated CD4+ cells (CD71+) and antigen presenting cells (CD80+) ($P<0.05$).

These preliminary findings suggest that choline is required for the maternal immune system during lactation, that there may be a detrimental effect of supplementing high amounts of free choline and that providing choline in the form of PC as compared to salt results in differences in maternal and pup body weight and in immune phenotypes. Further research will assess whether these phenotype differences affect characteristics of immune function in both the offspring and mother.

International Conference on Developmental Origins of Health and Disease: Metabolic Programming, Munich, Germany (oral presentation)

The Influences of Nutrient Restriction in Late Pregnancy and Accelerated Postnatal Growth on the Insulin and Leptin Systems and Hypothalamic Gene Expression Following Obesity in the Sheep.

Dellschaft N, Sebert S, Gardner D, Keisler D[1], Symonds M, Budge H (2010). Early Life Nutrition Research Unit, Academic Child Health, School of Clinical Sciences, University of Nottingham, Nottingham, UK; [1] Department of Animal Sciences, University of Missouri, Columbia, Missouri 65201 USA.

Objectives: Prenatal caloric restriction followed by accelerated postnatal growth influences later risk for metabolic diseases associated with obesity, raising the question as to whether long term programming of hypothalamic appetite control may be modulated by perinatal growth.

Methods: Pregnant twin-bearing sheep were either fed to requirements (R; n=8) or nutrient restricted to 60% of this amount (N; n=15) from 110 days up to term (~147 days). Twin offspring were reared to promote either accelerated (A) (RA, n=8; NA n=8) or standard (S) (NS, n=7) early postnatal growth. After weaning, offspring were then kept in a low activity environment until 17 months of age when insulin response to a GTT was assessed and entire hypothalami dissected for gene expression analysis by real-time PCR.

Results: Adult offspring body weight and composition were similar. Plasma leptin and insulin responsiveness were higher in N compared to R offspring. Whilst gene expression of hormone receptors i.e. insulin, leptin and ghrelin were unaffected, genes involved in insulin and leptin signalling (PTP1B), energy sensing (mTOR, AMPK, FTO), blood pressure regulation (AVP) and the glucocorticoid system (GCR) were overexpressed in N compared to R offspring.

Conclusions: Although maternal diet did not alter adult body weight or composition, long term systemic changes in leptin and insulin regulation were induced by fetal growth restriction. These adaptations were further associated with differential responses in the gene expression profiles within the hypothalamus, suggesting programmed alterations in energy sensing pathways.

International Conference on Developmental Origins of Health and Disease: Metabolic Programming, Munich, Germany (oral presentation by Sylvain Sebert)

Maternal nutrient restriction and early accelerated postnatal growth programs liquid chromatography–electrospray–high-resolution mass spectrometry (LC-ESI-HRMS) metabolomic profiles in the blood.

Alexandre-Gouabau M1, Moyon T[1], Antignac J[2], Dellschaft N[3], Budge H[3], Symonds M[3], Sebert S[3] (2010). [1] INRA, UMR Physiologie des Adaptations Nutritionnelles, Rue de la Géraudière, BP 71627, 44316 Nantes, Cedex 3, France; [2] Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), Ecole Nationale Vétérinaire (ENVN), USC INRA 2013, Nantes, France; [3] Early Nutrition Research Unit, University of Nottingham, Nottingham, UK.

Background: Consistent evidences suggest that alterations in energy metabolism perinatally programmed risks for metabolic diseases. Nonetheless, the alterations that induce and maintain the program are yet unrevealed. Metabolomic modelling can be critical upon that matter.

Design: Twin-bearing sheep were allocated to either fed a control (C, n=9) or a restricted diet (R, 60% of C, n=16) during late gestation (110 days to term ~ 145 days). Mothers delivered twins bred either in environment that favored accelerate (A) or restricted (R) postnatal growth (CA; n=9, RA, n=7, RR, n=7). Weaned offspring were raised indoor up to 17 months of age. At 130 days of gestation in mothers and 17 months in the offspring, fasted blood samples were collected and assessed by liquid chromatography–electrospray–high-resolution mass spectrometry (LC-ESI-HRMS).

Results: The term R offspring was 10% lighter than C ($P<0.001$). RA offspring grew faster than both the RR and CA ($P<0.01$). 2629 variables were analysed by partial least-square-discriminant analysis (PLS-DA) demonstrated a dichotomy between the maternal environments (predictability (Q2= 40%) a difference further exacerbated when comparing a selection of 171 metabolites (Q2= 51%),17 months after birth, metabolic profiling allowed the identification of the 3 groups of offspring by O (orthogonal)-PLS-DA analysis (Q=51 %, P= 2.310-5).

Conclusion: This novel "omic" approach demonstrated long term programming effects induced by perinatally. We are now moving towards a second phase: the identification of the metabolic classes involved in these models. They may help the discrimination of programmed metabolic pathways.

Neonatal Society scientific meeting, London, UK (oral presentation)

The Influence of Maternal Nutrient Restriction in Late Pregnancy and Accelerated Postnatal Growth on Plasma Insulin and Leptin and Hypothalamic Gene Expression Following Obesity in the Sheep.

Dellschaft N, Sebert S, Keisler D[1], Symonds ME, Budge H (2010). Early Life Nutrition Research Unit, Academic Child Health, School of Clinical Sciences, University of Nottingham, Nottingham, UK; [1] Department of Animal Sciences, University of Missouri, Columbia, Missouri 65201, USA.

Background: Prenatal caloric restriction, followed by accelerated postnatal growth, influences later risk for metabolic diseases associated with obesity. This may be associated with a resetting of hypothalamic appetite control and insulin sensitivity.

Aim: The aim of this study was to differentiate between the long term effects of nutrient restriction in late pregnancy and those of accelerated

early postnatal growth followed by obesity. Outcomes measured were plasma leptin, insulin sensitivity and hypothalamic regulation of energy balance and blood pressure regulation.

Methods: Pregnant twin-bearing sheep were either fed to requirements (R; n=8) or nutrient restricted to 60% of this amount (N; n=15) from 110 days up to term (~147 days). Twin offspring were either reared by their mother as singletons to promote accelerated early postnatal growth (A) (RA, n=8; NA n=9) or both offspring were reared together by their mother to promote standard early postnatal growth (S) (NS, n=7). After weaning, offspring were then kept in a low activity environment until 17 months of age when insulin response to a glucose challenge was assessed and entire hypothalami dissected for gene expression analysis by quantitative real-time PCR. Appropriate institutional Animal Ethics Committee approval was obtained.

Results: Although birth weight was reduced in N offspring, adult offspring body weight and composition were similar between groups. Plasma leptin and insulin responsiveness were higher in N compared to R offspring. Whilst gene expression of hormone receptors i.e. insulin, leptin and ghrelin were unaffected, genes involved in insulin and leptin signalling (protein tyrosine phosphatase, non-receptor type 1), energy sensing (mammalian target of rapamycin, adenosine monophosphate kinase, fat mass and obesity associated), blood pressure regulation (arginine vasopressin) and the glucocorticoid system (glucocorticoid receptor) were overexpressed in N compared to R offspring. None of these effects were significantly influenced by changes in postnatal growth.

Conclusion: Although maternal diet did not alter adult body weight or composition, long term systemic changes in leptin and insulin regulation were induced by fetal growth restriction. These adaptations were further associated with differential responses in the gene expression profiles within the hypothalamus, suggesting programmed alterations in energy sensing pathways.

Appendix C: Suppliers

Contacts used in Nottingham

AbD serotec	Oxford, UK
Anglia Scientific	Cambridge, UK
Applied Biosystems	Foster City, CA, USA
Bio-tek Instruments Inc	Potton, UK
Bioron	Ludwigshafen, Germany
Fisher Scientific	Loughborough, UK
Fujifilm	Tokyo, Japan
Hamamatsu	Welwyn Garden City, UK
IBM	Portsmouth, UK
Leica	Milton-Keynes, UK
Linton Instrumentation	Diss, UK
Manor Farm Feeds	Oakham, UK
Mercodia	Uppsala, Sweden
Packard Inc	Downers Grove, Australia
Perkin Elmer	Waltham, MA, USA
Premier Biosoft	Palo Alto, CA, USA
Qiagen	Hilden, Germany
Randox Laboratories	Crumlin, UK
Siemens	Camberley, UK
Sigma-Aldrich	Gillingham, UK
Techne	Burlington, NJ, USA
Thermo Scientific	Wilmington, DE, USA
VWR	Radnor, PA, USA
Wako	Neuss, Germany

Contacts used in Nantes

Interchim	Montluçon, France
Pall	Port Washington, NY
Sigma Aldrich	St Quentin-Fallavier, France
Solvent Documentation Synthesis	Peypin, France
Thermo Fisher Scientific	Bremen, Germany
Umetrics Inc	Umeå, Sweden

Contacts used in Edmonton

AB Sciex	Concord, ON, Canada

Agilent Technologies	Santa Clara, CA, USA
Alpco	Salem, NH, USA
Analtech Inc	Newark, DE, USA
BD	Franklin Lakes, NJ, USA
Charles River Laboratories	Montreal, QC, Canada
Fisher	Pittsburgh, PA, USA
Gold Top	Edmonton, AB, Canada
Harlan Teklad	Madison, WI, USA
Invitrogen	Burlington, ON, Canada
Leica	Nussloch, Germany
Lipid Nutrition	Channahon, IL, USA
Merck	Darmstadt, Germany
Millipore	Billerica, MA, USA
MP Biomedicals	Solon, OH, USA
North American Mogul Products	Chagrin Falls, OH, USA
PMI Nutrition International	Brentwood, MO, USA
Richardson Oilseed Ltd	Let bridge, AB, Canada
Roche	Laval, QC, Canada
Safeway	Calgary, AB, Canada
Sigma	St. Louis, MO, USA
Thermo Fisher Scientific	Edmonton, AB, Canada
Thermo Scientific	Nepean, UN, Canada
USB Corp	Cleveland, OH, USA
Varian Inc	Mississauga, ON, Canada
VWR International	Edmonton, AB, Canada
Zeiss Ltd	Toronto, ON, Canada