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**THE EFFECTS OF DIETARY FRUCTOSE AND SALT ON MATERNAL,  
FETAL AND ADULT OFFSPRING GROWTH, METABOLIC STATUS AND  
CARDIOVASCULAR HEALTH**

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Thesis submitted to the University of Nottingham  
For the degree of doctor of Philosophy

**July 2011**

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**Declaration**

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

**Signed:**.....

**Date:**.....

## DEDICATION

To

Abby, Finlay, Koda, Mason & Kira, perhaps without knowing, you are all the reason why I did this.

Children are natural mimics who act like their parents despite every effort to teach them otherwise. More often than not, your children will naturally become what you are; so achieve what you want them to achieve.

Never, never, never give up.

I love you all.....x

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## Acknowledgements

There are many people I need to acknowledge for the part played in the development of this thesis. First of all, I would like to thank my supervisors, David and Sheila, without their invaluable guidance this thesis would surely have not been the same. I would also like to thank my other unofficial, 'surgical supervisor' Julie March; her easygoing demeanour and humour made life a lot easier and I look forward to working with you again. I would like to acknowledge the BBSRC, Nutricia Foundation and School of Veterinary Medicine & Sciences for funding and financial support which made this research possible. Special thanks must go to David Gardner; his unique style of supervision and sense of humour, unlimited knowledge and an eye for a cheap travel deal made for a great period of my life and some unforgettable times in the camper van, as well as sharing hostel dormitories with Interpol's 'most wanted' and making conferences more like a re-run of Trains, Planes and Automobiles. I am grateful for the opportunity, your time, energies, enthusiasm and advices, for teaching me to be independent in my research, for helping me develop my critical spirit, and for also simply for being such good supervisor. It has been a great pleasure for me to work with you. Thank you.

I feel it appropriate to acknowledge some other people, my office mates, in particular Phil, Louise, Sarah, Jamie and Co. for tolerating my foul language and incessant moaning, thanks for being generous of your broad knowledge and helping me when I needed it. My experience as a PhD student at the Veterinary School would not have been so pleasant without the combined, daily efforts of all the people of the School. A special thanks to the scientific technical staff, Scott, Margaret and Ceri for helping make our School highly regarded, and for being keen to share your knowledge with me. It must be said that no matter how pleasant the School could be, it is also very good to have a life outside. I therefore wish to thank Phil, Rob, Andy, Tom and Jake, all ex-house mates but always mates, cheers gents it was an absolute pleasure and a good laugh living with you mentalists.

Finally, I would like to further dedicate this thesis to those who have had to endure me the most and for those I care for the most, and without whom, none of this would be possible, in no particular order, Abby, Lisa, Scott, Rachy, Dad, and my beautiful girlfriend Asli for putting up with me, and who, at times, has made the unbearable, bearable, and last but not least, my Mam; Margaret.

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## Related Publications

### Full papers

**Gray C**, Gardiner SM & Gardner DS (2011) Maternal salt or fructose intake independently program cardiovascular control in the adult rat offspring.

**Gray C**, Gardiner SM & Gardner DS (2011) The effect of dietary inclusion of salt and/or fructose on maternal and fetal metabolic status and metabolic and cardiovascular health of the offspring. (*Submitted, March 2011*).

Rhodes P, Craigon J, **Gray C**, Rhind SM, Loughna PT & Gardner DS (2009) Adult-onset obesity reveals prenatal programming of glucose-insulin sensitivity in male sheep nutrient restricted during late gestation. *Plos One* 4.

### Abstracts

**C. Gray**, Sheila M. Gardiner and David S. Gardner (2011). Maternal fructose and/or salt consumption programs cardiovascular hypersensitivity in the offspring. Physiological Society summer meeting 2011. (*Oral communication*).

**C. Gray**, Phillip Rhodes, Stuart Rhind and David Gardner (2008). Protein restriction during early gestation impairs renal function in sheep with adult-onset obesity. Proceedings of the Nutrition Society, 67, E385 doi:10.1017/S0029665108000591

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P. Rhodes, **C. Gray**, S. Rhind, D. S. Gardner (2008). Prenatal diet and adult-onset obesity influence plasma amino acids in adult sheep. *J Physiol Proc Physiol Soc* 11 C30

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C Bastone, **C Gray**, P Rhodes, S Rhind & D Gardner (2009). Maternal protein or energy restriction and offspring hpa axis response to an itt. *Endocrine Abstracts* 19 P198.

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**C. Gray**, M. E. Symonds, S. M. Gardiner, D. S. Gardner (2009). The effect of increased fructose and/or salt intake on maternal growth and fetal number in rats. Proc Nutrition Society - Oral Communications

**C. Gray**, M. E. Symonds, S. M. Gardiner, D. S. Gardner (2009). The effect of increased fructose and/or salt intake on maternal liver and plasma lipids in rats. University College Dublin Proc Physiol Soc 15, PC22 - Poster Communication

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D.S. Gardner, P. Rhodes., C. Bastone, **C. Gray**, C. Kyle and S. Rhind (2009) The effect of prenatal diet on adolescent and adult hormone kinetics in sheep. FNPS Annual meeting – Abstract 83.

**C. Gray**, M.E. Symonds, S. Gardiner and D.S. Gardner (2010) Effects of a “western” diet on maternal metabolism and fetal development in rats. FNPS Annual meeting – Abstract 33.

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## Abstract

The modern Western diet is typically high in salt and fructose. Variations in maternal diet can have delayed developmental effects on the adult offspring's cardiovascular function leading to acute or chronic hypertension. The aim of the work in this thesis was to determine the effect of moderate dietary salt and/or fructose intake on maternal and fetal growth, metabolic status and cardiovascular health of the adult offspring. Sprague Dawley rats were fed either 1) control diet (chow) with tap water, 2) salt diet, 4% NaCl, 3) fructose diet, purified chow plus 10% fructose in tap water or 4) fructose and salt diet for 28 days prior to conception, through gestation and lactation. Data were collected on the non-pregnant and pregnant dam, the fetus and neonate and the adult offspring. Cardiovascular data in adult offspring were recorded between the ages of 10-15 weeks by implanted radiotelemetry probes. Dams fed fructose prior to and during gestation increased caloric intake ( $P<0.001$ ) from fructose water with a consequential decrease in total energy intake ( $P<0.001$ ) from food. Increases in plasma glucose ( $P=0.04$ ) (without an effect on insulin), triglyceride ( $P<0.014$ ), non-esterified fatty acids ( $P\leq 0.05$ ), cholesterol ( $P<0.001$ ) and uric acid ( $P<0.004$ ) were all increased by the consumption of fructose in pre-gestational females. Dams consuming salt prior to and during gestation elicited an increase in cardiac ( $P<0.001$ ) and kidney tissue mass ( $P<0.001$ ). Fructose-fed dams also displayed a significant redistribution of regional fat depots i.e. visceral fat increased ( $P<0.001$ ) whilst gonadal fat decreased ( $P<0.008$ ). Fructose also increased liver weight ( $P<0.001$ ) and intra-hepatic triglyceride concentration was also observed to be increased ( $P<0.007$ ). However, few effects on the fetus but subtle effects on fetal and neonatal growth were observed at this stage. Fructose and salt combined reduced litter size (7 vs. 14 pups) ( $P<0.001$ ) without an effect on birth weight. Maternal fructose diet skewed the sex ratio in favour of males (60:40) ( $P<0.001$ ) and maternal salt influenced placental architecture (decreased labyrinthine ( $P<0.007$ ), increased trophoblast layer ( $P=0.03$ )) and had marked effects on maternal osmolality ( $P<0.001$ ). Male ( $P=0.07$ ) and female ( $P<0.02$ ) offspring from fructose-fed mothers had relatively heavier livers. In the adult offspring male and female offspring plasma osmolality was significantly increased in offspring fed prenatal salt ( $P<0.001$ ). In the offspring, maternal salt diet significantly increased ( $\sim 15\text{mmHg}$ ) basal mean arterial pressure (MAP) in the adult male offspring ( $P<0.001$ ), but significantly decreased basal MAP ( $\sim 8\text{mmHg}$ ) in the adult female offspring. Both fructose and salt diet had effects on the circadian variation in blood pressure and heart rate. Subsequent cardiovascular challenges revealed little beyond an altered cardiovascular set-point in these offspring. The study emphasizes the importance of *quality* rather than *quantity* when assessing maternal diet, particularly in terms of its mineral and simple sugar content. In conclusion, data within this thesis demonstrates for the first time a moderate maternal dietary intake of salt and fructose can affect offspring osmolality profile and blood pressure in a sex-specific manner and produce a pattern of symptoms resembling NAFLD which, in part, are passed vertically to the offspring.

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## Commonly used Abbreviations

<b>A</b>	ANOVA	analysis of variance
	AM	amplitude modulation
	am	<i>Ante Meridien</i>
	ACS	acyl-CoA synthase
	ACO	acyl-CoA oxidase
	Ach	acetylcholine
	ANF	atrial naturetic factor
	ADP	adenosine diphosphate
	AAP	alanine aminopeptidase
	AMP	adenosine monophosphate
	ATP	adenosine triphosphate
	ADH	antidiuretic hormone
	AT1R	angiotensin type 1 receptor
	AT2R	angiotensin type 2 receptor
	AGE's	advanced glycation end-products
	ACE	angiotensin converting enzyme
	Ang	angiotensinogen
	AngI	angiotensin I
	AngII	angiotensin II
<b>B</b>	11 $\beta$ -HSD	11 $\beta$ – hydroxysteroid dehydrogenase
	BCG	bromocresol green
	BMI	body mass index
	BHF	British Heart Foundation
	BP	blood pressure
	BSA	bovine serum albumin
	BUN	blood urea nitrogen

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<b>C</b>	Ca	calcium
	CD	control diet
	CH	carbohydrate
	CHD	coronary heart disease
	CO <sub>2</sub>	carbon dioxide
	CoA	coenzyme A
	COMA	Committee on Medical Aspects of Food Policy
	COX-2	cyclooxygenase-2
	Cm	centimetre
	CM	chylomicron
	CV	coefficient of variation
	CVD	cardiovascular disease
<b>D</b>	d	days
	DBP	diastolic blood pressure
	DoHaD	developmental origins of health and disease
	DPX	distyrene-xylene
	DRV	dietary reference value
	DSI	Data Sciences International
<b>E</b>	ECF	extracellular fluid
	EDTA	ethylenediaminetetraacetic acid
	ELISA	enzyme-linked immunosorbent assay
	ET-1	endothelin-1
<b>F</b>	F	female
	F-1-P	fructose 1 phosphate
	FK	fructokinase
	FFA	free fatty acids

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	FD	fructose diet
	FOAD	fetal origins of adult disease
	FSA	Food Standards Agency
	FSD	fructose and salt diet
<b>G</b>	g	grams
	G-3-P	glucose 3 phosphate
	G3PDH	glycerol-3-phosphate dehydrogenase
	GAPDH	glyceraldehyde-3-phosphate dehydrogenase
	GC	glucocorticoid
	GK	glucokinase
	GFR	glomerular filtration rate
	GLDH	glutamate dehydrogenase
	GLUT (1-16)	glucose transporter
	GR	glucocorticoids receptor
	GI	glycemic Index
	g/day	grams per day
	g/Kg	grams per Kilogram
<b>H</b>	h	hours
	H	hydrogen
	HDL	high density lipoprotein
	H&E	hematoxylin and eosin
	HK	hexokinase
	Hr	hour
	HR	heart rate
	HO	Home Office
	HFCS (45,55,95)	high fructose corn syrup (% fructose)
<b>I</b>	ICP-MS	inductively coupled plasma mass spectrometry

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	IKK/Nf-kB	nuclear factor Kappa-B inhibitor
	I.R	insulin resistance
	i.m.	intra-muscular
	i.v.	intra-venous
	IUGR	intra-uterine growth retardation
<b>J</b>		
<b>K</b>	K <sup>+</sup>	Potassium ion
	Kcal	calorie
	kHz	kilohertz
	Kg	kilogram
	Kg/M <sup>2</sup>	kilograms per metre squared
<b>L</b>	lb	pound
	L	litre
	LBW	low birth weight
	LDL	low density lipoprotein
	L-NAME	G-nitro-L-Arginine-Methyl Ester
<b>M</b>	m	male
	MAP	mean arterial pressure
	Min	minute
	Mg	milligram
	MJ	mega joule
	mmHg	millimetres of mercury
	MUFA	monosaturated fatty acid
	MR	mineral-corticoid receptor
	mRNA	messenger ribonucleic acid

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<b>N</b>	N	number
	Na <sup>+</sup>	sodium
	NAD <sup>+</sup>	nicotinamide adenine dinucleotide
	NADH	nicotinamide adenine dinucleotide
	Na-K-ATPase	sodium potassium ATPase
	NEFA	non-esterified fatty acid
	NAFLD	non-alcoholic fatty liver disease
	NDC	non-communicable disease
	NDNS	National Diet National Survey
	NO	nitric oxide
	NOS	nitric oxide synthase
	NS	non-significant
<b>O</b>	O <sub>2</sub>	oxygen
<b>P</b>	P	phosphorus
	PAD	peripheral arterial disease
	PBS	physiological buffer solution
	PFA	paraformaldehyde
	PFK	phosphofructokinase
	pm	<i>Post Meridiem</i>
	PP	pulse pressure
	PPi	pyrophosphate
	ppq	parts per quadrillion
	PAT	perirenal adipose tissue
	PCR	polymerase chain reaction
	<b>QR</b>	RAAS
RAS		renin angiotensin system
RNA		ribonucleic acid

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	RNI	reference nutrient intake
	ROS	reactive oxygen species
<b>S</b>	s.d	standard deviation
	SD	salt diet
	SED	standard error of the difference
	SEM	standard error of the mean
	SACN	Scientific Advisory Committee on Nutrition
	\$	dollar
<b>T</b>	TAG	triacylglyceride
	TD	Teklad diet
	TG	triglyceride
	TOOS	N-ethyl-N (2hydroxy-3-sulphopropyl-m-toluidine)
<b>U</b>	UK	United Kingdom
	US	United States
<b>V</b>	vLDL	very low density lipoprotein
<b>W</b>	WHO	World Health Organisation
	w/v	weight/volume

XYZ

"The history of man for the nine months preceding his birth would, probably, be far more interesting and contain events of greater moment than all the three score and ten years that follow it."

**Samuel Taylor Coleridge - 1885**

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## 1. Introduction

### 1.1 Cardiovascular disease

The World Health Organization (WHO) describes cardiovascular disease (CVD) as a non-communicable disease that affects the heart and/or blood vessels (W.H.O, 2010). The term CVD is generic in nature and describes a cluster of often related pathologies. These can include coronary heart disease (CHD, affecting blood vessels supplying the heart muscles), cerebrovascular disease (affecting blood vessels supplying the brain) and peripheral arterial disease (PAD, affecting blood vessels supplying the limbs). CHD, PAD and cerebrovascular disease tend to be preceded by, and a consequence of, atherosclerosis (a build up of intravascular plaque formation). Other pathologies also include congenital heart disease (malformation of heart structure), rheumatic heart disease (damage to heart muscles/valves by rheumatic fever) and hypertension (chronic elevation of blood pressure, greater than 140 mmHg (systole) and 90 mmHg (diastole)). In the UK, CVD is still the main cause of death among adults (BHF, 2010b). Over 192,000 deaths occur each year (34% of all deaths). Of those deaths 49% are from CHD and 23% from stroke. CHD by itself is the commonest cause of death in the UK (90,000 per year), and the most common cause of premature death (20%). Nearly all deaths from CHD are caused by heart attacks. In the UK there were 227,000 reported heart attacks of which 30% of patients died (2007) (BHF, 2010a).

### 1.1.1. HYPERTENSION & OTHER RISK FACTORS

Hypertension is recognised as the overall major risk factor for CVD (Vasan, *et al.* 2001). Current WHO recommendations define hypertension as a resting blood pressure above 140mmHg systolic and 90mmHg diastolic in under 50's, and 160mmHg systolic, 95mmHg diastolic in people aged >50 years of age (Table 1). The risk of CVD increases with increased blood pressure (even within normal ranges) (W.H.O, 2010). It is known that >35% of the adult population in the UK have hypertension, and this has not changed significantly over the past forty years (BHF, 2010a).

**Table 1. Classification of blood pressure in adults.**

<b>Category</b>	<b>Systolic (mmHg)</b>	<b>Diastolic (mmHg)</b>
<b>Hypotension</b>	<90	or <60
<b>Normal</b>	90 - 119	60 – 79
<b>Pre-hypertension</b>	120 – 139	80 – 89
<b>Stage 1 hypertension</b>	140 – 159	90 – 99
<b>Stage 2 hypertension</b>	≥ 160	≥ 100

**Table1.** Not taking antihypertensive drugs or underlying pathology. When systolic and diastolic pressure falls into different classes, the higher class should be selected to classify the blood pressure classification. In addition to classifying stages of hypertension on the basis of average blood pressure levels, specific target organ disease and additional risk factors should be identified. This specificity is important for further risk classification/treatment. Adapted from (Guyton-Hall, 2001c).

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Hypertension is classified by the underlying cause or progression and primary (essential) hypertension is thought to account for 90% of all cases of hypertension (W.H.O, 2010). Secondary hypertension accounts for the remaining 10% of all cases. The progression of hypertension can be classified as benign or malignant. Benign hypertension usually involves a steady increase of blood pressure over many months or year which is usually asymptomatic. Malignant hypertension occurs rapidly with a severe rise in blood pressure seen over days or weeks. Primary and secondary hypertension can be, and are usually, asymptomatic until a time when the hypertension is so severe, symptoms are manifested. In primary hypertension there is no known cause for the rise in blood pressure, making treatment difficult (Vasan *et al.* 2001).

The exact cause is unknown but can be multi-factorial in nature; risk factors include age, obesity, excessive alcohol intake, high salt intake, and genetic susceptibility (BHF, 2010b; W.H.O, 2010). Further risk factors can include race, sex and age. The risk of developing CVD in males is much more prevalent than in females with a progressive increase with age (W.H.O, 2010). However, the increased risk of hypertension in males relative to females is equalised when women become post-menopausal. Post-menopausal women have been shown to have increased risk of hypertension in comparison to pre-menopausal women (Vazquez *et al.* 2009; Harrison-Bernard *et al.* 2000). It has been shown that circulating oestrogens may be cardioprotective in nature and may contribute to reducing the risk of CVD in premenopausal women (Sudhir *et al.* 1996). In all races, risk of CVD increases with age, for example Afro-Caribbean descendency shows a lower risk and South-East Asians have

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a much greater risk of developing CVD in comparison with the UK average (BHF, 2010c). Other risk factors, mainly environmental, can be manipulated to decrease risk of CVD. Smoking, sedentary lifestyle and an unbalanced diet can all increase risk of CVD. However hyperlipidemia and diabetes, together with high blood pressure, tend to be the major risk factors for CVD. High low density lipoprotein (LDL) and high triglycerides (TG) caused by a diet high in saturated fats have been shown to increase CVD risk (Ascherio *et al.*, 1996). High salt intake is also another important risk factor for development of CVD (Kempner, 1948; Cook, *et al.* 2007; Stamler, *et al.* 1996). Other dietary induced risk factors involved in the progression of CVD can include Type 2 diabetes and obesity (BHF, 2010d).

Since hypertension and CVD have a multifactorial pathogenesis, effective cardiovascular prevention needs to include a multifactorial approach to diet change, exercise development, smoking cessation and existing treatment of hypertension, diabetes and dislipidemia. The kidneys and the renin angiotensin aldosterone system (RAAS) play a central role in blood pressure regulation and the interaction with macronutrients and ions have also been implicated in the progression of the hypertensive state and therefore could be targeted as potential mechanisms of treatment (Ichiki *et al.* 2002). The secondary effects of diet, such as, obesity and diabetes, can play a major role in the pathogenesis of hypertension (Banegas, *et al.* 2007).

In summary, hypertension is a prevalent condition in the Western world and is one of the most commonly identified risk factors associated with increased risk of developing cardiovascular/coronary heart disease. Research that aims to elucidate potential causes and

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consequences of hypertension is therefore of utmost importance and potentially offers significant benefit in terms of risk reduction and economic alleviation.

### **1.1.2. DEVELOPMENTAL PROGRAMMING OF HYPERTENSION**

The developmental origins of health and disease (DoHaD) hypothesis was originally based on a number of studies which observed the inverse relationship between low birth weight and increased risk of CVD. Evidence also suggested that poor fetal growth and subsequent small size at birth could increase risk of coronary heart disease, stroke, hypertension and Type-2 diabetes (Barker *et al.*, 1989; Barker *et al.*, 1993; SACN, 2010). The concept of unbalanced or under nutrition during gestation or early life having adverse effects on the offspring's health as an adult (i.e. the 'developmental origins' hypothesis), states that disease or metabolic disorders can be 'programmed' *in utero* by under nutrition or nutritional insult during critical periods of early development (Barker, *et al.* 1989). The hypothesis suggests that a fetus encountering under-nutrition or a deficiency in micronutrients during development will adapt to the immediate environmental short comings to ensure its immediate survival causing permanent alterations in tissue architecture, cell number and function. Therefore, the newborn may be metabolically disadvantaged at times of dietary fluctuations as an adult (Lucas 1991; Barker, *et al.* 1989).

The developmental programming concept is not new. In 1934 researchers showed an association between living conditions, socioeconomic status and death rates from all causes in the UK and Sweden (Kermack *et al.*, 1934). Forsdahl also reported a correlation between

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infant mortality rate and CHD related deaths during 1964-67 in a cohort of 40-69 year old men (Forsdahl, 1977). More recently Barker *et al* (1989) showed geographical regional differences in stroke and CVD rate. Barker proposed that the causes of CVD may lie in the effects of poverty and undernutrition on the mother resulting in undernutrition of the fetus and neonate. A follow up to this study, the Hertfordshire cohort (birth records 1911-1930), showed an association between lower birth weight and risk of stroke and CVD mortality in men. Data from this cohort also revealed that weight at one year was inversely related to CHD and left ventricular mass (Fall *et al.*, 1995). Further observations made in cohorts from Preston (1935-43) and Sheffield (1907-23) (Barker, 1990) considered further measurements such as head circumference and ponderal index (Barker, 1992), rather than birth weight alone, as reported in the Hertfordshire cohort study. The Sheffield cohort showed CVD was related to babies that were thin at birth with a small head circumference (Barker, 1990). The Preston cohort strengthened the findings from the Hertfordshire cohort, showing a stronger inverse relationship with birth weight and CVD risk. Further observations made in two Adelaide cohorts involving 830 newborn children in 1975-76 measuring systolic and diastolic pressures up to 8 years of age showed blood pressure at 8 years was positively related to placental weight and inversely related to birth weight (Moore *et al.*, 1996). Studies have also shown low birth weight to increase risk factors that may predispose to CVD, such as diabetes (Lithell *et al.*, 1996), plasma clotting factors and hyperlipidemia (Osmond & Barker, 2000). These epidemiological studies provided the fundamental human evidence to suggest that cardiovascular responses may be programmed prenatally and potentially exacerbated

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postnatally in relation to low birth weight and cardiovascular health in later life. A number of factors could be involved with linking the phenomenon of low birth weight and later life CVD, but the extent to which nutrition was the primary driver has yet to be defined. However, further epidemiological evidence linking nutrition and health in later life has been shown in data from the Dutch hunger winter (Roseboom *et al.*, 2000b). The Dutch hunger winter was a severe famine which occurred between November 1944 and June 1945. The famine primarily affected the North Western Netherlands and was the result of a blockade imposed by the German military occupation during the Second World War. The Dutch hunger winter was unique as it was brief, defined in length and the population affected kept detailed rationing and health records before, during and subsequent to the famine. The effect of intrauterine malnutrition on the offspring has been the focus of many studies since. One study shows the effect of famine on the fetus is largely dependent on the trimester of pregnancy during which the malnutrition occurred. Malnutrition during the first trimester of pregnancy tended to show the greatest adverse effects, resulting in increased weight of mothers nearing term and children with low birth weight and subsequent obesity (Stein *et al.*, 1993). Intrauterine malnutrition during the first trimester was also shown to significantly increase cardiovascular morbidity in later life, and cause alterations in plasma lipid profiles (Roseboom *et al.*, 2000a). Children affected by famine in the second trimester of pregnancy were found to have a 3-fold higher incidence of microalbuminuria, and creatinine clearance was shown to be ~10% lower in affected groups (Painter *et al.*, 2005). In addition to these findings, the authors also went on to conclude that the ratio of protein and carbohydrate

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may be a determinant of the effects observed. In addition to the Dutch hunger winter, cohorts from other famines have been studied showing similar associations, such as the siege of Leningrad (Stanner *et al.*, 1997).

Not all studies have shown such an association between undernutrition, birth weight and adult cardiovascular outcomes. One extensive review of 55 studies observed a clear trend ( $P < 0.001$ ) towards a weaker association in those with a higher sample number (Huxley *et al.*, 2002). Huxley concluded that the claims for a strong inverse relationship between birth weight and subsequent blood pressure may chiefly reflect the impact of random error, selective emphasis on results and inappropriate adjustment for current weight and confounding factors (Huxley *et al.*, 2002). Others too have criticized the blood pressure measuring techniques (self-reported), the inclusion of subjects taking anti-hypertensive drugs and the lack of adjustment for other variables such as, diet, smoking and physical activity (Paneth & Susser, 1995). In addition to this, they also suggested that lower weight babies born from a restricted nutritional environment would also be exposed to a poor postnatal environment casting doubt on the associations of birth weight and adult disease (Paneth & Susser, 1995). Socioeconomic status can increase the exposure to possible risk factors such as smoking, poor diet, physical activity and alcohol abuse. Failure to account for these variables over the lifespan of the subjects seriously undermines and confounds the strength of studies focussing on birth weight and subsequent BP in adult life (Kramer & Joseph, 1996). Having demonstrated some marked effects on subsequent later life health in offspring as well as the limitations in epidemiological studies. A number of animal models

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have been developed to investigate the proof of principle of nutritional programming (Section 1.2).

## **1.2 Animal models of diet-induced hypertension**

A large variety of animal models utilising very different techniques have resulted in a wealth of information on hypertension. Animal models of hypertension have yielded observations that may be useful in elucidating molecular mechanisms and pathways involved in the aetiology of hypertension. When used correctly, animal models can be an important tool for scientists to investigate human CVD, especially in order to conduct time course studies or when studying early disease pathogenesis. In addition to this, hypothesis-driven experimental manipulation of an animal model is permissible which would be impossible in humans. Although animal data cannot be directly compared with human disease, animal studies are essential for understanding molecular mechanisms, metabolic pathways during gestation and elucidating 'critical' time points during early growth and development of disease states. The concept of programming of hypertension and related CVD is supported by a large number of animal studies, although the timing of elevated BP, its persistence and severity differs with the animal model studied. However, it has been shown that a variety of nutritional manipulations during gestation can program a rise in resting blood pressure in adult sheep (Sinclair *et al.* 2008) and rats, (Gardner *et al.* 1996; Harrison *et al.* 2010).

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### **1.2.1. TOTAL CALORIC RESTRICTION**

Observations by Woodall *et al* (1996) show a restriction of 70% overall energy intake from food throughout pregnancy resulted in significant programming of BP in offspring (Woodall *et al.*, 1996b). This level of restriction is excessive and other groups have shown a programming of hypertension at 20 – 30% reduction in maternal energy intake from food during late pregnancy (Vehaskari & Woods, 2005). The exact reduction in nutrient, calories or imbalance in ratio of nutrients and the mechanisms that contribute are largely unknown in such trials. Global nutrient restriction could arguably be more relevant to developing countries which, in recent years, have seen the largest growth in CVD related illnesses.

### **1.2.2. SPECIFIC NUTRIENT INTERVENTION**

Specific nutrient deficiencies are more likely to occur within the western populations. This may predispose pregnant mothers to excessive or unbalanced levels of macronutrients which can lead to an imbalance and a deficiency in other specific micro or macronutrients. Animal models of specific nutrient restriction include protein, iron, vitamin C or B. The maternal low protein model is the most extensively studied model of all specific restricted models. This model is useful in comparing protein intakes in developing countries, where protein is restricted in the diet due to famine or geographical location and availability of animal or plant proteins; furthermore, the modern western diet has seen a gradual decrease in the amount of protein eaten over time.

Normal protein levels in rat chow have been set at approximately 19% (Nelson and Evans,

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1953) of total weight (21% casein), and commonly studied restricted levels range from 12% (mild) (Langley and Jackson, 1994), 9% (modest) (Brawley and Itoh, 2003; Langley and Jackson, 1994) and 5% (severe) (Tonkiss, 1998; Lamireau and Nuyt, 2002) of total caloric intake. All three restricted levels have shown increases in BP in adult offspring, although the range between 12 – 19% of total weight could be considered normal as studies show 12% to be sufficient protein availability to sustain a pregnant dam (Nelson & Evans, 1953; Gahl *et al.*, 1991). Both studies showed that 8 – 12.5% was a perfectly adequate level of protein to maintain nitrogen balance and sufficient protein for a pregnant rat to sustain adequate fetal nutrient supply without adversely effecting litter size or birth weight. The majority of low protein diets are supplemented with sucrose or glucose to make up the deficiency in calories. In a balanced rat diet, sucrose would normally only make up 5-6% of total caloric intake. The majority of studies, with a few exceptions, show ranges from 4% (Athauda, 2004), 21% (Langley & Jackson, 1994) and 66.7% (Snoeck *et al.*, 1990). This addition of simple sugars could be confounding the effects of a low protein diet. Therefore, differences in nutrients (carbohydrates/fats) may be also confounding experimental findings in nutritional studies (Endoh *et al.*, 2001). In the fructose diets used by Hwang and Reavel *et al* alterations in macronutrient ratios (to enable 66% fructose in the diet) also could have confounded the specific effect of fructose per se, e.g. the change in fat content (from 2% to 9%) make the study a multi-factorial dietary intervention and not just a change in level of fructose.

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It must be noted that all of the studies mentioned used the tail cuff method which involves restraint of the animal and thus may suffer from artefacts of stress. Radio telemetry which is widely considered the gold standard technique. In the current study radio-telemetry was the preferred method. A review of both direct and indirect methods of blood pressure monitoring carried out by Kurtz and colleagues lists the advantages, disadvantages and recommendations for blood pressure measurements in freely moving and restrained animals. They concluded there is no overall superior technique, rather than the objectives of the study determine the chosen method of monitoring (Kurtz *et al.*, 2005; Pickering *et al.*, 2005). However, radio-telemetry is generally considered to be the 'gold standard' of techniques to monitor blood pressure in freely moving unrestrained animals (Tonkiss *et al.*, 1998; Kurtz *et al.*, 2005). Radio-telemetry does not involve the heat and restraint stress inherent with the tail cuff method and research has indicated rises in blood pressure associated with restraint stress (Tonkiss *et al.*, 1998; D'Angelo *et al.*, 2005). However, it would be wrong to conclude the tail cuff as a redundant method. More recently, a study by Swali *et al* directly compared the tail cuff and telemetry methods of blood pressure analysis in male rats simultaneously. They showed a good correlation between both methods in control rats but not low protein animals. Male maternally-fed low protein animals had higher blood pressures at weeks but displayed lower blood pressures at 12 weeks of age when measured by telemetry. Response to stress was measured, and similar increases in blood pressure and heart rate were observed without an effect of maternal diet and no evidence of an increased pressor response in low protein offspring (Swali, *et al.* 2010).

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Previous studies have shown when performed correctly, the tail cuff can be an accurate, reproducible technique, for determining blood pressure changes in conscious rats (Kubota *et al.*, 2006). It must also be noted that all techniques of blood pressure monitoring are subject to effects of external factors such as, habituation to new environment, temperature, light cycle, ambient noise (including very high frequency noise), group and non-group housing of animals and general husbandry. It is therefore of great importance to recognise and standardise these environmental factors to reduce the possible effects upon blood pressure response.

Currently, the overwhelming majority of studies focus on the impact of restricting one or more dietary components. However, few studies have considered maternal excess and the possible role excessive consumption of one or more nutrients might have on the pregnant dam and subsequent cardiovascular health of adult offspring. Many studies examining the effects of a 'western' diet focus mainly on high fat intake and the resulting effects on weight gain, obesity, diabetes and hypertension. A model of maternal obesity developed by Khan *et al.* (2003) involved feeding female Sprague Dawley rats a control (5% fat) or high fat (25% lard) before, during and after gestation causing hypertension in female, but not male, offspring of high maternal fat diet (Khan *et al.*, 2003).

Additionally, a few other studies have considered the hypertensive effect of maternal dietary fat intake (LangleyEvans *et al.*, 1996a), high salt (Contreras, 1993), high fructose (Fergusson & Koski, 1990) and sucrose (Soria *et al.*, 1996). Furthermore, combinations of differing

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macronutrients in excess or deficiency have also been studied such as salt, fructose and/or fat. In addition, the studies mentioned all used the Sprague-Dawley rat. SD rats are generally not genetically as salt sensitive as other strains of rat, making them a more appropriate strain to study dietary salt interventions (Giardina *et al.*, 2001) (Ogihara *et al.*, 2001).

Rawana *et al* showed the effects of low dose fructose ingestion during gestation and lactation causes hyperlipidemia in offspring of dams fed fructose (10%) (Rawana *et al.*, 1993). The dams also had heavier livers, greater weight gain, higher plasma triglyceride and glucose levels than glucose fed dams. Body weights of pups were not significantly different (Allen *et al.*, 1966). Further studies have shown high carbohydrate diet during the weaning period showed an increased adiposity in the offspring and transfer of this phenotype to the second generation (Srinivasan *et al.*, 2008). To date, very few studies have given consideration to the perinatal feeding of a salt and fructose supplemented diet and the subsequent health of the adult offspring and their physiological response to a high salt diet.

### **1.2.3. CRITIQUE ON THE USE OF EXPERIMENTAL DIETS IN NUTRITIONAL ANIMAL RESEARCH**

Most scientific studies involving animal models of nutrition investigate the effects of specific nutrients on genotype, metabolism, phenotype and ultimately, impacts on health. Unfortunately, many nutritional studies draw conclusions from unbalanced, nutritionally different dietary regimes. The use of non-purified diets in animal studies will add to a varying general phenotype and diet is, at times, considered rather as a tool than a factor that produces differing models of nutritionally induced phenotypes. Although some research

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requires the use of experimental diet as a tool to produce a specific end-point or phenotype, without investigating effects of specific nutrients to identify 'active' components of the diet. The major concern is that of researchers developing nutritional models, particularly in the field of developmental programming and specific nutrient intervention. e.g. the use of 'High-fat-diet (HFD)' in knock-out mouse studies. Study of some of these models will commence without consideration of how the phenotype was created and differences seen between control and experimental group diets. For the purpose of this discussion, emphasis will be placed on the three main energy providing components (protein, fat and carbohydrate) commonly used in nutritional intervention studies in rats.

The low protein and high fat models are two extensively studied models of specific nutrient intervention studies. The caloric deficit created by reducing a 20% protein diet to 10-12% (total energy) has to be made up with additional macronutrients to balance the diet making it isocaloric and comparable to the control diet. The majority of low protein and high fat diets are supplemented with carbohydrates (sucrose or glucose) to make up deficiencies in total energy intake. High and low fat diets increase (>66%) (Boullu-Ciocca *et al.*, 2008) or reduce (<28%) (Gao *et al.*, 2002) levels of carbohydrates in the dietary regime and normally protein would remain constant (~18%) (Sinclair & Crawford, 1973)). Low carbohydrate diets tend to have an increased protein content (>30%) (Lloyd & Wang, 2007) or increased fat content (>60%) (Omagari *et al.*, 2008). High carbohydrate diets are predominantly made up with higher sucrose content. This inevitably results in a reduction of fat to achieve isocaloric

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values comparable to that of the control diet. All of these nutritional alterations would therefore induce a collateral effect, rather than that of the specifically reduced or increased macronutrient primarily being studied.

In a balanced rat diet, sucrose (fructose/glucose 50:50) would normally make up 4-6% (Nelson & Evans, 1953) total energy of the diet, with most manufactured chows containing 7-10% total energy (Harlan UK). Low protein and high fat diets show ranges from 21% (Khan *et al.*, 2003) to >66.7% (Harris R. Liebermana, 2005) of carbohydrate from sucrose. In addition, it has been shown that substitution with carbohydrate has a dominating effect over the influence of low protein (Endoh *et al.*, 2001).

Some diets will inevitably produce more than one phenotype, for example, fructose feeding has been shown to cause hypertriglyceridemia (Turner *et al.*, 1979), hyperuricemia (Feig *et al.*, 2004), hyperglycaemia (Gray, 2009), hypertension and obesity (Bray *et al.*, 2004). The addition or reduction of macronutrients, for example of sucrose to make up the deficit in calories by the reduction of another macronutrient, is therefore highly likely to confound the effects of an experimental diet. This is essentially making the diets a multifactorial dietary intervention such as low protein diets with added high carbohydrate or fat (Warden & Fisler, 2008). Therefore, differences in nutrients such as increases in carbohydrates and/or fats will be contorting the diets to such a degree that experimental findings are hugely difficult to attribute to any particular macronutrient addition or exclusion.

An example of misguided design of a nutritional intervention is a recent paper published in Cell by Zhang *et al.* (2008), showed a hypothalamic Ikk/NF- $\kappa$ B and endoplasmic reticulum

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stress linked over nutrition to energy imbalance and obesity. Although an excellent paper, it shows major disparity between experimental diets. A model of obesity was developed using a high fat diet manufactured by one company and the control diet by a different supplier. On closer inspection of the dietary contents of the aforementioned diets, all three macronutrients were significantly different between diets. Protein, carbohydrate and fat were all different, starch and sugar ratios were hugely different. At least nine differences were found apparent the experimental diets, possibly more given the limited micronutrient data available. Ingredients such as sodium, potassium and methionine were different in the high fat diet when compared to the control diet. Soybean products were also used within the high fat diet. Limited information on mineral, vitamin, fatty acids and individual amino acids within the high fat diet prevented further comparisons (Kozul *et al.* 2009) (Zhang *et al.* 2008).

In order to develop appropriate models of nutrition, specially formulated diets are needed to reduce easily avoidable variables. Purified diets are diets manufactured with a highly refined and specific amount of ingredients. The process gives the diet a low variation between batches. Purified diets are ideal for researchers to manipulate certain aspects of experimental diets without the addition of extraneous compounds and excessive or inappropriate macronutrient substitution. Although, when changing specific macronutrients it is still difficult to determine true isocaloric values between experimental diets and consideration must be given. However, though the use of purified diets and inclusion of fibrous compounds, the overall effects can be offset to a degree. These diets enable

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research to be easier to attribute cause and effect and are easily modifiable and highly reproducible. Absolute batch-to-batch consistency with precise nutrient composition results in accurate, highly reliable data which can be reproduced to develop robust models of nutritional disease. Typically, amounts of vitamins, minerals, fibre and at least one of the three main energy sources will remain constant within these purified diets.

Additionally, standard chows which are often used as a control diet are formulated from natural ingredients. Bioavailability of nutrients in standard chow diets have been shown to be diluted compared with purified diets due to nutrient-nutrient interactions and degradation during the manufacturing process. These differences in micronutrients in purified diets when compared to non-purified diets have also been shown to influence the outcome of studies (Kanno *et al.*, 2002). In diets which use non-animal products, soy protein or vegetarian based diets contain increased levels of phytoestrogens, resulting in an increased oestrogenic activity in experimental species. Researchers must be aware of the confounding effects of phytoestrogens. Phytoestrogens have been shown to have an influence on fertility (Casanova *et al.*, 1999; Dillingham *et al.*, 2005), bone density (Draper *et al.*, 1997), cholesterol metabolism (Hirose, 2006), energy intake from food, water intake (Hartley, 2002), and alterations in the hypothalamic-pituitary-gonadal axis have all been reported in rats. More recently, a comparative dietary study by (Kozul, 2008) showed marked differences in gene expression in mice fed purified chow when compared to control chows.

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The type of diet used could have as much effect in experimental design as choice of strain, age, techniques and statistics. All of which in their own right can affect the power of an experiment. Powerful experiments are defined as studies which have the maximum likelihood of detecting a true treatment effect. Increased power is achieved by controlling for and eliminating variation, which should include careful design of experimental diets.

With growing pressure to reduce the amount of animals used in research we must, at every opportunity, try to reduce the avoidable variation seen within the majority of nutritional animal studies to date. This may, given the use of appropriate statistical models, help reduce animal usage. Furthermore scientists have a responsibility to report on accurate, non-biased findings, which in part can be helped by the use of the best dietary regimes possible to ensure clearly attributable cause and effect in specific nutrient intervention studies. Because of the multi-faceted nature of nutritionally-induced pathologies, and the complex interactions between nutrients and the metabolome, it is of importance to increase the use of purified diets to eliminate unnecessary variation and enable comparison across studies.

Thus, in order to identify specific roles for individual nutrients such as fructose or salt (or any potential interaction) on later blood pressure control. Without confounding the study by also having large changes in other dietary components within the experimental diets. We have employed a specially-formulated purified diet to be used eliminating the risk of unforeseen nutrient-interactions which may have occurred that was not otherwise captured by the dietary design.

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#### 1.2.4. MECHANISMS OF PROGRAMMED HYPERTENSION

Having demonstrated proof of principle, animal models have since been utilised to examine the mechanisms of DoHaD. Programming can be defined as ‘influencing behaviour or phenotype in a predetermined way’. Lucas first proposed the possibility of a stimulus or insult during development establishing a permanent physiological response (Lucas, 1991). The suggestion of an early insult, stimulus or change in environment having such a profound effect upon the fetus during critical periods of development has changed how we interpret the original DoHaD hypothesis, putting more emphasis upon the periods of development and timing and type of insult. Development of tissues and organs can continue after birth into the first 2-3 weeks of postnatal life (species dependent) (Witsch, *et al.* 1962). For example, in the rat, kidney development can continue up to postnatal d12 (Okada *et al.*, 2001), whereas sheep and human kidney development is largely complete at birth. Similar to the rat, the human liver can still be developing until day 18 (Si-Tayeb *et al.*, 2010). Therefore, programming can operate both pre and postnatally, and the fetal origins of adult disease (FOAD) hypothesis has been made less specific and is more readily referred to as the DOHaD hypothesis (Gluckman & Hanson, 2004b).

Developmental programming of hypertension appears to be a consequence of nutrition and metabolic imbalances during a critical or vulnerable period of development. A vulnerable or critical period can be defined as a window of time in fetal development where physiological systems are developing and are sensitive to specific stimuli (Widdowson & McCance 1975). These critical windows may largely depend on the stage and speed of growth. Cells appear

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to be more vulnerable during critical periods of rapid growth. Programming of function and structure is more likely to be permanent during rapid cell growth and division in organs and tissues rather than hypertrophic growth (Winick *et al.* 1966), and genetic potential may only be fulfilled if the environment is optimal (Widdowson & McCance 1966). In sheep a nutritional stimuli in late gestation impacts more upon the rapidly growing fetus (Harding *et al.* 1992). In addition, the timing of nutritional stimuli has also been shown in rat dams fed high salt diets (2-8% NaCl in chow) throughout pregnancy and/or lactation produce offspring with increased mean arterial blood pressure (Grollman & Grollman, 1962; Contreras *et al.* 2000; Swenson *et al.* 2004; Porter *et al.* 2007). Although continuation of the dietary stimuli through lactation appears important as pre-natal-only exposure to salt diet [2.3%, (Myers *et al.* 1985), 8%, (Porter *et al.* 2007)], had no effect on blood pressure of adult offspring.

Alterations in morphology and functionality of tissues and/or metabolism may be influenced by maternal nutrition and hormonal environment. This may be a consequence of programming at a cellular or sub-cellular level, e.g. low protein models have shown permanent reduction in cell number and alterations in liver (Zenman & Stanbourn 1969), placental size (Hastings-Roberts *et al.* 1977), kidney size and nephron count (Langley-Evans *et al.* 1996; Welham *et al.* 2002). Changes in enzyme activity and gene expression studies reveal changes in overall availability. Microarray data and gene expression from aortas of maternal high fat-fed offspring show differences of over 200 genes relative to controls. Differential gene expression was shown to include genes involved in collagen, elastin and endothelial nitric oxide synthase (Taylor *et al.* 2005). Programmed alterations in receptor

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populations (Meaney *et al.* 1988) due to inter-uterine environment may programme the activity of steroid hormones or steroid dependent enzymes. Similarly, low protein rats have been shown to have altered glucocorticoid receptors in prenatally fed offspring as well as the mother (Lunn & Austin 1983; Mulay 1982). This may be important in determining the set-points or range at which the offspring's hypothalamic-pituitary-axis (HPA) responds during adulthood. A variety of mechanisms have been proposed to underlie the effects of programming including over exposure to glucocorticoids (Gardner, *et al.* 1996), altered kidney development (Whelham, *et al.* 2002) and subsequent functionality, maladapted vascular function (Puyo, *et al.* 2010) and epigenetic imprinting (Sinclair, *et al.* 2008). However, cellular and molecular mechanisms that underlie the nutritional programming paradigm are not fully understood but are increasingly studied and may present a fuller understanding of the progression and understanding of hypertension and CVD.

#### **1.2.4.1 Programmed alterations to kidney structure & function**

Guyton first proposed that sustained hypertension required the involvement of the kidneys and a right shift in the pressure natriuresis curve had been widely shown in most cases of hypertension (Guyton, 1991). The role of kidneys and the RAS in programming of hypertension has been studied extensively. Studies have shown intra-uterine growth restricted (IUGR) offspring in low protein (LP) rat offspring are born with smaller kidneys with a reduction in nephron number (Welham, *et al.* 2002). The reduction in nephron number and impairment of growth could play a role in the developmental origins of hypertension. More recently, these effects have been shown to be inter-generational, with

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decreased nephron endowment and hypertension being transferred to the second generation (Harrison & Langley-Evans, 2009). Impaired nephrogenesis has also been shown as a result of exposure to low protein diet and excess glucocorticoids in rats (Langley-Evans *et al.* 1996) and sheep (Dodic *et al.* 2002).

Additionally, programming of offspring renal function has been shown to be altered in later life. Lopez-Bermejo (2008) observed associations between estimated glomerular filtration rate (eGFR), serum creatinine excretion and low birth weight in 35 boys and 38 girls aged 9.5 years. Serum creatinine was significantly reduced and eGFR was increased in low birth weight individuals (Lopez-Bermejo *et al.* 2008). Similarly, Keiger-Veen and colleagues have shown that preterm birth may be a risk factor for kidney disease. Renal function was assessed by urinary albumin creatinine ratio, eGFR and plasma creatinine. They showed plasma creatinine and creatinine:albumin ratio was inversely proportional to birth weight with decrease in eGFR and increase in microalbuminuria in preterm babies (Keiger-Veen *et al.* 2005). Animal data has also shown evidence of programming effects on altered renal function during adult life. In sheep, increases in plasma osmolality, sodium and mean arterial blood pressure in offspring from dehydrated-induced anorexic mothers (Ross *et al.* 2004). Likewise, in rats Hoppe (2006) observed alterations in renal structure, function and increased arterial blood pressure in animals fed low protein (8%) and high salt (8%) relative to controls. Renal function was assessed by eGFR and creatinine clearance. They reported a decrease in mean arterial pressure, reduction of nephrons, renal plasma flow and eGFR and creatinine clearance.

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Intrarenal RAAS has been studied considerably in the context of altered renal development. Angiotensin II (Ang II) is an important factor during renal development, and low protein may alter the RAAS (Woods *et al.*, 2001). Studies have shown lower expression of angiotensin I receptor ( $ATR_1$ ) mRNA, reduced Ang II expression or concentration and lower nephron counts in offspring which went on to be hypertensive as adults (Balbi *et al.* 2004). Altered kidney development and reduced nephron endowment may also cause an increase in sodium retention, by the resetting of sodium reabsorption via an increase in tubular sodium expression of sodium transporters, such as Na-K-ATPase in the proximal ascending limb of the distal tubule. Rats born to mothers receiving a maternal high salt diet have a suppressed renin and  $ATR_2$  distribution within the kidney in the early postnatal period (Ingelfinger *et al.*, 1986; Balbi *et al.*, 2004). During this period renin concentrations would usually be high to facilitate the production of Ang I and Ang II which are required for development and maturation of the kidney (Gomez *et al.* 1995). Furthermore, experimental suppression of the RAAS components during development can permanently alter the offspring's nephron number, salt/water homeostatic ability, blood pressure and salt sensitivity (Contreras *et al.*, 2000). Sodium and potassium levels have also been shown to be altered in milk by maternal high/low salt diets, without an effect on plasma sodium in the dam but with a tendency to increase fetal/offspring plasma sodium concentrations (Deloof *et al.*, 2000). Thus, any programming effects on renal function in the current study will be assessed by creatinine, albumin clearance, albumin:creatinine ratio, plasma osmolality, plasma and urinary sodium, and total osmolar clearance.

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#### 1.2.4.2 Programmed alterations to vascular sensitivity

Blood is distributed throughout the body by a network of branching, progressively smaller blood vessels. All blood vessels have an internal lining of squamous epithelial cells called the endothelium. Additionally, as the blood vessels get smaller there is a gradual transition from having walls with large amounts of elastic tissue (e.g. aorta) to walls with larger amounts of smooth muscle such as small arteries and arterioles. Arterioles are highly reactive to changes in circulating hormones and autonomic nerves, in particular adrenergic innervations, and respond accordingly by constricting or dilating. These vessels are the main resistance to blood flow and are often referred to as resistance vessels or the microvasculature. Resistance vessels are primarily involved in the regulation of arterial blood pressure and control of local blood flow. Dilatation of resistance vessels lowers resistance to increase blood flow (vasodilatation) and constriction of resistance vessels increases resistance and decreases blood flow (vasoconstriction). Therefore, vasodilatation/constriction can cause increase or decrease blood pressure within the local and/or systemic circulation influencing the rate of exchange by governing blood flow to exchange vessels (Guyton-Hall., 2001b).

In low birth weight babies, elastin composition within the walls of the aorta and large arteries is reduced (Levy *et al.*, 2008; Struijker-Boudier *et al.*, 2009). Hypothesised to be a major risk factor leading to arterial stenosis and hypertension, elastin synthesis occurs largely in the prenatal stage with around 95% of lifelong elastin complement being present at birth (Song *et al.* 1992; Bertelsen, 1960). Pulse wave velocity measured in adults who had

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a low birth weight suggests a lower arterial compliance and impaired microvascular function. Capillary recruitment was shown to be associated with an altered systemic renin angiotensin aldosterone system (RAAS) and angiotensin II (Ang II) and its subsequent inhibitory role in rarefaction and angiogenesis (Alves *et al.*, 1999). Furthermore, animal models have also shown altered vascular function in adult offspring. Programming of vascular dysfunction in animals has shown alterations in both enhanced vasoconstrictive response and endothelial-mediated vasodilation, the degree at which changes occur are dependent on vessel bed and animals studied. Programmed increase in resting blood pressure has been associated with increased sensitivity to AngII in female and male adult offspring of low protein-fed mothers with an increase in superoxide production in mesenteric arteries (Hadoke *et al.* 2006). Similar observations in programming studies showing impaired endothelium-dependent vasodilation have been reported. Mesenteric arteries from offspring of a maternal 50% nutrient restriction diet showed an altered response to acetylcholine administration (Ozaki *et al.* 2006). Offspring from maternal high fat-fed dams (Taylor *et al.* 2004) and offspring from hypoxic dams have also shown blunted response to acetylcholine in carotid but not femoral arteries indicative of a decreased endothelium-dependent vasodilation (Williams *et al.* 2005). In addition to these factors, oxidative stress is associated with many adverse fetal conditions such as pre-eclampsia and gestational hypertension, hyperglycaemia, inflammation, smoking and alcohol intake. Programming of hypertension by oxidative stress and vascular dysfunction has been shown to be characterised by enhanced vascular superoxide production (Li & Shah, 2004). Reactive oxygen species (ROS) are considered as a major

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component of the pathogenic element of CVD, atherosclerosis, vascular/endothelial dysfunction and tissue remodelling. The major mechanism for this is when the presence of ROS overwhelms antioxidant defence mechanisms in local or systemic tissues (Li & Shah, 2004). Furthermore, nitric oxide is one of the major bio-active vasodilator molecules and since the constitutive production of nitric oxide within the vascular endothelium is key for determining baseline arteriolar tone, and increased salt intake has been reported to inhibit nitric oxide synthase activity (Li et al., 2009). Thus, in the current study we have tested whether maternal salt and/or fructose intake may influence offspring vascular nitric oxide balance by administering nitric oxide blockade using N<sub>(G)</sub>-nitro-L-arginine methyl ester (L-NAME) and monitoring subsequent cardiovascular responses.

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### 1.3 Evolution & diet

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The early life environment may act upon many mechanisms to influence this risk but the kidney and vasculature are key targets given their role in cardiovascular maintenance. Developmental programming of hypertension has largely been concerned with maternal dietary influences on later risk of increased blood pressure and therefore it is pertinent to assess what is understood about diet and disease and what constitutes a 'Western Diet'. The modern human body and genome has evolved over the last 250,000 years and is genetically programmed to sustain life on diets similar to that of the Paleolithic era (Cordain, 2002; Schick, 2007). The term Palaeolithic, meaning 'of age of stone', is a period of time that covers the majority of human existence on earth. The Palaeolithic era extended from 2.5 million years ago to around 10,000 B.C. (Toth, 2007). During this period the transition by evolution from early *hominid*, such as *Homo Erectus* and *Homo Habilis*, into *Homo Sapiens* (modern human) occurred (Cordain *et al.*, 2005). 10 – 12,000 years ago humans lived a predominantly nomadic hunter-gatherer lifestyle. The Neolithic revolution around 10,000 years ago prompted by the progression of agriculture and animal husbandry, leading to stable settlements, food surplus and the transition from hunter-gather to farmer and trader (Cordain *et al.*, 2005). When compared to a modern western diet, the early Palaeolithic diet contained much more meat, fish, vegetables and fruits (Eaton & Cordain, 1997; Cordain *et al.*, 2005). Macronutrient characteristics of the diet such as protein (38% of total energy), carbohydrate (23% of total energy) and fat (39% of total energy) are very similar to the values reported in modern hunter-gather societies, but hugely different from values

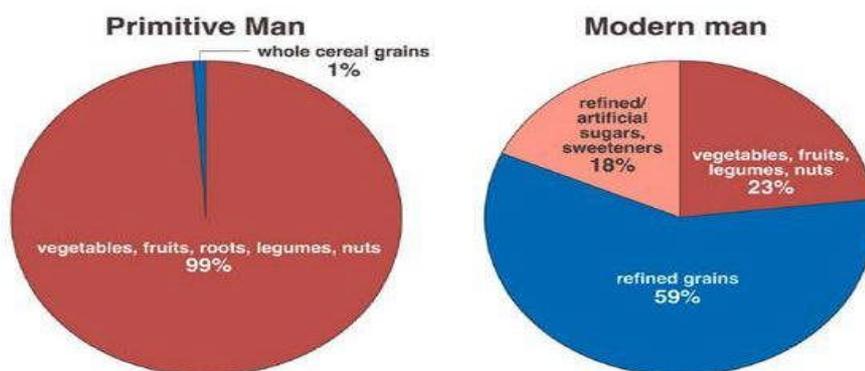
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reported in modern western diets (16% protein, 49% carbohydrate, 34% fat) (FSA, 1990; FSA, 2003; FSA, 2004). Palaeolithic diets were low in carbohydrates (23% energy) and consisted of high fibre intake of which 42.5g was plant fibre (Table 2, Figure.1). In the modern western diet this trend is reversed (>49% carbohydrate and <24g fibre).

The Palaeolithic diet has been reported to include more fat (39% energy) than the average (34% energy) western diet (FSA, 1990; FSA, 2003; FSA, 2004). This is a consequence of ingesting a greater amount of polyunsaturated (PUFA) and monounsaturated (MUFA) fats. Surprisingly, more than 50% of the Palaeolithic diet is of animal origin and saturated fat levels are within healthy ranges (<10% energy). However, the cholesterol levels are higher in the Palaeolithic diet than currently recommended levels (300mg). Hunter-gathers also consumed very little sodium compared with modern western man (Cordain 2005).

**Table 2. Mean daily intake of macronutrients and other compounds of the Palaeolithic diet**

Constituents/day	Total
Protein (g)	217
% energy	38
Carbohydrate (g)	129
% energy	23
Fat (g)	100.3
% energy	39
Sugars (g)	76.5
Fibre (g)	42.5
Cholesterol (mg)	461
Sodium (mg)	726
Saturated fat (g)	18
Monosaturated fat (g)	44.3
Polyunsaturated fat (g)	26.7

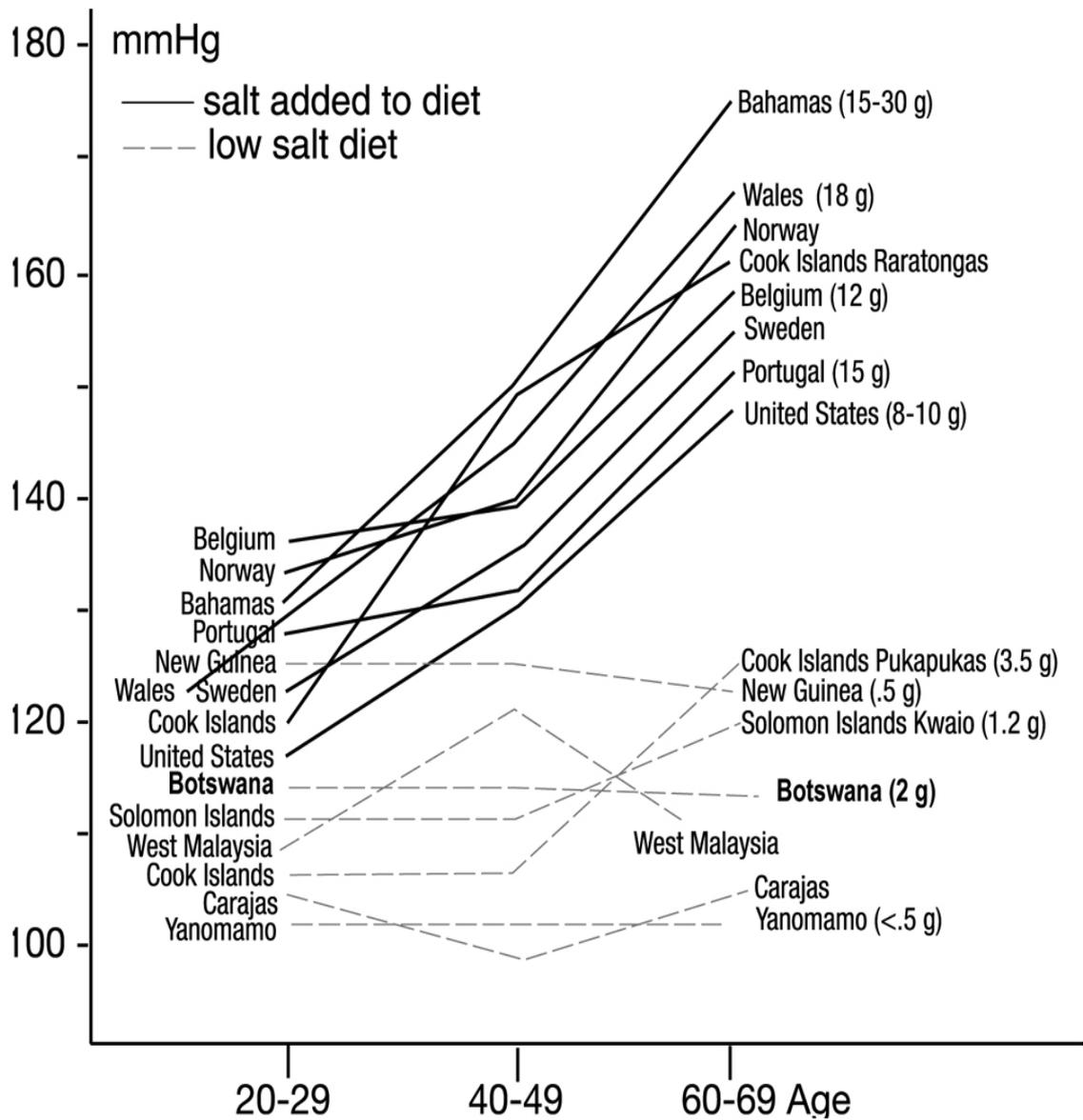
**Table2. Representation of mean daily intake of macronutrients and other compounds of the paleolithic diet. Adapted from: (Eaton *et al.* 1997).****Figure 1. Comparative carbohydrate intake in primitive man vs modern man****Figure1.** Represents the average daily intake of Palaeolithic man vs. contemporary western man.

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Taken from: (Eaton *et al.* 1997).

Renal systems studied in various species show evolutionary adaptation. Mammals originally descended from reptiles; however, the majority of mammals are terrestrial and have a much greater need for water conservation. In mammals, renal medullary thickness has a positive relationship with habitat aridity (Al-kahtani *et al.*, 2004). In early man, the need for an increased capacity to retain water was crucial to survival and people with increased ability to retain salt may have had a greater survival advantage. The kidney, through salt and water retention, achieves this in an environment when the major source of sodium would have been intrinsic sodium contained in meat, fish and plants; water availability would have also been limited. The evolutionary advantage of an increased ability of renal salt and water reabsorption has progressively decreased since humans have migrated to cooler environments and the advent of settled self sufficient communities and improved food production. Over time we have increased our salt intake, forcing the natural selection of a pre-existing beneficial trait into an unbeneficial, possibly dormant, pathological trait. It could be argued that evidence for this can be shown in modern day hunter-gatherer societies or communities whom, without the influence of additional dietary sodium, only eat intrinsically available sodium and additional extrinsic salt is extremely rare. These communities sustain lower blood pressures than similarly aged groups of western peoples; neither do they present age-related or essential hypertension (Eaton & Cordain, 1997; Cordain *et al.*, 2005)(Figure 2).

**Figure 2. Comparison of modern day hunter gatherers and westernised societies.**



**Figure 2.** Graph shows examples of average salt intake in both western and isolated communities and age-related changes in blood pressure. (Cordain et al 2005)

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### 1.3.1 PRESENT DAY DIETARY TRENDS

Dietary intake has altered only slightly from the proposed dietary intakes from around 5000 to 3000 years ago (Eaton & Cordain, 1997). However, during the last 200 years carbohydrate and sodium have become much larger constituents of the modern diet (Johnson *et al.*, 2007). The last eighty years has seen some of the largest changes in ratio of macronutrients within our diets, defining what we now refer to as the 'western' diet. Advances in agriculture and food technology during this time have seen a definitive shift into the 'age of excess' (FSA, 1990; FSA, 2003; FSA, 2004). In particular, the Food Standards Agency has carried out comprehensive studies on national dietary trends including, food and macronutrient intakes of children and adults from the 1920's to the present day. Other researchers have expanded on these national diet and nutritional surveys (NDNS) comparing 1950's intake with 1990's intake in children and 17 year-olds to adult nutrient intake (Prynne *et al.*, 1999; Prynne *et al.*, 2005). They showed further increasing macronutrient changes over a shorter time period to that seen since Eaton defined the Palaeolithic diet and made comparisons with present day macronutrient intakes (Eaton, 2006; Prynne *et al.* 2005). The study also showed that carbohydrate had now replaced dietary fat as the major source of calories (Table 3). Fat has also reduced slightly during this period and reductions in protein intake are also evident. The ratio of complex carbohydrates has decreased around 15% in the amount of % energy from starch and an increase of >50% of % energy from sugar. Interestingly the authors report a decrease in overall caloric intake; this could be explained by the replacement of dietary fat by lower caloric yield carbohydrates and decreases in overall physical activity. The authors

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also show a large increase in the amount of fruit juices and soft drink consumption within the 1990's cohort. The study shows soft drinks have taken over from drinks such as tea and water during meal times since the 1950's (Prynne *et al.* 1999; 2005; FSA 1990; FSA 2004).

Thus, a proportion of the variation in blood pressure between individuals may be attributable to the developmental environment and, in particular, diet at this time. In terms of the relationship between variation in nutrient exposure and variation in blood pressure then the evidence is strongest for a role for increased fructose or increased salt. Furthermore, when considering what constitutes a 'Western Diet' or a diet representative of the typical intake in current developed societies vs. those of hunter-gatherer societies (i.e. Palaeolithic diet) then further investigation of a role for these nutrients is warranted. In order to this in a controlled and mechanistic environment then one has to turn to animal models of nutritional programming.

**Table 3. Daily macronutrient and total energy intake in the United Kingdom. 1950-2004.**

<b>Constituents (% energy)</b>	<b>NDNS (1950)</b>	<b>NDNS (1990)</b>	<b>NDNS (2004)</b>
<b>Protein</b>	13	13	12
<b>Carbohydrate</b>	47	52	49
<b>Fat (% energy)</b>	40	35	38
<b>Sugar (% energy)</b>	16	28	33.5
<b>Starch (% energy)</b>	31	24	15.5
<b>Total energy intake (Kcal) (MJ)</b>	2294.5	2194	2198
	9.6	9.1	9.2
<b>Subjects/study</b>	4599	493	2087

**Table3.** The data shown was compiled from FSA NDNS studies 1990, 2003, 2004. Slight changes are seen in total protein, carbohydrate and fat intakes. Composition of carbohydrate shows the greatest alterations with % energy from sugar increasing consistently from 16% (1950), 28% (1990) and more than double 33.5% (2004). Similarly, total % energy from starch has decreased since the 1950 from 31% to 15.5%. In addition, total energy intake has decreased approx 100Kcal or 0.4MJ/day to 2198Kcal (9.2MJ) (2004) from 2294.5Kcal (9.6MJ) (1950).

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## 1.4 Fructose

### 1.4.1 DIETARY FRUCTOSE

Carbohydrates such as starch have been the major energy source within the diet of man since the advent of agriculture 8000 – 10000 years ago and very few simple sugars would have been included within our ancestral diet (Eaton & Cordain, 1997). As glucose and fructose are found in only small quantities in fruit, and honey is seasonal, amounts of dietary fructose were far lower than the proportion of glucose derived from starch found in the diet eaten by humans for thousands of years (Figure 1) (Eaton *et al.* 1997). Westernization of diets has seen significant increases in simple sugars (Tables 1,3), especially sucrose (a disaccharide of fructose and glucose) and fructose. Further studies of Palaeolithic man and modern day hunter-gatherers, show that the average intake of fructose per annum was around 5lb (2kg) the majority coming from honey, fruit and vegetables. Current typical intake per annum is around 69kg (Cordain *et al.*, 2005). Surprisingly, during the 1700's, individual sugar/fructose consumption was not very different to that seen in the Palaeolithic era (2kg). An increase to around 8kg was seen over the following 100 years up to 1800. Significant increases seen after the abolition of sugar tax in the 1870's saw sugar/fructose consumption increase to 45kg by 1950. However, by 1997 consumption had risen consistently to 69kg per annum (Figure 5) (Johnson *et al.*, 2007). This can also be partly attributed to the introduction and first mass commercial production of high fructose corn syrup (HFCS) in Finland (1969). There are three main varieties of HFCS (HFCS-45, HFCS-55

and HFCS-95), the numerical value indicates the percentage of fructose relative to glucose within the HFCS.

Figure 3. Sugar intake per capita in UK and US (1700 – 2000)

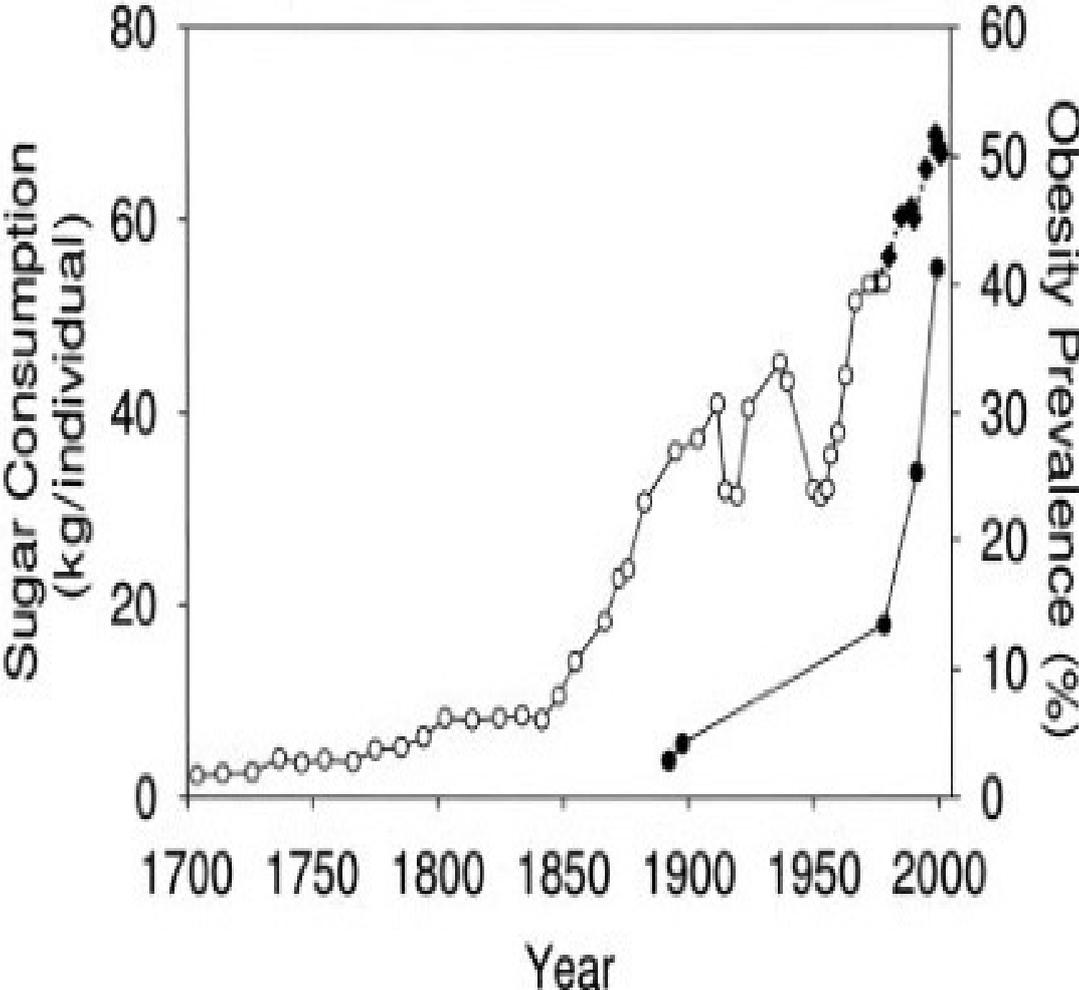


Figure3. Sugar intake per capita in the United Kingdom from 1700 to 1978 (O) & in the United States from 1975 to 2000 (●) compared with obesity rates in the United States 1890 to 2000 (◆). Adapted from: (Johnson *et al* 2007).

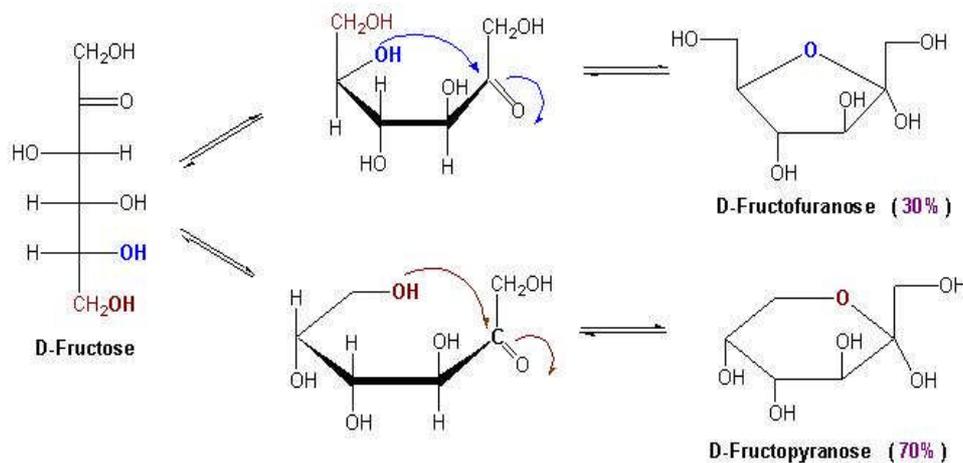
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Sucrose, fructose, glucose and HFCS are now added to a huge range of food products such as fruit juices, soft drinks, cereals and other pre-packaged foods as sweeteners to enhance flavour and palatability of products. The use of HFCS has increased by 1000% between 1970 and 1990, subsequently increasing consumption of added or extrinsic sugars (Johnson *et al.*, 2007; Sanchez-Lozada *et al.*, 2007). The use of HFCS and fructose has increased due to its sweeter taste than other sugars. Yudkin first argued the importance of the sweetness of fructose and how it could make foods more palatable (Yudkin, 1964). Fructose has been shown to be 2.5 times sweeter than glucose and does not inhibit satiety as other carbohydrates (Choi *et al.*, 2003).

### 1.4.2 CELLULAR UPTAKE & FRUCTOSE METABOLISM

Fructose is a mono-saccharide, it has a ketone functional group and it shares the same chemical formula and with is an isomer of glucose. In equilibrium, fructose can be found existing as around 70% fructopyranose and 30% fructofuranose (Figure 4).

**Figure 4. Isomeric forms of fructose.**



**Figure4.** Diagram of the Isomeric forms of fructose in equilibrium within solution. Taken from: (Elliott, 2005).

Fructose is absorbed by the small intestine and is transported across the epithelial barrier into cells and the blood stream by glucose transporters (Cook, 1971). Entry of fructose into cells is not insulin dependent and does not promote insulin secretion unlike glucose. Moreover, up to 90% of all ingested fructose is absorbed intact (Zhao & Keating, 2007).

Fructose and other hexoses are not small enough to diffuse directly into the cell. They are

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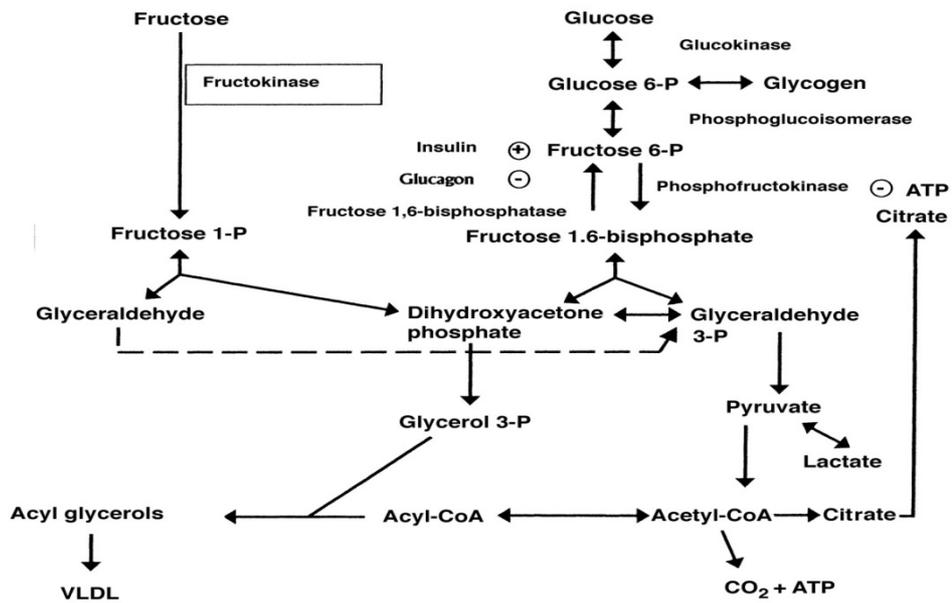
transported across cellular membranes by glucose transporters. There are two types of glucose transporters, the Na<sup>+</sup>-dependent (facilitated diffusion transport system), and the Na<sup>+</sup> monosaccharide co-transporter system (Crane *et al.* 1960). In mammals, the facilitated transport of hexoses is mediated by the GLUT protein family (glucose transporter isoforms). This family of glucose transporters is comprised of at least 14 isoforms (GLUT-1 – GLUT-14). GLUT proteins exist in the membrane in two conformational states; extracellular hexoses bind to the transporter, which then alters its conformation, shunting the substrate across the membrane (Muekler *et al.* 1985).

Glucose transporters display tissue specificity of expression, e.g. GLUT-1 is abundant in erythrocytes and brain and very low in skeletal muscle and adipose tissue (McAllister *et al.* 2001). Glucose transporters also show specialized functions amongst differing GLUT isoforms and substrates. For example, GLUT-1, GLUT-3 and GLUT-4 are involved in substrate uptake from blood, whereas GLUT-2, found in high levels in liver, kidney and pancreas, can transport glucose and fructose in and out of cells. GLUT-5, GLUT-7 and GLUT-2 are currently known to be the primary transporters of fructose, and are found in high concentrations within the intestine, kidneys and testis (Zhao & Keating, 2007; Sheperd *et al.* 1992). Evidence of active transport of fructose has been shown as fructose can be found in high concentrations in specific tissues, suggesting movement against the concentration gradient (Ogawa *et al.* 1996). Absorbed fructose is transported in plasma via the hepatic portal vein to the liver where fructose is predominantly metabolised; only a small amount of fructose is metabolized by hexokinase in muscle and adipose tissue (Heinz *et al.* 1962). Fructose enters

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glycolysis at a stage where the fructose molecule has already skipped a major rate limiting step (Figure 4). When fructose is transported into a cell a phosphate group is added by fructokinase to produce fructose-1-phosphate (F-1-P) (Alberts, 2002). Phosphorylated fructose (fructose-1-phosphate) has no specificity to any glucose transporters; the molecule is essentially 'trapped' within the cell (Sillero *et al.* 2005). Fructose can be phosphorylated by either fructokinase (FK) (ketohexokinase) or hexokinase (HK). Hexokinase has a low affinity for fructose and several other hexoses and, under normal conditions, hexokinase would be responsible for specifically phosphorylating glucose in all tissues of the body. Unless high intracellular concentrations of fructose are present, very little fructose is metabolized to F-1-P by hexokinase. Thus, fructokinase is the primary mechanism of fructose phosphorylation (Figure 5) (Sillero *et al.* 2005). Fructokinase can be predominantly found within the cells of the liver with lower concentrations in kidney and intestinal mucosa. Therefore the majority of fructose is only metabolised by the liver (Heinz *et al.* 1962). Additionally, by forming F-1-P and entering glycolysis on a tri-phosphate level, F-1-P skips phosphofructokinase (PFK), where continued glucose metabolism is limited by feedback inhibition of citrate and ATP build-up. Thus, the kinetics of fructose metabolism are more rapid than that of glucose, allowing fructose to serve as an unregulated source of glycerol-3-phosphate (G-3-P) and acetyl-CoA for de novo hepatic lipogenesis (Alberts, 2002).

Figure 5. Glycolysis and fructose metabolism



Fructose metabolism in detail

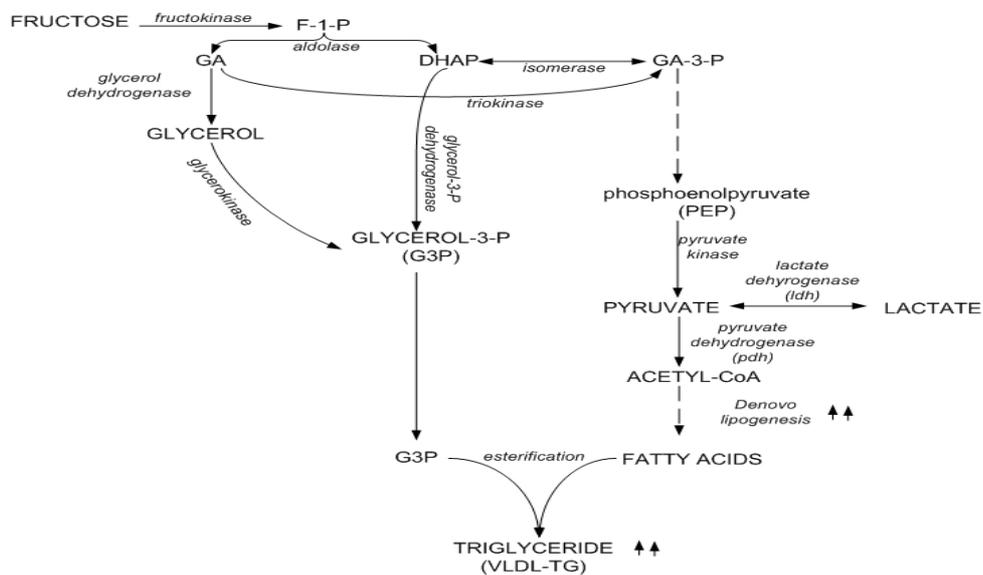


Figure 5. Glycolysis and the citric acid cycle provide the metabolites required to synthesize biological molecules. Amino acids, carbohydrates, lipids, nucleotides as well as other products, in turn serve as metabolites for intra and extracellular macromolecules. Black arrows represent an enzyme reaction leading to the indicated product. Taken from: (Elliott, 2005).

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### 1.4.3 ADVERSE EFFECTS OF FRUCTOSE

Keys and co-workers (1952) were the first to be credited with reporting the association between the western diet and cardiovascular disease (Keys, 1952). It was in the 1960's however, that the first correlations between high sugar intake and the role it may play in the rise in cardiovascular disease were made (Yudkin, 1964; Yudkin, 1967; Yudkin, 1970; Yudkin, 1972). More recently, fructose and its role in the widespread increases of obesity and heart disease have been proposed (Stanhope & Havel, 2008; Stanhope & Havel, 2009; Stanhope & Havel, 2010).

It is widely acknowledged that high intake of refined and simple sugars can have a negative impact on health (Hu & Malik, 2010; Sanchez-Lozada *et al.*, 2010; Stanhope & Havel, 2010). Excessive consumption of refined sugars alters the ratio of macronutrients and can replace more beneficial nutrients and reduce intake of vitamins, minerals, amino acids and other essential nutrients. Fructose has a very low glycemic index rating (G.I) in comparison with glucose (glucose = 100, fructose = 20) and has been shown to have no effect on short term blood glucose levels (Foster-Powell *et al.* 2002). For this reason, people with diabetes have been advised by clinicians to sweeten with fructose rather than sucrose or glucose.

A number of animal and human studies have reported associations with excessive fructose consumption and adverse effects upon metabolic status and health. Hypertriacylglyceridaemia, hyperuricemia, hypertension, endothelial dysfunction, hyperlipidemia and atherosclerotic blood profile, obesity, metabolic syndrome, non-

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alcoholic fatty liver disease (NAFLD), gout and renal microvascular damage, insulin resistance, hyperinsulinemia, increased advanced glycation end-products (AGE's) and unregulated appetite have all been reported as a consequence of excessive fructose intake in both humans and animals studies (Coelho *et al.*, 2010; Ferder *et al.*, 2010; Hu & Malik, 2010; Sanchez-Lozada *et al.*, 2010; Stanhope & Havel, 2010). Clinical studies have shown fructose and sucrose can induce weight gain and altered blood chemistry (Reiser *et al.* 2000). For example, men and women fed fructose to 17% of total energy intake for 6 weeks have been shown to have higher fasting and postprandial triglycerides (TG) (Bantle *et al.*, 2000). Other studies have fed fructose for 5 weeks and 20% of total energy intake and showed increased TG's in men with and without hyperinsulinemia (Reiser *et al.*, 1988; Reiser *et al.*, 1989). In addition, TG's increased in both groups with fructose but not in those replaced with cornstarch. Similar studies in humans showed a dose dependent relationship in males with hyperinsulinemia being fed fructose at 0%, 7.5% and 15% of total energy intake for 5 weeks. Moreover, TG's in the hyperinsulinemic group rose with increasing fructose intake (Hallfrisch *et al.*, 1986). Stimulated TG synthesis can lead to hepatic accumulation of TG's, which has been shown to reduce hepatic insulin sensitivity (Adeli *et al.*, 2001). Ideally, TG's are stored in adipocytes as a reserve of available energy when needed. However, during periods of increased production or higher substrate availability, TG's have been shown to accumulate within ectopic depots e.g. hepatocytes (Kawano *et al.*, 2007). Subsequently, this increases the production of VLDL and intra- and extra-hepatic fat deposition which has been implicated in the progression of NAFLD. Excess TG's can also be stored in skeletal muscle

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myocytes and visceral adipocytes, and may cause insulin resistance in muscle and liver (Adeli *et al.* 2001). Hypertriglyceridemia, caused by excessive fructose intake has also been reported as a precursor of insulin resistance (Thorburn *et al.*, 1989).

In addition, researchers have reported that regular excessive consumption of fructose can cause a rise in mean arterial pressure (MAP) in humans (Hallfrisch, 1990) and rats (Dai & McNiell, 1995). The mechanisms for such a rise in blood pressure or progression of hypertension are not fully understood, but increased TG's (Kawano *et al.* 2007), hyperinsulinemia (Adeli *et al.* 2001), excessive uric acid production (Mazzali *et al.* 2007), endothelial dysfunction (Puyo *et al.* 2010) and renal damage may all play a role. Fructose induced hypertension was first shown in the rat using the tail cuff method (Hwang *et al.*, 1987). Similarly, Hallfrisch showed a systolic blood pressure increase in men fed fructose at 7.5% and 15% of total energy intake after just 5 weeks (Hallfrisch *et al.*, 1982). Additionally, blood pressure increases in rats fed fructose at 10% of energy (Sanchez-Lozada, *et al.* 2007), the study used radiotelemetry BP monitoring for one week. However, the majority of studies tend to involve feeding excessively high levels of fructose (60, 68, 70% of total energy intake), making it difficult to compare against human dietary fructose consumption as 30% - 70% of total energy intake is not reflective of average human intake (Thorburn *et al.*, 1989; D'Angelo *et al.*, 2005; Rawana *et al.* 1993). These studies would normally be used in a pharmacological context. A number of studies have implicated the involvement of insulin resistance and hyperinsulinemia without the development of hypertension in the fructose

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fed rat (D'Angelo *et al.*, 2005; Thorburn *et al.*, 1989) Although fructose feeding in the short term does not significantly increase insulin secretion, long term feeding can cause hyperinsulinemia (Thorburn *et al.*, 1989). Thus, in the current study we have employed 10% fructose fed in water.

#### **1.4.3.1 NON-ALCOHOLIC FATTY LIVER DISEASE**

Non alcoholic fatty liver disease (NAFLD) is a condition in which early signs are observed in up to 74% of people with obesity (Erickson, 2009). NAFLD currently affects 10-24% of the population and is so-called because it cannot be explained by excessive alcoholic consumption. When left untreated NAFLD will progress to cirrhosis and liver failure (Clarke *et al.* 2003). Additional risk factors for NAFLD include hypertriglyceridemia and insulin resistance, both of which have been shown to be major side effects of excessive fructose consumption (Vuppalanchi *et al.* 2009). Individuals with hereditary fructose intolerance also present with liver cirrhosis and damage; if fructose is continually eaten, liver failure and kidney damage can develop (Lim *et al.*, 2010). Studies using the fructose fed rat have also shown similar results (Fan, 2008). However, the extent to which fructose consumption and NAFLD is playing a role in the increasing cases of liver damage and failure has not yet been investigated fully.

#### **1.4.3.2 SERUM URIC ACID**

Increased serum uric acid concentrations have also been associated with increased risk of CVD; and uric acid increases after fructose feeding in the rat and human. Uric acid was

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thought to be an inert metabolite or an antioxidant. However, moderate levels of serum uric acid have been shown to have a deleterious effect on the bioavailability of NO and the stimulation of intrarenal renin to raise blood pressure in rats (Mazzali *et al.*, 2001). Other studies have shown a progressive renal arteriosclerosis, renal disease, vasoconstriction and glomerular hypertension associated with increased plasma uric acid (Feig *et al.*, 2004; Feig *et al.*, 2006).

### **1.4.3.3 ADVANCED GLYCATION END PRODUCTS**

Fructose is a potent reducing sugar and has the capacity to glycate proteins at a rate 17 times faster than glucose (Uribarri *et al.*, 2005). Advanced glycation by fructose occurs in solution at room temperature (23°C), but increased temperature naturally speeds up the reaction. AGE's form when fructose is nonenzymically linked to proteins (Maillard browning reaction). Dietary AGE's, caused by fructose, have been shown to interact with tissues and serum proteins that may reduce tissue elasticity and interfere with cellular mechanisms (Uribarri *et al.*, 2005).

### **1.4.3.4 FRUCTOSE & OBESITY**

Historically, nutritionists have associated high intake of fat with obesity, but studies show that the greatest proportion of macronutrient change over time is carbohydrate, very little change other than percentage of saturated and unsaturated fats has occurred within fat intake over time (FSA, 1990; FSA, 2003; FSA, 2004) (Prynne *et al.*, 1999). Other than energy imbalance, there is still huge debate regarding the causes of obesity. Undoubtedly, fructose

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does have some obesity-promoting effects and the metabolic changes during fructose feeding seem to play more of a role than actual increased fat consumption (Hallfrisch *et al.* 1982; Bantle *et al.* 2000). Studies show that feeding fructose leads to increased visceral adiposity relative to glucose fed mice (Allen *et al.*, 1966). Additionally, studies in baboons showed an increase in abdominal fat accumulation when fed sucrose in comparison to glucose (Wagner *et al.*, 2006). Human studies have also shown a link between obesity and fructose feeding. A study of 548 children over 19 months showed an association between sugar-sweetened soft drink consumption and obesity, increasing body mass index by 0.24 kg/m<sup>2</sup> (p=0.03) with a 60% rise in obesity (p=0.02) (Berkey *et al.*, 2004). More recently, a review hypothesised that just a 33oz. soft drink containing 35g of sugars as well as the current mean intake of macronutrients and the proposed sedentary lifestyle may be enough to produce a positive energy balance causing excessive fat accumulation over a sufficient period of time (Bray *et al.*, 2004; Malik *et al.*, 2010). Although there are many gaps in knowledge regarding the onset of obesity more studies are needed to elucidate the exact role of fructose within the obesity conundrum.

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## 1.5 Sodium

### 1.5.1 DIETARY SODIUM

Diets of western communities tend to contain amounts of salt (sodium) which are hugely excessive to normal human physiological needs. Like most natural foods, salt is generally considered safe. Although the use of salt in foods is essentially unregulated, there is extensive guidance by dietary research from government bodies (COMA, FSA, SACN) in the UK on limiting the addition of salt in processed and packaged foods (SACN 2004; FSA, 2004; NDNS 2008). Approximately 11% of daily intake of salt is by the addition of salt when cooking and eating foods. 12% of dietary sodium per day comes from natural sources such as meats, animal products and plant matter. 77% of all daily intake of salt is derived from processed and prepared foods; up to 35% of this 77% can be contained in cereals and cereal products (SACN, 2004).

In the UK, the Committee on Medical Aspects of food Policy (COMA) set Dietary Reference Values (DRV) and Reference Nutrient Intakes (RNI) for the population. Currently, the RNI for salt is 4.0g (1.6g sodium) per day, with an achievable DRV of 6.0g per day. The Scientific Advisory Committee on Nutrition (SACN) has recently reviewed evidence of salt intake and hypertension (SACN 2004). The report finds evidence of a link, stronger now, than when reviewed by COMA in the early 1990's and recommends maintaining the achievable DRV of 6.0g per day.

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The FSA and COMA had a target to try and reduce salt intake to the RNI of 6g or less per day by 2010. The FSA Nutritional Data National Survey (NDNS) in 1986/87, 2000/01, 2003/04 and 2006 shows mean intakes of salt exceeded the 6g per day recommendation in all age groups and sexes. Intake was higher for men (11.0g) than for women (8.1g). Only 15% of men and 31% of women had intakes of 6g or less during the survey. Mean salt intake for men and women has increased only slightly from 9g per day 1986/87, to 9.5g per day in 2000/01 up to 9.6g per day in 2003/04 and 2006 (FSA 1987; 1990; 2001; 2004). More recently, a report by the FSA (NDNS) released in September 2008 reported that salt intake among adults in the UK had fallen to around 8.6g per day (for both men and women). The study was based on 294 men and 398 women (9.7g/day for men) (7.7g/day for women).

### **1.5.2 PHYSIOLOGICAL SODIUM**

The average adult human needs around 500mg of sodium (Na) per day. Since most animals consume a varied omnivorous diet, these are unlikely to be deficient in sodium. A 70Kg body contains approximately 3.5mol sodium. Half the sodium is within the ECF, where it makes up 90 % of the cations, and 10% intracellular, the remainder is within the bone (Seeley, 1992).

Sodium is an inorganic element and the major monovalent electrolytes within the body fluids and is primarily responsible for the osmolarity and volume of extracellular fluid (ECF).

The balance of sodium within the body is intimately linked with water balance. Together with potassium and chloride, sodium plays a role in conduction of electrical nerve impulses, excitation of muscle cells (including cardiac muscle) and in combination with the bicarbonate system regulates the pH of the body and excess or deficiency can be the basis of alterations

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in acid – base balance. Sodium and chloride are also linked to the transport of many molecules across cell membranes, and their presence is also necessary for the absorption of a number of nutrients from the gut. The plasma membrane enzyme, sodium-potassium – ATPase ( $\text{Na}^+/\text{K}^+$  ATPase) plays a pivotal role in water balance, nerve conduction and active transport. This enzyme pumps sodium out of the cell and returns potassium into the cell simultaneously whilst adenosine triphosphate (ATP) is hydrolysed. Nerve impulses along nerve cells, active transport of nutrients into enterocytes and muscle cells all are dependent on the  $\text{Na}^+/\text{K}^+$  - ATPase pump (Seeley, 1992).

Absorption of sodium in the gut during good health is >97%, some sodium is secreted into the gut in bile, pancreatic juice and small intestine, no more than 2% of excretion is via the faeces, approximately 2% is by the skin, unless during active sweating when sodium excretion increases. Communities living in tropical climates have been shown to excrete a more dilute sweat to prevent excess sodium loss (Taubes *et al.* 1998). The main excretory route for sodium is the urine (>95%). Most healthy individuals would be described as being in a state of sodium balance (i.e. urinary excretion is matched to dietary intake), so for this reason urinary excretion (over 24hr) is generally accepted as the most reliable estimate of sodium intake (Elliott, 2005).

### **1.5.3 SODIUM & WATER HOMEOSTASIS**

Dependent on the availability of water, the healthy adult kidney is able to excrete as much or as little sodium and fluid as required (Guyton 1967). Urinary sodium excretion during good health is a finely balanced myriad of homeostatic processes. Plasma sodium balance

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can be influenced by numerous mechanisms. Physical forces such as perfusion pressure and colloid osmotic pressure, neural and behavioural mechanisms like thirst and salt hunger as well as renal filtration rate, endocrine and hormonal can all affect  $\text{Na}^+$  homeostasis. All of which are highly sensitive to changes in ECF volume or blood pressure. Several mechanisms, both neural and hormonal, appear to play a role in this regulation. Deficient (hyponatraemia) and excessive (hypernatraemia) amounts of sodium can occur in the blood as a result of a defect in excretion of sodium. Some of the major mechanisms that regulate the sodium ion levels in the ECF.

Increased blood pressure in the right atrium of the heart causes secretion of atrial natriuretic factor (ANF), which increases sodium ion excretion and hence water loss in the form of urine. This results in decreased blood pressure as blood volume decreases because water is lost; blood osmolality is maintained because both sodium and water are lost (Guyton *et al.* 1964). The mechanism works in reverse with a decrease in ANF decreasing water loss and increasing BP when a decrease of BP is detected within the right atrium of the heart (de Bold *et al.* 1985).

The renin angiotensin aldosterone system (RAAS) is a major component of homeostatic regulation. The RAAS acts primarily by regulating renal reabsorption of sodium and responds to decreased BP within the afferent arterioles of the kidney. The liver, and to a lesser extent adipose tissue, produces and secretes angiotensinogen (Ang) into the circulation. Renin is a proteolytic enzyme released from specialised cells called the juxtaglomerular apparatus in response to decreased arterial pressure, sympathetic nervous activity and decreases in distal

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tubular NaCl at the macula densa. Renin hydrolyses Ao to yield angiotensin I (AngI). AngI is then cleaved by angiotensin converting enzyme (ACE), which is mainly produced and situated locally in the lung capillaries, to yield angiotensin II (AngII). AngII is a pivotal bioactive molecule of the RAAS and acts as an endocrine, autocrine, paracrine and intracrine hormone. It is a potent vasoconstrictor and binds to specific receptors (AT1R) on the cell surface of tissue and organs such as the liver, kidney, heart, brain and throughout the systemic vasculature. There are a number of AngII receptors, however, AT1R has been shown to be the most important in blood pressure control (Coffman, 2009). In the kidney, AngII vasoconstricts the glomerular arterioles, having a greater effect upon the efferent in comparison to the afferent arterioles and thus maintaining glomerular pressure and glomerular filtration rate (GFR) despite a reduced renal blood flow. Angiotensin II stimulates increased aldosterone secretion from the adrenal cortex. Aldosterone acts upon the distal convoluted tubules and collecting ducts of the kidney to increase  $K^+$  excretion in exchange for increased reabsorption of Na, facilitating water retention under the influence of ADH. The increased reabsorption will increase blood volume, increasing blood pressure, maintaining blood osmolality and excretion of concentrated urine (Guyton *et al.* 1964). AngII levels in the presence of a high expression of AT1R in the kidney may cause hypertension to a greater extent than any other local RAS such as in the brain or heart.

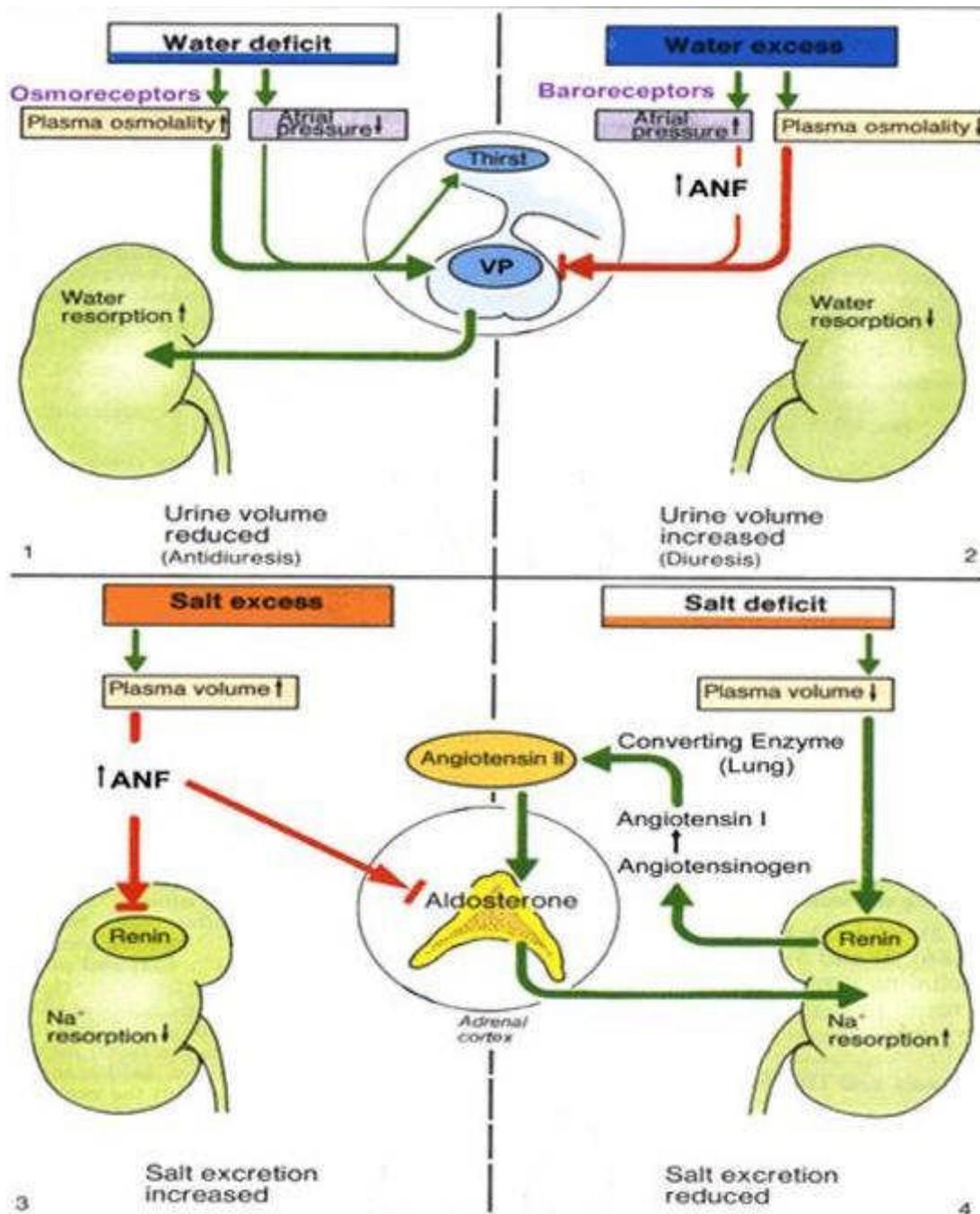
Utilising genetically modified mice, Coffman *et al* have shown the RAAS is essential for blood pressure control. Elevation in blood pressure can also be determined in part, by the quantity and distribution of the AT1R. Thus, null A1TR mice show significant reduction in blood

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pressure. Further studies have shown a correlation between plasma angiotensinogen and renin levels in patients with hypertension (Jeunemaitre *et al.*, 1992). In addition, AT1R receptors in Sprague Dawley rats may also be influenced by salt intake and plasma Na levels (Schmid *et al.*, 1997).

Significant decreases in arterial BP will also elicit a rise in anti-naturetic hormone (ADH) secretion. ADH is secreted from the posterior pituitary as a result of increased afferent activity from peripheral baroreceptors (e.g. in the large arteries). The increase in ADH will increase water reabsorption within the kidney, resulting in a small volume of concentrated urine being produced. An increase in arterial BP will be caused by an increase in blood volume and a decrease in blood osmolality (a dilution effect), the resulting increase in blood volume will act to restore blood pressure. Antidiuretic hormone (ADH) is considered the most important regulator of blood osmolality. An increase in blood osmolality (e.g increased sodium ion concentration) causes an increase in ADH secretion from the posterior pituitary, mediated by osmoreceptors. This results in increased water reabsorption in the kidneys, thus diluting the blood and restoring normal osmolality (Figure 6). Hence, blood osmolality is usually maintained to within +/- 3% through the precise control of extracellular ion concentration and concomitant fluid balance.

Figure 6. Mechanisms of salt and water homeostasis.



**Figure 6.** Role of the kidney in salt and water homeostasis. 1. Kidney & hypothalamic, pituitary response to a water deficit. 2. Kidney & hypothalamic, pituitary response to excess water. 3. Kidney & adrenal gland response to salt excess. 4. Kidney & adrenal gland response to a salt deficit. Adapted from: (Despopoulos & Silbernagl 1986).

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#### 1.5.4 ADVERSE EFFECTS OF SODIUM

The first implication that salt may be a factor in raising blood pressure was made in the early 1900's. Studies showed the positive relationship between chloride and blood pressure, during salt feeding (they did not know how to measure sodium at that time) (Ambard & Beaujard, 1904). Dietary salt intervention and a lowering of blood pressure was reported by Kempner *et al*, reductions in blood pressure of 50% of hypertensive patients placed on a low salt diet were seen (Kempner, 1948). More recently, epidemiological, clinical and animal studies show evidence of a correlation between salt intake and blood pressure (Stamler *et al.* 1996; Folte *et al.* 1989; Cooke *et al.* 2007). Clinical and animal trials have shown that reducing salt intake reduces BP levels in normotensive and hypertensive cohorts and may prevent hypertension. A more recent trial involving >3000 subjects aged 30 -54 with pre-hypertensive blood pressure, reported that a decreased salt intake reduced the risk of cardiovascular disease by up to 35%, one for 18 month, the other for 2-3 year (Cook *et al.*, 2007). The sodium-restricted subjects were placed in two groups, both of which reduced their sodium intake by 25 to 35%. The control group did not reduce sodium intake. The subjects were followed for a further 10 – 15 year and health status recorded. The results showed both sodium restricted groups were less likely to suffer cardiovascular disease and a >20% decrease reduced risk of dying from any other cause (Cook *et al.*, 2007). Another long-term intervention study, examined the salt intake of two villages in Portugal. Salt intake was reduced by >50% in one village population and assessed by 24hr urinary sodium excretion. A progressive decrease in blood pressure and a significant fall in systolic and diastolic

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pressures were seen when compared to the control village after the first and second years (Forte *et al.*, 1989). There is now a plethora of studies on undeveloped communities whom, historically, have a very low salt intake (<1g). These communities predominantly exhibit low blood pressure, and show no age-related hypertension as seen in western countries. Studies on two Nigerian village communities, one of which had long utilised salt from a nearby lake and the other which does not, but both population's diet (in macronutrient terms) being virtually the same, show differences in salt intake and blood pressure (Cappuccio *et al.*, 2006). The Qash'qai, a modern day hunter-gather tribe from northern Iran, because of local salt deposits, has a high intake of salt. The tribe, although consuming essentially a 'Palaeolithic' diet show increases in blood pressure and age-related hypertension as seen in western communities (Cordain *et al.*, 2005).

One of the largest salt studies (Intersalt) involved a controlled study of 52 communities in 32 countries around the world. This included data from over 10,000 adults in total, all with varying levels of salt intake (0.5g >25g per day). The study showed a weak positive relationship between salt intake and blood pressure and non-significant relationships between salt intake, age and increasing blood pressure; they estimated that an increase of 6g per day over 30 year would lead to a 9mmHg rise in BP (Stamler *et al.*, 1996; Stamler, 1997). The associations made in the study are as mentioned weak at best, a 9mmHg rise in blood pressure over 30 years could be described as negligible considering all other confounding factors involved during 30 years of adult life. In addition to the Intersalt study,

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the internationally recognised Cochrane Collaboration performed a meta-analysis on 32 existing studies involving individuals with elevated blood pressure (n=802) and normotensive individuals (n=2220), and examined the relationship between lowering salt intake and reducing blood pressure. The analysis found that in individuals with already elevated blood pressure, reducing salt intake by 2g/day (on average) reduced blood pressure by 5.06 mmHg for systolic and 2.7mmHg for diastolic after a four week intervention. In contrast, whereas in normotensive subjects, a reduction in blood pressure of 2.03mmHg for systolic and 0.99 mmHg for diastolic was found, suggesting a greater effect of salt reduction in hypertensive patients (Duley *et al.* 1999). In addition to these findings, a significant relationship between reduction in urinary sodium excretion and reduced blood pressure was observed (He & MacGregor, 2000). Given that the majority of studies analysed involved an average of 2g/day reduction in salt intake and current recommendations are to reduce salt by 5-7g/day, the authors argue that a more controlled and larger intervention than 2g/day would likely see a greater reduction in blood pressure, similar to the dose dependent responses seen in animal studies (see below). Clearly, there needs to be more epidemiological analysis carried out in this area before we can determine the true effects of salt on blood pressure. Animal studies more strongly suggest a positive and dose dependent relationships with salt intake and blood pressure. For example a study in chimpanzees' involved one group fed a normal diet which includes 0.5g of salt compared to a second group with a salt intake of 10 – 15g per day for 20 months. The high salt diet group showed significant rise in blood pressure and a return to control group levels upon cessation of the salt treatment (Denton *et al.*, 1995).

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High salt intake and relationships with blood pressure have also been shown in the cat (Buranakarl *et al.*, 2004), dog (Chandler, 2008) and the pig (Corbett *et al.*, 1979), although the majority of studies have been in rodents. In groups of adult rats fed varying concentrations (0.15, 2.8, 5.6, 7.8 and 9.8%) of salt within the diet, mean systolic blood pressure rose in proportion to the amount of dietary salt ingested but there was a large variation between individuals and sexes in response to high salt diets. This suggests the importance of population studies rather than individual studies of hypertension, to override a role for genetics, salt sensitivity/resistance and environment in the correlation between salt and blood pressure (Reed *et al.*, 1994).

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## 1.6 Rationale for the programme of work

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Ancestral man is predicted to have eaten a diet high in fibre, potassium, complex carbohydrates and protein and low in sodium, refined sugars and energy density i.e., a Palaeolithic diet. Since this time, when physiological and metabolic systems were evolving, there has been a gradual transition away from this Palaeolithic diet, with the emergence of agriculture (*ca.* 7 to 5,000 years ago) through to the industrial revolution (*ca.* last 100 years). The 'Modern diet' has rapidly become low in fibre and high in sodium, simple sugars and energy density. When superimposed on the Palaeolithic genotype and physiology, this has resulted in an increased incidence of non-communicable diseases (NCD), estimated to account for 60% of all deaths worldwide. Modification of diet offers an achievable and economically beneficial prevention strategy for NCD. For example, short-term consumption of a 'Palaeolithic' diet produces favourable and significant reductions in blood pressure, cholesterol, triglyceride and insulin resistance (Cordain *et al.* 2002; Eaton *et al.* 1987). Earlier dietary intervention, for example to pregnant mothers or those considering pregnancy, may have added benefit since adverse periconceptual and/or prenatal nutritional exposure increases the risk of NCD (e.g. cardiovascular or metabolic disease) in the adult offspring – a paradigm referred to as the developmental programming of health and disease. Animal studies have begun to elucidate mechanistic aspects of the DOHaD phenomenon, with variation in diet during development being central to the induced phenotype in the offspring. While, the majority of developmental programming studies aim to recapitulate either a 'Westernized' or under/over nourished diet using a low protein, or a high-fat and/or

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a high sugar paradigm, the dietary trends in the UK suggest that a high simple sugar and salt intake may be more relevant. In the US a similar dietary pattern of high fructose and high salt intake has been observed (USDA 2009). Whilst a few previous studies have considered the programming effects of a maternal diet high in either simple sugars (e.g. fructose) (Bell *et al.*, 2002; Alzamendi *et al.*, 2010) or salt (Contreras *et al.*, 2000; Porter *et al.*, 2007), none have considered any possible interaction between the two. This is important because increased intake of salt in (or added to) food tends to engender increased intake of simple sugars (e.g. from sugar sweetened beverages) (He *et al.* 2008; Moses *et al.* 2009; Malik *et al.* 2010). Since each is known to influence cardiovascular and metabolic health, then it is important to consider their interaction in an experimental design. Given that sex-specific effects are widely observed in developmental programming studies then gender-specific responses are also an important outcome for consideration. No study has considered the delayed cardiorenal and metabolic consequences for adult offspring (male and female) of combined intake of fructose and salt by the dam. Hence, the aim of the present study was to characterize the effect of moderate inclusion of dietary salt (as NaCl in food, to represent increased consumption of sodium in the Western world) and/or fructose in the drinking water (to represent increased consumption of sugar-sweetened beverages in the Western world) on growth and metabolism of the non-pregnant and pregnant dam, on fetal growth and on the metabolic status and growth of the male and female offspring to adulthood.

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## 1.7 Aims and Hypotheses

### 1.7.1 HYPOTHESES

- Pregnant Spague Dawley rats consuming excess fructose will develop a non-alcoholic fatty-liver phenotype.
- Pregnant Spague Dawley rats consuming excess salt will transmit a hypertensive phenotype to the male and female offspring resulting in altered cardiovascular function.
- Pregnant Spague Dawley rats consuming excess fructose and salt will transmit both a non-alcoholic fatty-liver and hypertensive phenotype to the male and female offspring as adults.
- Further adult exposure of these maternally nutrient-programmed offspring to either fructose or salt will exacerbate the programming effects observed.

### 1.7.2 AIMS

To first characterise the effects of moderate dietary inclusion of salt and/or fructose on maternal and fetal growth and metabolic status, through assessment in:

- the **non-pregnant dam** (*Chapter 3*).
- the **pregnant dam** (*Chapter 3*).
- the **fetus and neonate** (*Chapter 4*).

and to subsequently determine any potential effects of fructose and/or salt on baseline and stimulated cardiovascular health and sensitivity of the adult male and female offspring, through assessment of:

- **adult offspring resting blood pressure and heart rate** (*Chapter 5*).
- **adult offspring cardiovascular responses to pressor challenges** (*Chapter 5*).
- **adult offspring renal function** (*Chapter 5*).

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## 2 Methods

### 2.1 Study Design

This thesis consists of 3 studies (Figures 7,8,9), each using the same dietary protocol but different with respect to experimental outcomes. Purified diets (Table. 4) were used in combination with a 10% fructose solution to achieve the required dietary groups: 1) control (CD), fed purified control chow 1% NaCl (TD.08164 Teklad Harlan, Maddison. WI.) and fresh water; 2) Salt (SD), fed purified control chow with added 4% NaCl (TD.08162 Teklad Harlan, Maddison WI.) and fresh water; 3) Fructose (FD), fed purified control chow 1% NaCl (TD.08164) and fresh water with 10% fructose (Sigma-Aldrich, UK) added; Fructose and salt (FSD), fed purified control chow with added 4% NaCl (TD.08162) and fresh water with 10% fructose added.

### **2.1.1 STUDY 1**

During study one, food, water intake, pre-gestational and gestational weight gain were monitored daily throughout the time course of the experiment. Tail vein blood and urine samples were taken from non-pregnant animals at day 14 and pregnant animals at day 19 of gestation. Insulin, plasma and urinary metabolite concentration and osmolality were analysed. When fetal weight was maximal (day 20), dams were euthanized, placentas, maternal and fetal blood were collected, all tissues were weighed and collected for further analysis. Individual fetuses were weighed and sexed, litter size and total weights were also recorded (Figure 7).

Study 1.

Figure 7. Study one: An investigation of the effect of maternal diets with or without added salt and fructose on maternal, fetal growth and metabolic status.

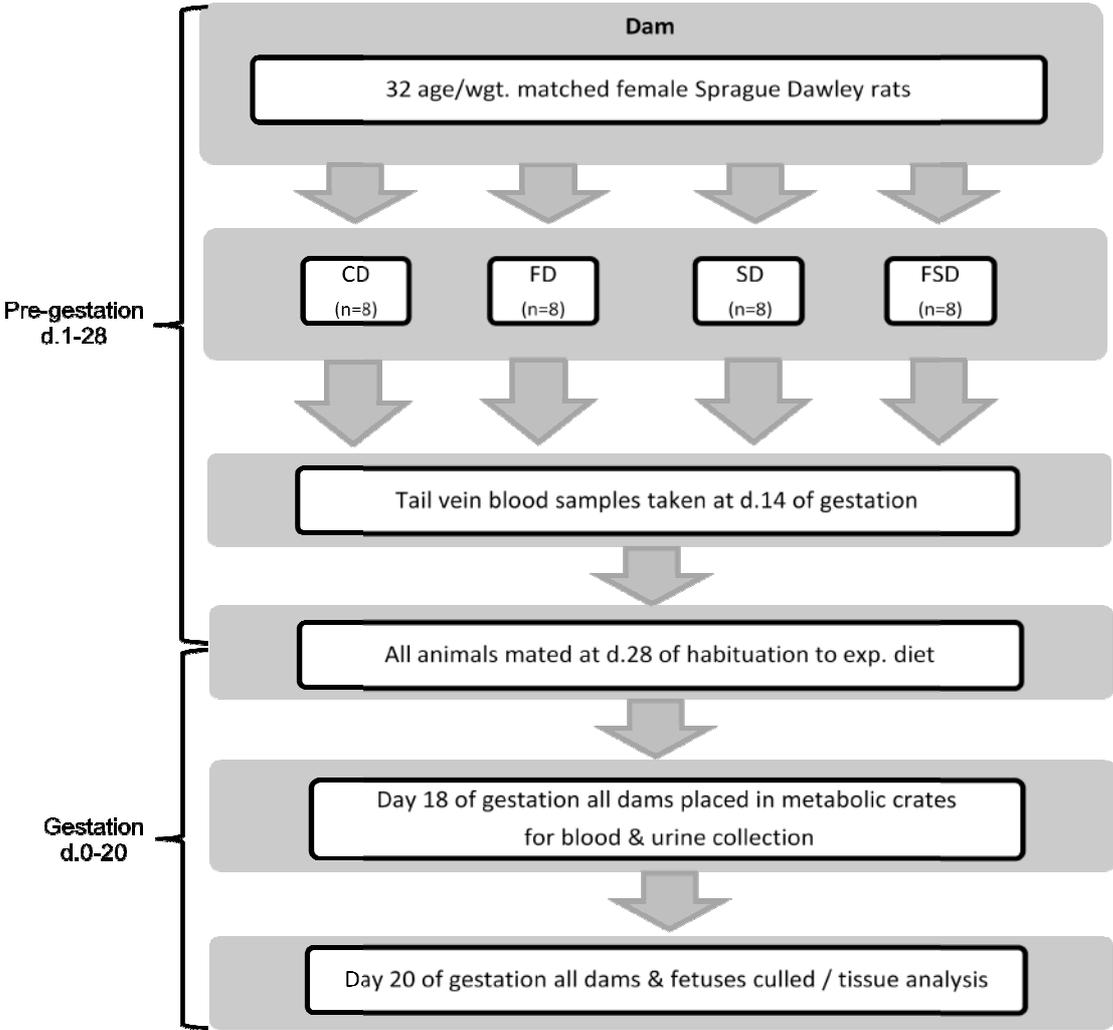


Figure7. Diagram represents the flow of work undertaken throughout study one. Four groups (n=8/group) of weight and age matched female Sprague Dawley rats were randomly assigned to the different dietary groups (CD=7, SD=8, FD=8, FSD=8). Rats were fed experimental diets for four weeks prior to mating and throughout gestation to day 20 when all animals were culled and tissues, blood were taken.

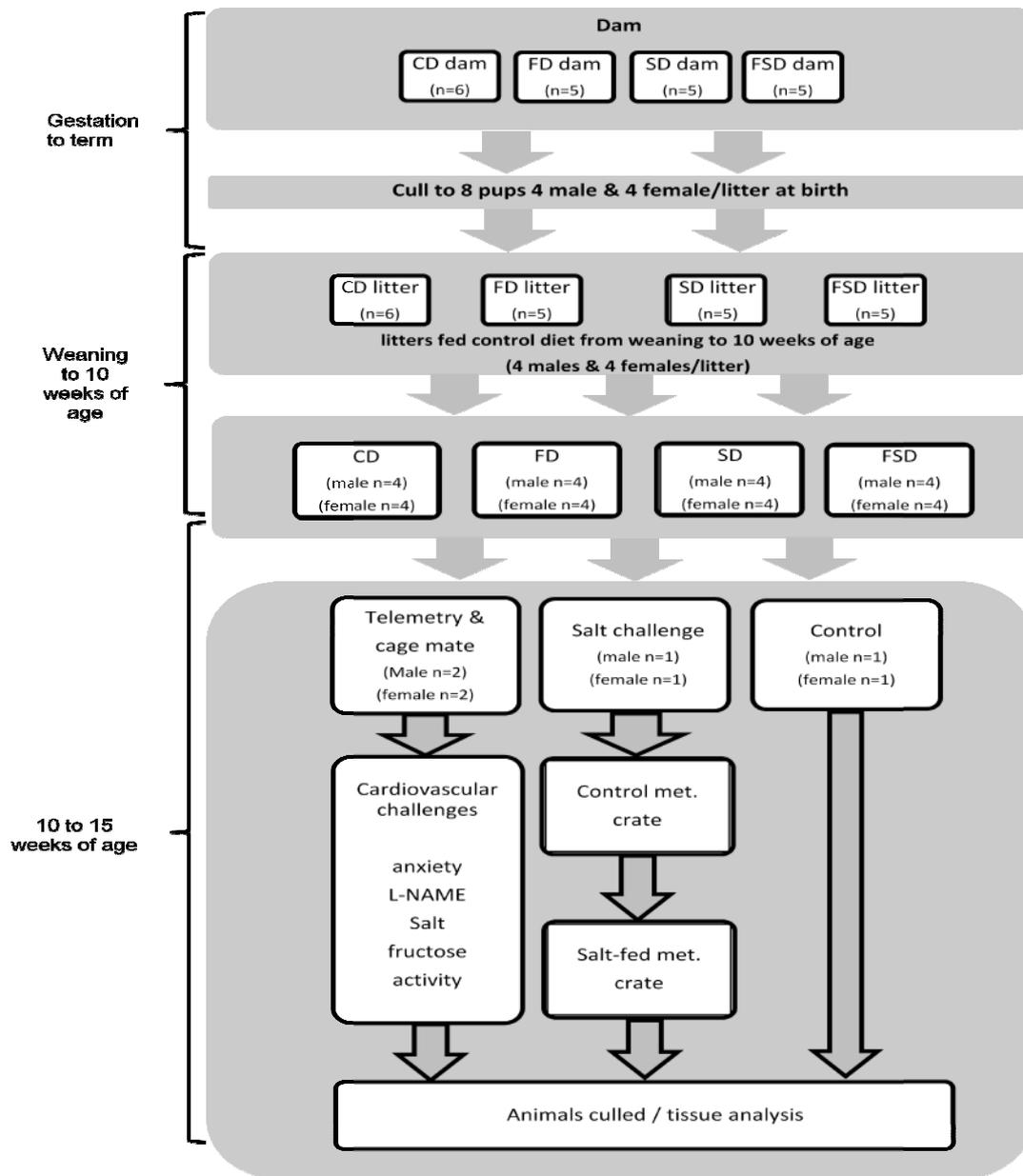
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### 2.1.2 STUDY 2

During study 2, the effect of a maternal salt and/or fructose diet on cardiovascular variables (systolic, diastolic, MAP, pulse pressure, heart rate and diurnal rhythms) and adult metabolic status (reflected in the variation in plasma metabolites) was determined. The maternal protocol employed in study 1 (Figure 7) was reproduced, but dams were maintained on experimental diets up to term (day 21) and throughout lactation (postnatal day 21). After birth, litter sizes were recorded, pups sexed, weighed and culled to eight pups/dam (male = 4, female = 4) and allowed to wean. At weaning, pups were weighed, separated by sex and fed purified control diet until 10 weeks of age. Dams were euthanized at time of weaning. At 10 weeks of age one male and one female per litter (5 male, 5 female/group in total) had radio telemetric probes surgically implanted. Basal blood pressure was monitored and further cardiovascular challenges were administered to assess cardiovascular health in the adult offspring (*Section, 2.4*). On completion of the cardiovascular challenges telemetry animals and cage mates were culled and tissues collected. The remaining non-telemetered animals (male = 1, female = 1) were placed in metabolic crates and continued on their control diet for 3 days and urine samples collected. The same animals (male = 1, female = 1) were then placed in metabolic crates and fed salt diet (4%) for three days, with urine output recorded and urine collected for metabolite and osmolality analyses. All animals were euthanized at 15 weeks of age blood samples and tissues were collected for further analysis (Figure 8).

## Study 2.

Figure 8. Study two: Metabolic status and cardiovascular health in adult offspring.



**Figure 8.** Diagram represents the flow of work during study two. The same habituation and mating protocol was followed as per study one (Figure 8). Age & weight matched female Sprague Dawley rats were fed experimental diets throughout gestation and lactation. Litters were culled to 8 at term. At weaning, 4 male and 4 female pups from each diet were surgically implanted with radio telemetry probes at 10 weeks of age. Each subsequent cardiovascular challenge (5-7 days) was followed by a recovery or 'washout' period. Both females & males from the same litters were run parallel and subjected to each stage of the above experimental protocol. (i.e. one telemetered animal, one cage mate, one control/salt met. crate animal and one control animal). Under normal conditions offspring were culled at 15 weeks of age.

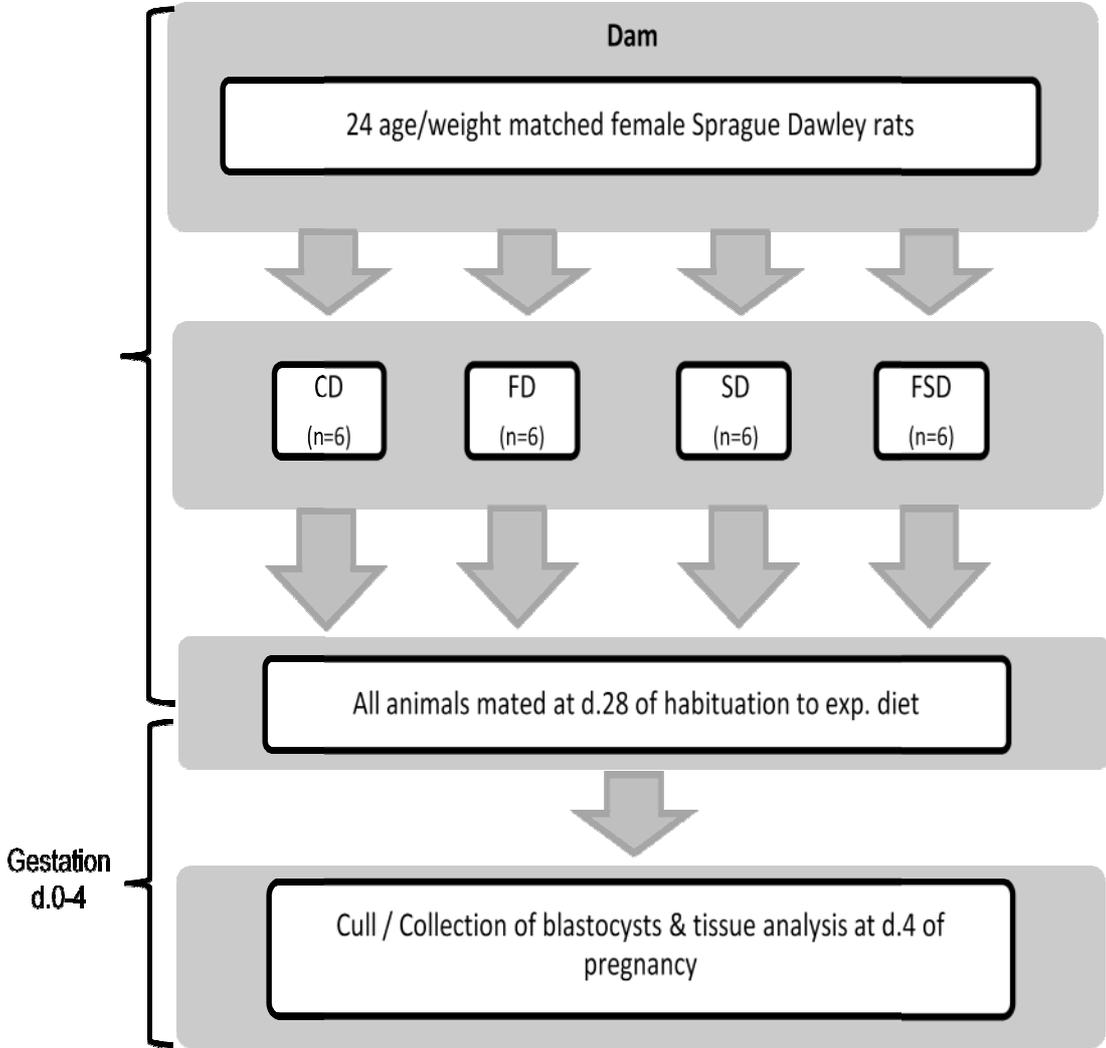
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### 2.1.3 STUDY 3

Four groups (n=6/group) of weight and age matched female Sprague Dawley rats were randomly assigned to the different dietary groups (CD, SD, FD, FSD). The maternal protocol employed in study 1 (Figure 7) was reproduced, but dams were maintained on experimental diets up to day 4 of gestation. Food, water intake and pre-gestational and gestational weight gain was monitored daily throughout the time course of the experiment. Prior to implantation of blasocysts (day 4), dams were euthanized, reproductive tracts were excised and blastocysts collected (figure 9). Maternal blood, kidney, heart and liver were also collected and stored (-80°C) for further analysis.

**Study 3.**

**Figure 9. Study three: Investigating the effects of fructose and/or salt maternal diet on ovulation and total pre-implantational blastocysts.**



**Figure9.** Diagram represents the flow of work undertaken throughout study one. Four groups (n=6/group) of weight and age matched female Sprague Dawley rats were randomly assigned to the different dietary groups (CD=6, SD=6, FD=6, FSD=6). Rats were fed experimental diets for four weeks prior to mating and throughout gestation to day 4 when all animals were culled. Tissues, plasma and day 4 blastocysts were taken.

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## 2.2 Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (UK), unless otherwise stated.

## 2.3 Animals

All experimental animal procedures were carried out under license and in accordance with the Home Office Animals (Scientific Procedures) Act 1986. Sprague Dawley rats (8 weeks, 190-200g) were purchased from Harlan, UK. Animals were group housed according to sex and were acclimatized for 7 days. During this period all animals (male & female) were fed standard non-purified laboratory chow (AIN-93G, Harlan Teklad, Maddison WI.) Animals were kept in a temperature (20-22°C) and humidity (55-65%) controlled environment and subjected to a 12 hour light/dark cycle (07-00-19-00). Bedding was changed on a weekly basis and food and water was fed *ad libitum* before and during the studies. On commencement of animal studies, animals were individually housed in wire mesh and plastic cages.

### 2.3.1 EXPERIMENTAL DIETS

Females were assigned randomly to 4 different experimental groups. Purified diets (Table. 4) were used in combination with a 10% fructose solution to achieve the required dietary groups: 1) control (CD), fed purified control chow 1% NaCl (TD.08164 Teklad Harlan,

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Maddison, WI.) and fresh water; 2) Salt (SD), fed purified control chow with added 4% NaCl (TD.08162 Teklad Harlan, Maddison WI.) and fresh water; 3) Fructose (FD), fed purified control chow 1% NaCl (TD.08164) and fresh water with 10% fructose (Sigma-Aldrich, UK) added; 4) Fructose and salt (FSD), fed purified control chow with added 4% NaCl (TD.08162) and fresh water with 10% fructose added. In all studies virgin female Sprague Dawley rats weighing 190-210g were initially habituated to experimental diets for >28 days. Animals were fed experimental diets and water *ad libitum* during the habituation period (>28d), mating (1-5d) during pregnancy (>20d) and lactation (>21d). Habituation to diet and experimental end points may have differed between experiments and will be stated where necessary. Energy intake from food, water intake and body weight were measured daily, water bottles and chow were weighed every 24h. giving a representation of 24h food and water intake for the previous day. Total caloric values from food, fructose solution and total calorie intake were calculated using the following formulae.

**Food intake:**

$$\text{g/day} \times \text{Kcal/g} = \text{Kcal/day (food)}$$

**Water intake:**

$$\text{ml/day} \times \text{Kcal/ml} / 10 = \text{Kcal/day (10\% fructose water)}$$

**Table 4. Comparison of experimental purified diets**

<b>Ingredients (g/Kg)</b>	<b>Control diet</b>	<b>Salt diet</b>
<b>Protein</b>	17.7	17.7
<b>Carbohydrates</b>	61.9	61.9
<b>Fat</b>	5.2	5.2
<b>Kcal/g</b>	3.7	3.7
<b><u>Selected Micro-Macronutrients</u></b>		
<b>Casein</b>	200	200
<b>L-Cystine</b>	3	3
<b>Corn starch</b>	417.49	417.49
<b>Maltodextrin</b>	132	132
<b>Sucrose</b>	100	100
<b>Cellulose</b>	50	50
<b>Na</b>	0.103	1.57
<b>P</b>	2.96	2.96
<b>Ca</b>	5.04	5.04
<b>K</b>	3.6	3.6

**Table4.** Modification of purified diet AIN-93G with fat reduced to 5%. Salt diet contains 4% total NaCl (0.26% provided in mineral mix and 3.74% from added NaCl).

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### 2.3.2 MATING

Animals were mated in HO approved wire mesh floored mating cages. Male and female animals were fed experimental diets and water *ad libitum* during the mating protocol. Mating was confirmed by the presence of vaginal plugs on cage floors. This was accepted as day 0 of gestation. Females were placed back in experimental cages and continued their experimental diets throughout gestation to 20d (Study 1) and lactation to 21-22d (study 2) and gestation to 4d (Study 3). Food intake, water intake, weight gain were recorded and total calorific intakes calculated.

### 2.3.3 METABOLIC BOWLS

At day 14 of habituation and day 19 of gestation (study 1), animals were placed in metabolic crates for a period of 24 hours. Animals were given food and water *ad libitum* during this period. Total urinary excretion, energy intake from food and water intake values were collected during this 24 hour period. Urine samples were placed in 2.5ml Eppendorfs and stored at -80°C. All animals were then placed back into experimental cages and continued their experimental diets to day 20 of gestation (study 1).

### 2.3.4 BLOOD SAMPLING

Animals were placed in a temperature-controlled crate for 15 minutes at 35°C. Animals were then restrained in a restraining tube and covered with a towel to minimise stress. A gauge 6 butterfly clip catheter was then inserted into the lateral tail vein and 1.5-2ml of blood was collected in a K+ EDTA tube and spun immediately. Blood samples were spun at 12000g for 8

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minutes using a bench top centrifuge. A glass Pasteur pipette was used to extract the supernatant plasma layer which was placed in a 2.5ml eppendorf and stored at -20°C.

## **2.4 Radiotelemetry**

### **2.4.1 RADIOTELEMETRY BLOOD PRESSURE TRANSDUCER IMPLANTATION**

Rats were anaesthetised with a mix of 2.4ml of sublimaze (Jansen-Cilac Ltd.) and 0.12ml of domitor (Pfizer, UK) at a dosage of 300ug/kg bodyweight. Animals were identified as being adequately under the effects of anaesthesia when the righting reflex was lost (>5min.) and they become non-responsive to the toe pinch. Rats were then shaved around the midline on the abdominal side in preparation for surgery. All shaven areas, feet and tail were cleaned with hydrex (Adams Healthcare, UK) to remove hair clippings and loose debris. The animals were then placed on a heated small animal surgical plate. The legs of the animal were taped to the heat plate, to position the animal shaven side up. A spray-on temporary adhesive was then applied to the shaven areas of the animal and veterinary surgical covers stuck in place over the site of the prepared surgical area.

A ventral midline abdominal incision was made extending from the xiphoid cartilage (10-12cm) to the lower abdominal area. Straight scissors were then used to extend the incision through the abdominal wall following the *linea alba*. Jansen retractors were inserted and opened carefully to expose the abdominal cavity and organs. A strip of transpore tape was used to hold the retractors in place. An autoclaved blue towel was then soaked in warm

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sterile saline and laid along the midline. The intestines, stomach and spleen were fully removed and placed onto the tissue to reveal the abdominal aorta. The tissue was then wrapped around the organs to keep moist. Two areas of the aorta were identified and sterile cotton buds used to carefully remove any connective tissue. The first area was just below the renal artery and the second just above the lower bifurcation. The aorta was lifted using blunt curved forceps and further connective tissue removed so as to free the artery completely. A sterile thread was then passed under and around the artery at both sites. The telemetry implant was then prepared by carefully removing the catheter tip. All animals were implanted with TA11PA-C40 telemetry pressure units (DSI, St-Paul, MN USA). Blood flow in the aorta was occluded by gently lifting the upper thread located next to the renal artery. A pair of curved artery forceps were placed on the lower thread and positioned so the weight of the forceps occluded the lower section of the aorta under their own weight. Tightening the threads around the isolated vessel in this order ensured the vessel was still plump with blood. A hypodermic needle (gauge 6) bent to 45° was used to make a small incision around 5-10mm up from the bifurcation. Lifting the needle and stretching the incision allowed passage of the catheter tip into the aorta. The catheter was fed into the aorta so that the collared portion (10mm) of the catheter was full inserted into the aorta. The area was cleared of any blood and fluid that may have leaked from the occluded vessel and Vetbond (3M Animal care Ltd. UK) applied to the site of catheterization. The catheter was held in place (30s) to allow the Vetbond to dry. The upper thread was slowly released to ensure no leaks presented themselves. The threads were then cut and removed from the

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vessel allowing a return of unrestricted blood flow.

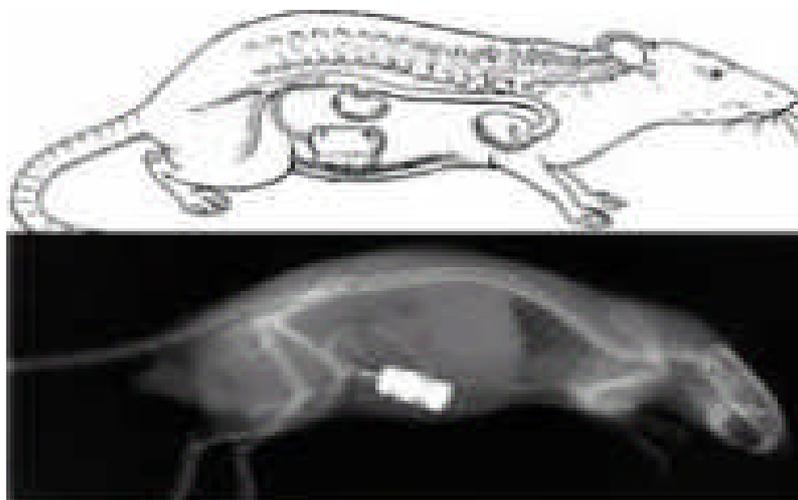
The signal and operation of the implant were tested by turning the implant on with a magnet and listening for a signal on an AM radio tuned to 550kHz. The implant was then placed against the body wall and using 3/0 Mersilk suture (Ethicon W502), the implant was sutured to the left side abdominal wall (Figure 11). The Jansen retractors and tissue was then removed and any displaced internal organs returned to their original positions. Sterile saline, warmed to body temperature was added to the abdominal cavity to prevent any dryness. The body wall was sutured using 2/0 vicryl (Ethicon W9181). Abdominal skin was then sutured using 3/0 Mersilk (Ethicon W622) and Michel clips. The wound was then dusted with Veterinary wound powder (Hayward & Bower Ltd.)

Antibiotics were then administered with an intramuscular injection of 0.05ml of Amoxycare LA (Animalcare Ltd.). Reversing agents, 0.4ml (1/10) buprenorphine ( $0.02\text{mg Kg}^{-1}$ ) Bupracare (Alstoe Animal Health, UK.) and 0.8ml (1/10) atipamezole ( $1\text{mg Kg}^{-1}$ ) Antisedan (Pfizer, UK) were administered by subcutaneous injections. To counter any dehydration, 10ml of sterile saline was injected into two different sites subcutaneously. The animal was placed into a cage containing Vetbed and then the cage placed on a heat pad with a sheet covering the top of the cage to prevent light penetration and undue stress. The animal was then observed for adverse signs of surgery. When the animal was conscious and mobile, water bottles and a small amount of food of high palatability (cocktail sausages) were offered. Animals were

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observed over the next 24 hours and then returned to the standard wire mesh and plastic cages. When the animals had recovered sufficiently, the telemetered animals were pairhoused with an uninstrumented mate to minimise stress throughout the duration of recording.

**Figure 10. Radiotelemetry implant placement.**



**Figure10.** Radiotelemetry probe placement (above). X-RAY of telemetry probe *in-situ* (below).

#### **2.4.2 HAEMODYNAMIC DATA COLLECTION**

All rats were allowed 7-10 days to recover from implantation surgery. Blood pressure, heart rate and activity were telemetrically recorded using the Dataquest A.R.T data acquisition system (DSI, St-Pauls MN USA). Raw data was converted and saved using the Dataquest A.R.T acquisition & analysis (GOLD version 4.02) software (DSI, St-Pauls MN USA). The

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hardware and software were installed to run on a standard desktop computer (Dell, N.York, ON, Canada).

## **2.5 Administration of haemodynamic challenges**

Blood pressure showed a significant diet\*sex interaction and also varied significantly with the time of day, therefore results are presented mainly as graphs with time on the x-axis. For all challenges except the anxiety challenge the telemetered animal had a companion in the cage and group *n* were as follows: CD, male *n*=6, female *n*=6, FD male *n*=5, female *n*=5, SD, male *n*=5, female *n*=5 and FSD, male *n*=5, female *n*=5.

### **2.5.1 RECOVERY RECORDING**

A 7-day period was allowed and rats were left alone with cage mates and minimal disturbance in sound-proofed room on a 12h (07.00am-07.00pm) light-dark cycle. During this period blood pressure and heart rate measurements were recorded using Dataquest A.R.T data acquisition system (DSI, St-Pauls MN USA) on a scheduled recording format (30s/15min.). This was done to ensure a reduction in blood pressure from the date of surgery and a levelling out of blood pressure observed. Over the course of seven days a levelling out of blood pressure and heart rate was observed in all animals with clear circadian rhythmicity. This was assumed to be the best representation of basal blood pressure and heart rate post surgery.

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### 2.5.2 BASAL RECORDING

All animals were allowed to recover for seven days before commencing basal blood pressure recording. Telemetry animals and cage mates were fed standard non-purified chow (AIN-93G, Harlan Teklad, Maddison WI.) and water *ad libitum*. Telemetry data acquisition software (DSI) recorded data continuously for 48 hours to measure day and night basal blood pressure variables, heart rate and activity. Rats were recorded at 30sec intervals over this period and data were analysed either by fitting separate fourier curves to all data points (2880 data points per animal per day) within treatment groups, or by compressing the data to hourly means over a representative 24h period.

### 2.5.3 ANXIETY CHALLENGE

To induce a stress response upon the telemetered animals, an anxiety challenge was devised. Cage mates of all telemetered animals were removed and placed into separate wire mesh and plastic cages for 24 hours. Telemetry data acquisition software (DSI) was set to record data continuously for 24 hours immediately after the telemetered animal and cage mate had been separated, to provide a measureable stress response in the telemetry animals. Rats were recorded at 30s. interval for 24h (starting at ~10.00am) and data analysed as above. Once the 24 hours had elapsed, all telemetered animals were reunited with the same cage mate.

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#### 2.5.4 N<sub>(G)</sub>-NITRO-L- ARGININE METHYL ESTER (L-NAME) CHALLENGE

N<sub>(G)</sub>-nitro-L- arginine methyl ester (L-NAME), is a non-specific inhibitor of nitric oxide synthase and hence, causes vasoconstriction by removing the vasodilator influence of NO and is widely used to induce hypertension in mammals. L-NAME was provided in drinking water at 150 mg L<sup>-1</sup> (150 µg ml<sup>-1</sup>). 150mg of L-NAME was weighed and added to a 1L flask of fresh water and made fresh, daily. Once the solution had been mixed adequately the water was poured into small animal drinking bottles ready for feeding. Telemetry animals and cage mates (untelemetered) were fed standard non-purified chow (AIN-93G, Harlan Teklad, Maddison WI.) *ad libitum*. A seven day feeding regime was decided to be the best experimental approach to ensure a consistent intake of the L-NAME treated water. Adult rats were found to drink on average 27ml day<sup>-1</sup> (Section 5.5.1), with no effect of prenatal dietary group. Therefore during the challenge the rats were consuming on average 4.12 mg L-NAME day<sup>-1</sup>. Telemetry data acquisition software (DSI) was set to record data at scheduled intervals (30s/15min) for 7 days to measure day and night basal blood pressure variables, heart rate and activity. Rats were recorded at 15min intervals for 7 days. Data were analysed either by fitting separate fourier curves to all data points (672 data points per animal [96 per day]) within treatment groups, or by compressing the data to hourly means. All animals were given a 7 day wash out period after L-NAME feeding.

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### 2.5.5 SALT CHALLENGE

Telemetry animals and cage mates were fed purified control chow with added 4% NaCl (TD.08162 Teklad Harlan, Madison WI.) and fresh water *ad libitum*. Telemetry data acquisition software (DSI) was set to record data at scheduled intervals (30s/15min) for 7 days to measure day and night basal blood pressure variables, heart rate and activity. All animals were given a 7 day wash out period after salt feeding. Data were analysed either by fitting separate Fourier curves to all data points (672 data points per animal [96 per day]) within treatment groups (Fourier figure), or by compressing the data to hourly means.

### 2.5.6 FRUCTOSE CHALLENGE

A 10% fructose solution was prepared by weighing 50g of fructose and adding to a 500ml of fresh water. Having mixed adequately, the solution was poured into small animal drinking bottles for feeding, fructose solution was prepared fresh, daily. Telemetry animals and cage mates (untelemetered) were fed standard non-purified control chow (AIN-93G, Harlan Teklad, Madison WI.) and 10% fructose solution *ad libitum*. Telemetry data acquisition software (DSI) was set to record data at scheduled intervals (30s/15min) for 7 days to measure day and night basal blood pressure variables, heart rate and activity. Data were analysed either by fitting separate Fourier curves to all data points (672 data points per animal [96 per day]) within treatment groups, or by compressing the data to hourly means.

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### 2.5.7 VOLUNTARY ACTIVITY CHALLENGE

Telemetry animals were removed from group housed cages and individually housed in activity analysis cages (Lafayette Instruments USA) for 7 days. While housed in the activity cages, animals had unrestricted access to the running wheels and any activity was voluntary. Every 24 hours total activity was measured in wheel revolutions by an electronic counter and recorded. All animals were fed standard non-purified chow (AIN-93G, Harlan Teklad, Maddison WI.) and water *ad libitum* for the duration of the activity challenge. Telemetry data acquisition software (DSI) was set to record data continuously for 24 hours to measure day and night basal blood pressure variables, heart rate and activity. Data were analysed either by fitting separate fourier curves to all data points (672 data points per animal [96 per day]) within treatment groups (Fourier figure), or by compressing the data to hourly means. On completion of the experiments all animals were euthanized by subjecting the animal to a rising concentration of CO<sub>2</sub> and cervical dislocation to confirm death. The telemetry implant units (TA11PA-C40) were then dissected from the animals. The implant units were then cleaned and returned to Data Sciences International Ltd. for reconditioning before reuse.

<b>2.6 Determination of plasma &amp; urinary metabolites</b>
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#### 2.6.1 PLASMA & URINE

Plasma samples were analysed for concentrations of (all mmol L<sup>-1</sup>) glucose, urea, creatinine, albumin, triglycerides (TAG), non-esterified fatty acids (NEFA) and cholesterol. Urinary

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samples were analysed for concentrations of urea ( $\text{mmol L}^{-1}$ ), creatinine ( $\mu\text{mol L}^{-1}$ ) and albumin ( $\text{g L}^{-1}$ ). All samples were analysed using a Randox autoanalyser (Randox RX Imola, Co. Antrim UK).

### 2.6.2 GLUCOSE ANALYSIS

This assay is based on the glucose hexokinase method. It is intended for the qualitative in vitro determination of glucose concentration in serum, plasma and urine. The method is an UV, enzymic method utilising hexokinase and glucose-6-phosphate dehydrogenase enzymes. A 0.41% inter-assay variation was accepted.

#### Reaction:

Glucose + ATP      *hexokinase*>      G-6-P + ADP

G-6-P +  $\text{NAD}^+$       *G-6-P-DH*>      Gluconate-6-P + NADH +  $\text{H}^+$

The minimum detectable concentration of glucose was determined as 0.64mmol/l.

### 2.6.3 NON-ESTERIFIED FATTY ACIDS

This assay is an enzymic colorimetric method utilising Acyl CoA synthase (ACS), Acyl CoA oxidase (ACO) and peroxidise. It is intended for the quantitative determination of Non Esterified Fatty Acids (NEFA) in serum and plasma. A 1.80% inter-assay variation was accepted.

#### Reaction:

NEFA + ATP + CoA      *ACS*>      Acyl CoA + AMP + PPi



\*4-AAP = 4-aminoantipyrine

\*TOOS = N-ethyl-N-(2-hydroxy-3-sulphopropyl)-m-toluidine

#### 2.6.4 TRIGLYCERIDES

The assay is based upon the GPO-PAP method. It is an enzymic, colorimetric assay for the quantitative in vitro determination of triglyceride in serum and plasma. Triglyceride is determined by the production of quinoneimine from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol catalysed by peroxidase. A 0.1% inter-assay variation was accepted.

##### Reaction:



#### 2.6.5 CHOLESTEROL

The assay is an enzymic endpoint method for qualitative determination of cholesterol in serum and plasma. Cholesterol is determined from enzymic hydrolysis and oxidation of hydrogen peroxide and 4-aminoantipyrine to quinoneimine. A 2.86% inter-assay variation was accepted.

##### Reaction:



Chol. + O<sub>2</sub> Chol.                      *Oxidase*>                      Cholestene-3-one + H<sub>2</sub>O<sub>2</sub>

2H<sub>2</sub>O<sub>2</sub> + phenol + 4-aminoantipyrine   *peroxidise*> quinoneimine + H<sub>2</sub>O<sub>2</sub>

### 2.6.6 URIC ACID

The assay used is for the quantitative determination of uric acid in serum, plasma and urine.

It is an enzymic, colorimetric method utilising uricase and peroxidise. A 1.75% inter-assay variation was accepted.

#### Reaction:

Uric acid + O<sub>2</sub> H<sub>2</sub>O                                      *uricase*>                      Allantoin + CO<sub>2</sub> + H<sub>2</sub>O<sub>2</sub>

2H<sub>2</sub>O<sub>2</sub> + H<sup>+</sup> + TOOS + 4-aminophenazone   *peroxidise*>      quinonedi-imine dye + 4H<sub>2</sub>O

### 2.6.7 CREATININE

The assay is a colorimetric method measuring the rate of complex formation. Creatinine in an alkaline solution reacts with picrate to form a coloured complex. A 1.61% inter-assay variation was accepted.

### 2.6.8 ALBUMIN

This assay is based on the bromocresol Green method and is used for the quantitative determination of albumin in serum, plasma and urine. The method measures the formation of albumin-BCG-complex. Albumin will bind to the indicator 3,3',5,5'-tetrabromo-m cresol sulphonphthalein or bromocresol green (BCG) which absorbs at 578nm. A 0.65% inter-assay variation was accepted.

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### 2.6.9 UREA

The assay is an enzymic kinetic method utilising urease and GLDH to yield glutamate and  $\text{NAD}^+$ . It can be used for the determination of urea in serum, plasma and urine. A 1.15% inter-assay variation was accepted.

#### Reaction:



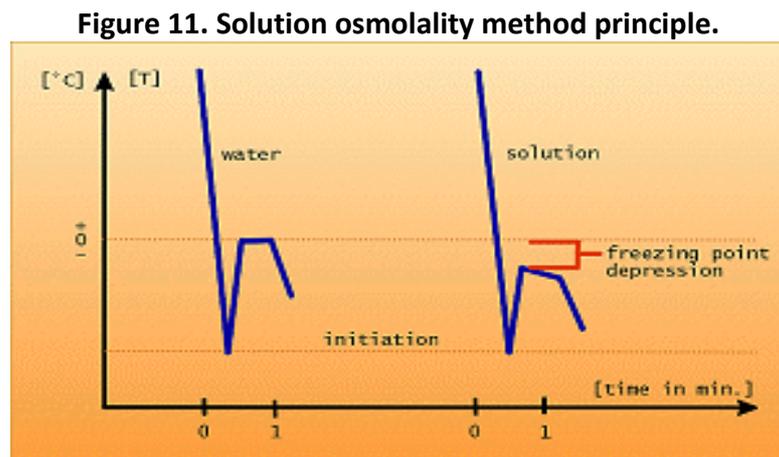
### 2.6.10 INSULIN ELISA

Plasma insulin levels were assessed with a rat specific insulin ELISA (Merckodia Ultrasensitive RIE). Samples and calibrators were all pipetted in duplicate. Two pooled plasma samples of varying insulin concentration were also pipetted in duplicate to assess inter-assay variation. Samples were pipetted onto the prepared plate, plates were then read at 450nm (Labsystems Multiskan Ascent plate reader plus Ascent software 2.6). A 4.69% inter-assay variation was accepted.

### 2.6.11 PLASMA & URINE OSMOLALITY

Plasma and urine sample osmolality was analysed using an Osmolality analyser (Osmomat 030, Gonotech, UK). The Osmomat 030 is used for the determination of osmolality in solution, it requires relatively small sample volumes (30-50 $\mu\text{l}$ ) and is routinely used in human medical testing and research. Results are highly reproducible ( $<\pm 0.5\%$ ,  $<\pm 2$  digits (4 digit display) for 50 $\mu\text{l}$  sample) and a resolution of 1 mOsmol/Kg. Total osmolality of a solution is

measured by comparing the freezing point of pure water and freezing points of the samples by injection of ice crystals, e.g. Pure water has a freezing temperature of  $0^{\circ}\text{C}$  whereas a saline solution with a concentration of 1 mOsmol/Kg has a freezing point of  $-1.858^{\circ}\text{C}$  (Figure11).



**Figure11** Diagram of solution osmolality method principle. X axis represents time, y-axis represents temperature. Blue lines show the difference in freezing point of water and a given solution, from which freezing point depression is analysed. Taken from: [www.gonotech.com](http://www.gonotech.com) (UK).

### 2.6.12 ICP-MS URINE ELECTROLYTE ANALYSIS

Urinary electrolytes Na, K and Ca were analysed using inductively coupled plasma mass spectrometry (ICP-MS). The quadrupole ICP-MS instrument used was a Thermo Fischer Scientific XSeries 2 ICP-MS (Thermo Fischer, UK) in combination with a dedicated PC and analysis software.

Samples are decomposed to neutral elements in high temperature argon plasma and quantified on their mass to charge ration. The ICP-MS has a high sensitivity and can detect trace elements in solution to less than 10 ppq (parts per quadrillion). In this instance rat urine metabolites were detected in parts per million (ppm). The final value represents the

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mean of five replicates from each sample, on average the inter-assay variation was less than 2%.

Following ICP-MS analysis all ppm values for Na, K and Ca were converted to and data expressed as mmol L<sup>-1</sup> by dividing ppm value (mg L<sup>-1</sup>) to molecular weight.

e.g.

**Molecular weight of Ca = 40.08g/mol (periodic table value)**

**Result 68 ppm Ca. = 68/40.08 = 1.69 mmol/L**

## **2.7 Determination of hepatic triglyceride**

Extraction of lipid for determination of triglycerides in non-adipose tissues was carried out using a modified version of the Folch method of lipid extraction (Folch *et. al*). 50mg of liver tissue was obtained from each rat and placed in a dispomix tube with 2 mL of cold chloroform-methanol (2:1) on ice. A dispomix homogeniser was used to homogenise the liver sample. Samples were then agitated for 20 min at room temperature. The homogenate was filtered through a funnel and folded filter paper to recover the liquid phase of the homogenate. To ensure all of the liquid had been recovered, 8ml of 0.9% NaCl solution was added to the filter paper to ensure all of the filterate was collected. The solvent was then

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washed using 3ml of dH<sub>2</sub>O and vortexed for 5-10 sec. The liquid phase (13ml) was then vortexed for 5-10 sec. The liquid samples were then centrifuged for 10 min at 2400rpm to separate the lipid and the 0.9% salt solution. The upper phase and the gel-like interphase were then removed using a Pasteur pipette and discarded appropriately. The remaining solvent was vortex for a further 10 sec. The samples were then centrifuged again for 10 min at 2400rpm and the upper layer assumed to be any impurities left within the solvent. This upper phase was syphoned off using a Pasteur pipette and discarded. 0.6ml of the chloroform layer was taken and placed in a 5ml tube ready for drying. This lower chloroform phase is where the extracted lipids are suspended. Samples were placed into a tube rack and onto a heat block (35°C) inside a fume cupboard to aid evaporation. A sample concentrator using a nitrogen stream was applied to the tops of all sample tubes and evaporated (20-25min). The concentrated samples were then redissolved in 60ul of tert-butanol and 40ul of triton X-100. The resuspended sample was then stored at -20°C until analysis. Samples were allowed to defrost to room temperature before concentrations of (mmol L<sup>-1</sup>) TAG and NEFA were analysed. All samples were analysed using a Randox autoanalyser (Randox RX Imola, Co. Antrim UK).

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## **2.8 Tissue collection**

### **2.8.1 FETAL TISSUE AND PUPS**

In study 1 at day 20 of gestation pregnant dams were euthanized by a rising concentration of CO<sub>2</sub> and cervical dislocation to confirm death. Day 20 fetuses were excised from the mother weighed and then sexed by genito-anal distance. Fetuses were separated from placentas. Placentas were weighed and placed in 2.5ml eppendorfs and stored at -80°C. Fetuses were then killed by decapitation. Fetal blood samples were taken by extracting the blood through a capillary tube by capillary action from the neck area. Capillary tubes were then sealed and put on ice until spun. Blood was spun for 2 min at 7000g using a capillary centrifuge. Haematocrit was calculated using a haematocrit reader. Plasma supernatant was then identified in with the capillary tube and the tube snapped. Plasma was then blown into a 0.5ml eppendorf and stored at -20°C. Liver, kidneys, heart, lungs and brain were carefully excised and weighed before being snap frozen in liquid nitrogen then later stored at -80°C. All term pups were euthanized by cervical dislocation.

### **2.8.2 ADULT TISSUE COLLECTION AND ANALYSIS**

All animals at weaning age and over were euthanized by subjecting the animal to a rising concentration of CO<sub>2</sub> and cervical dislocation to confirm death. All animals were killed by HO schedule 1 killing by trained technical staff. Blood samples were taken immediately after killing by cardiac puncture. 2ml of blood was collected and placed in K+ EDTA tubes. Blood was spun immediately for 8 min at 12000g using a centrifuge. Plasma was then collected and

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placed into a 2.5ml eppendorf and stored at  $-20^{\circ}\text{C}$ . Brain, tongue, heart, liver, kidney, pancreas, brown adipose, gonadal fat and visceral fat depots were quickly removed trimmed of fat and connective tissue when necessary and weighed. All tissues were snap frozen in liquid nitrogen immediately after dissection and stored at  $-80^{\circ}\text{C}$  for further analysis.

## **2.9 Placental analysis**

### **2.9.1 WET AND DRY PLACENTAL MASS ANALYSIS**

In study 1, placental wet weight was recorded at the time of dissection (d20). Each placenta was placed in a 2.5ml eppendorf tube and snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Placentas were placed in a freeze dryer making sure the lids were open or removed. The freeze dryer was set to infinity and the tubes left for 24h. The placentas were removed and placed on a fine balance and the weight recorded. The placentas were placed back into the freeze dryer for a further 24h. This process was carried out until a constant weight was achieved.

### **2.9.2 FIXING & SECTIONING**

Placentas were defrosted in physiological buffer solution (PBS) for 4h and placed in paraformaldehyde (PFA) for 24h at  $4^{\circ}\text{C}$ . Placentas were then washed and stored in 0.2M PBS for a further 24h. Each placenta was then cut into quarters. Two quarters were placed largest surface area up into a cassette tray and placed into a tissue processor (LEICA TP 1020) (The remaining two quarters were placed into 70% ethanol for further analysis).

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Placentas were placed into embedding trays largest surface area down and filled with approximately 1ml of embedding wax. Prepared placentas were then placed into an embedding machine (LEICA EG 1160). The embedding trays were then placed into cassettes and placed upon a cold plate (5°C) and the wax allowed to set. Wax blocks containing the prepared placentas were then stored in a 4°C fridge overnight. A microtome (LEICA RM2255) was used to finely section the fixed placentas. The microtome was set to cut at a thickness of 10µm with every fourth section mounted upon a slide (ensuring sections of only 30µm apart were analysed to ensure no single cells were observed and counted twice). Sections of placenta to be used were placed in a water bath (LEICA HI 1210) at 43°C and carefully placed on microscope slides. The slides were then placed on a heat plate (LEICA HI 1220) at 42°C for 1h. Slides were then placed in an oven at 55°C overnight to dry.

### **2.9.3 SAMPLE STAINING**

All dried slides were stained using hematoxylin and eosin (H&E) stain in accordance with the established protocol. When staining of the slides was complete, cover slips were mounted onto the slides using DPX mountant, care was taken so the mountant did not dry out. Slides were then laid flat over night to allow the mountant to set before imaging.

### **2.9.4 SLIDE IMAGING & ANALYSIS**

Placental sections, 10 from each dietary group (CD, SD, FD & FSD), were imaged using a slide scanner (PathScan Enabler IV) at x5 magnification. The scanner was linked to a desktop PC

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(Dell XPS 430) and the images stored for analysis. Images were analysed using an image analysis program, Image Pro (Media Cybernetics Inc.). A minimum of 10 fields of view per placenta were used. Systematic random sampling according to the published method of stereology was used (Mayhew & Burton 1988). Proportions of labyrinthine and trophoblast tissue were then determined in each section by 5 random measurements of width. Width of total placenta was measured and width of trophoblast and labyrinthine tissues were measured and calculated as a proportion of total area (Figure 12).

**Figure 12. Analysis of placental physiology.**



**Figure12.** Photomicrograph of a control (CD) rat placenta at day 20, stained with H&E at X5 magnification. Labels indicate the structural physiology of the placenta.

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**2.10 Collection of pre-implantational blastocysts**

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Mating was confirmed by the presence of vaginal plugs on cage floors. This was accepted as day 0 of gestation. Females were returned to cages and continued with experimental diets until day four of gestation. Animals were euthanized by a rising concentration of CO<sub>2</sub> and cervical dislocation to confirm death. The animal was dissected and ovaries and uterine horn identified. Fat and connective tissue were removed from the ovaries, horn and cervix then removed from the animal and placed in a Petri dish containing approximately 2ml of PBS and 4% bovine serum albumin (BSA).

One of the ovaries was removed and 1ml syringe with 26 gauge needle containing 0.5ml of 0.5 mM PBS and 4% BSA was inserted into the open end of the fallopian tube. The needle was held firmly in place and flushed through the fallopian tube to eject the blastocysts from the cervix. The procedure was also carried out on the other fallopian tube and once again on both tubes to ensure the greatest chance of all blastocysts being flushed from the uterine horn into the Petri dish. The resulting fluid was examined using a light microscope (LEICA 12.5 (M2)) at 4.2x magnification. A micro capillary pipette with aspirator was used to pick out the blastocysts. Isolated blastocysts were transferred to a clean Petri dish and the number recorded. Individual blastocysts were transferred to a 0.2ml Eppendorf tube, labelled and snap frozen in liquid nitrogen. Blastocysts were then stored in -80°C freezer for further sex determination analysis.

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## 2.11 Statistics

### 2.11.1 POWER CALCULATIONS

Although large numbers of offspring are generated in the experiments, to avoid litter effects an adequate number of dams needed to be used in the study. Power calculations have thus used previous variance values based upon maternal treatment rather than offspring effects.

D is the magnitude of the effect expected; SD is previous standard deviation of the variable.

The equation assumes 5% significance with 95% confidence intervals.

**Equation:** 
$$N = 2.05^2 (1.94 + 1.64)^2 / 10^2$$

In haemodynamic studies using a minimum 5 dams/group (5 male, 5 female offspring/dam), a power of 88% and a P value of 0.05, this will give a standardised difference of interest of 2, translating into a difference of >10mmHg in blood pressure.

### 2.11.2 FACTORIAL STUDIES

All statistical analysis was carried out using Genstat v11 (VSNi, UK). The study was designed to investigate the effects of four different maternal diets upon the cardiovascular health of the resulting adult offspring. The statistics used mainly employ a two-way (2X2) factorial analysis of variance. The experiments have four treatments (diets), the diets differ in terms

of two different factors, the addition of fructose and/or salt. Between them, the factors distinguish all four diets (Table 5). The two-way factorial design determines main effects (differences observed involving one factor) and interaction effects (difference between differences observed in one factor with the addition of a second factor).

**Table 5. Interpretation of factorial effects**

Observed statistical effect	groups			
	CD	SD	FD	FSD
Salt only	0	2	0	2
Fructose only	0	0	2	2
Interaction (salt*fructose)	0	2	0	0
	0	0	2	0
	0	0	0	2

**Table5.** An effect of just salt (one factor) would show difference in salt fed groups (SD, FSD), the same applies for an effect of fructose (FD, FSD). An interaction effect can be identified as an effect observed only in either salt and/or fructose fed animals (FD, SD, FSD). *i.e* interaction effect may be present in FSD fed animals but in neither FD or SD animals. Thus, fructose and salt are interacting to cause an effect, but as a single factor (fructose or salt) no difference is observed. O, represents no effect observed, whereas; 2, represents an effect.

### 2.11.3 NON-PREGNANT AND PREGNANT DAM ANALYSES

Pre-gestational and maternal energy intake from food, water intake and weight gain was recorded daily for 28 days, total energy intake was calculated using the resulting data. All data were analysed as a 2x2 factorial design with fructose\*salt\*time included (repeated measures analysis).

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Adult female pre-gestational plasma metabolite data at day 14 of habituation were analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance). Data are presented as predicted means with the average standard of error of the difference (s.e.d) with 32 degrees of freedom in each case.

Adult female gestational data (day 20) was analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance) with 29 degrees of freedom in each case with diet+littersize as fixed effects.

#### **2.11.4 OFFSPRING ANALYSES**

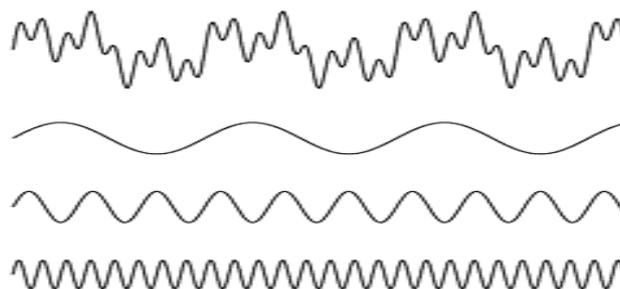
Fetal and pup data were analysed by mixed models (Restricted maximum likelihood (REML)) with diet+gender+littersize+diet\*gender as the fixed effects and dam+dam/pup as the random effects. Male and female rats weaned from mothers fed a maternal fructose  $\pm$  salt diet were subsequently maintained on a postnatal control chow diet. All data were analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) and dam as the random effect. Data are shown as predicted means with the average standard of error of the difference (s.e.d) with 20 degrees of freedom in each case. Adult offspring data represents adult male and female offspring at 14/15 weeks of age. Blood pressure in telemetered male and female offspring at 10 – 15 weeks of age showed a significant diet\*gender interaction and also varied significantly with time of day due to diurnal rhythms, therefore results are presented as graphs with time on the x-axis. Continuous cardiovascular data were recorded at 30s intervals for 24h, whereas scheduled noncontinuous cardiovascular data was recorded for 30s every 15 min for up to 7 days. Data

were analysed either by compressing to hourly means or by fitting separate fourier curves to all data points (2880/animal/day) within groups and analysed as a 2X2 factorial design with fructose\*salt\*gender included. Male & female offspring activity data were measured every 24h for 7 days. All data were analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance).

### 2.11.5 FOURIER ANALYSIS OF ADULT OFFSPRING CARDIOVASCULAR DATA

Fourier analysis is a type of statistical analysis that can be used to identify patterns and cycles in time series data sets, where there is a lot of random variation such as circadian cardiovascular data. Fourier analysis can detect wave forms or signals apparently otherwise lost within variable data sets and simple wave forms can be constructed from specifically identified sets of sine wave components (Figure 13).

**Figure 13. An example of wave decomposition by Fourier analysis**



**Figure13.** Fourier analysis: The complex wave at the top can be decomposed into the sum of the three simple waves below. As discussed in *Methods*, the Fourier equation derives 4 parameters from the model in which Y represents the measured outcome variable (e.g. pressure or heart rate): 1)  $a$ , the set-point around which pressure or heart rate show circadian oscillation e.g.  $\approx 350 \text{ beats.min}^{-1}$  for average heart rate, 2)  $b$ , the average amplitude of oscillation away from the set-point e.g.  $\pm 50 \text{ beats.min}^{-1}$  would represent an average heart rate of  $400 \text{ beats.min}^{-1}$  at night and  $300 \text{ beats.min}^{-1}$  during the day, 3)  $w$ , the average wavelength of the circadian oscillation, and 4)  $e$ , is a mathematical term representing the average offset necessary to standardize a starting point for each circadian curve.

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### 2.11.6 PLACENTAL AND BLASTOCYST ANALYSES

The proportion of the placental area made up by the labyrinth was calculated by dividing it by the total area and multiplying by 100. The average was taken from the 5 measurements per section and then the average per placenta was calculated. Widths of the trophoblast were compared using a two-way ANOVA. The ANOVAs were blocked by animal to allow for the repeated measurements (x5) of placental structures from the same individual. Proportion of the total placental area made up of labyrinthine tissue was also tested for significance using a two-way ANOVA. Data were then used to plot the means, allowing the effect of the interaction between salt and fructose to be visualised. Time allowed only n=3 placentas per dietary group to be analysed (but on average 10 determinations per placenta for width and 5 for area) and so the data should be regarded as preliminary but formal statistical significance was investigated and accepted if  $P < 0.05$ . For blastocysts, the data were first tested for normality and then a non-parametric one way ANOVA (Friedmans test) was performed (excluding 0 values) in order to test for variation from control between groups. A parametric analysis of variance (ANOVA) could not be used as in the FSD group because the sample size was too low (n=3).

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### 3 Dietary effects on the non-pregnant and pregnant dam

#### 3.1 Introduction

The obvious ethical issues involved in the study of human nutrition and fetal development have led to a large number of small and large animal models being developed. These models are used to study the basic physiological principles of nutrient availability and maternal-placental-fetal metabolism. In recent years, a number of investigators have provided compelling evidence that the balance of micro- or macro-nutrients supplied to the fetus, and the timing of disturbances within the supply, are important in determining the trajectory of fetal development, growth and later physiological consequences.

The current study considered the combined effects of increased salt and/or fructose intake, a trend that is presently observed in the modern western diet (He *et al.*, 2008; Stanhope & Havel 2008). The aim of this study was to investigate the physiological outcomes of feeding a moderately increased fructose and NaCl 'western' diet to non-pregnant ('pre-gestational') and pregnant female rats prior to characterising the effects on the developing fetus and neonate. In the context of fetal development and the effects that increased maternal consumption of a 'westernized' diet may have on subsequent neonatal and consequential adult health and disease, the majority of animal studies have employed a high fat and/or high sugar feeding protocol (Khan *et al.*, 2005; Howie *et al.*, 2009). However, in the UK, whilst moderately higher than optimal (RNI) intake of saturated fat is observed, total fat

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intake is within acceptable ranges (NDNS) and data suggests total fat consumption is close to recommended levels (National Food Survey, 2007). In contrast, both sugar and salt are over consumed relative to RNI's at the current time, each known to adversely influence metabolic and cardiovascular health (Schulze *et al.*, 2004; Johnson *et al.*, 2009c; Sanders, 2009; Bibbins-Domingo *et al.*, 2010a) with the possibility of interactions between the effects of high salt and fructose in the diet (He *et al.*, 2008). Indeed, in contrast to the immediate metabolic consequences for maternal and fetal health, the effects of a more realistic maternal 'Western' diet and consequential health of the offspring remains largely unknown. Jen and colleagues have shown that pregnant dams fed a diet high in glucose, fructose or sucrose can develop hyperglycaemia (Jen *et al.*, 1991). As a consequence, hyperglycaemia during pregnancy is a risk factor for the progression of gestational diabetes (Bray *et al.*, 2004; Moses & Brand-Miller, 2009), which has been shown to cause macrosomia, birth injury and fetal and neonatal hypoglycaemia (Langer *et al.*, 2006). Moreover, fructose in particular has been shown to cause maternal hepatomegaly, insulin resistance (Zou *et al.*, 2009) and greater metabolic perturbations than seen in dams fed glucose or sucrose during pregnancy (Fergusson & Koski, 1990). In addition to fructose, different levels of dietary NaCl during pregnancy and lactation have also been shown to cause alterations in fetal growth and metabolism. Beausejour and colleagues (2003) reported that rats fed 1.8% NaCl (in water) caused a reduction in placental weight, decreased uterine artery diameter and increased maternal blood volume, resulting in an overall rise in blood pressure and decreased RAAS activity; symptoms which are all common to pre-eclampsia (Beausejour *et al.*, 2003).

However, the role of fructose and/or salt and in particular their interaction during gestation upon the dam and developing fetus and its subsequent health status remains poorly understood.

This chapter therefore characterises the short-term physiological effects of a diet moderately high in salt and/or fructose (i.e. attributes characteristic of a modern western diet) upon the non-pregnant and pregnant dam in terms of her growth, food intake, water intake and indices of her metabolism (plasma metabolite profiles) and renal function (urinary metabolite profiles) and general health (organ masses).

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### 3.2.Protocol

During study 1, animals (n=8/group, unless stated otherwise) were fed purified diets containing either additional 4% NaCl or control chow with and without a 10% fructose solution to drink for four weeks prior to and during pregnancy and throughout lactation (Section 2.1). Food, water intake and pre-gestational and gestational weight gain were monitored daily throughout the time course of the experiment. Tail vein blood and urine samples were taken from non-pregnant animals after 14 days on the diets and in pregnant animals at day 19 of gestation. Insulin, plasma and urinary metabolite concentration and osmolality were analysed. When fetal growth was maximal (day 20), dams were euthanized and maternal and fetal blood were collected together with placentas and all major maternal and fetal organs. Individual fetuses were weighed and sexed, litter size and total litter weights were also recorded. In addition, blastocysts were collected at day 4 of gestation (Study 3), fetal tissue collected at day 20 of gestation (study 1) and neonatal tissue collected at birth (study 2). Detailed descriptions of all measurements and protocols for analysis of plasma, hormones, metabolites, electrolytes and osmolality are given in *Methods* (Sect. 2.5,2.8, 2.9, 2.10).

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## 3.3 Results

### 3.3.1 PRE-GESTATIONAL GROWTH, FOOD, WATER & CALORIC INTAKE & PLASMA METABOLITES

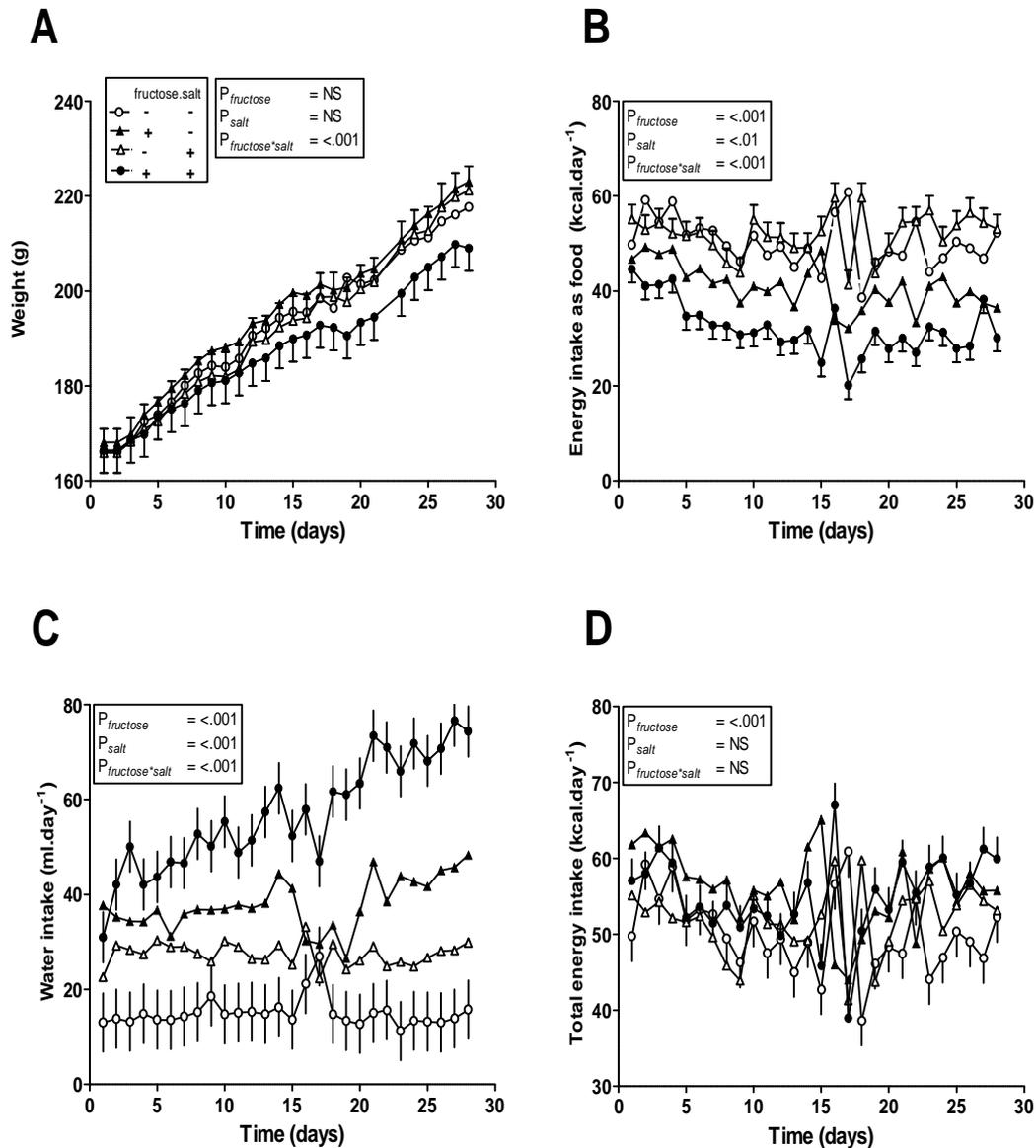
Pre-gestational female Sprague-Dawley rats were randomly assigned to four different experimental groups. No significant differences were observed in body weight between groups prior to commencing the experimental protocols (CD,  $172 \pm 13$ , SD,  $172.5 \pm 16$ , FD,  $172 \pm 13$  and FSD,  $171 \pm 11$ g). Initially the animals were habituated to the purified diets for 28 days prior to mating. During the habituation period, there was a significant interaction ( $P < 0.001$ ) between fructose and salt on bodyweight, reflecting reduced weight gain in FSD alone in comparison to all other groups. The divergence in weight was apparent from  $\sim$ d13 of feeding the diets (Figure 14A), and thereafter FSD gained on average  $1 \pm 0.3 \text{g} \cdot \text{day}^{-1}$  less than all other groups.

During the pre-gestational period a significant effect of fructose was observed ( $P < 0.001$ ) on energy intake from food with females drinking fructose having significantly lower energy intake from food than control animals (effect size,  $36 \pm 1 \text{ kCal/d}$  vs.  $51 \pm 1 \text{ kCal/d}$  fructose vs. no fructose intake); in those rats consuming both fructose and salt energy intake was further reduced ( $P < 0.001$  for interaction; Figure 14B). Energy intake from food in CD and SD females was not different.

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Water intake was significantly increased by intake of fructose and of salt ( $P < 0.001$  *in both cases*) and when combined water intake was further enhanced ( $P < 0.001$  for interaction), thus FSD consumed more water in comparison to all other groups (Figure 14C). With time, intake of 10% fructose water (i.e. FD and FSD females) increased gradually from day 1-28; thus, when combined (reduced energy intake from food but increased energy intake from fructose water) there was a significant effect of fructose on total caloric intake ( $P < 0.001$ ) i.e. FD and FSD females consumed, on average,  $12 \text{Kcal day}^{-1}$  more than CD and SD females (Figure 14D).

**Figure 14. Pre-gestational weight gain, energy intake from food, water and caloric intake in dams.**



**Figure 14.** Data represents adult female pre-gestational weight gain, energy intake from food, water and total caloric intake of non-pregnant rats. Females were fed a pre-gestational fructose  $\pm$  salt diet for 28d prior to mating. CD (n=8); FD (n=8); SD (n=8); FSD (n=8). Graph a) represents pre-gestational weight gain (1-28d), graph b) represents pre-gestational energy intake as food (1-28d), graph c) represents pre-gestational water intake (1-28d) and graph d) represents female total energy (Kcal) intake (1-28d) Weight gain, food and water intake was recorded daily for 28d, total energy intake was calculated using the resulting data. All data were analysed as a 2x2 factorial design with fructose\*salt\*time included (repeated measures analysis) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d).

Effects on non-pregnant and pregnant dam

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### 3.3.2 PRE-GESTATIONAL PLASMA METABOLITES

During the pre-gestation period (1-28 days) plasma was taken to assess the effect of salt and/or fructose on metabolic status. After 14 days of feeding, dietary fructose led to a significant increase ( $P=0.04$ ) in plasma glucose (CD,  $8.128 \pm 0.151$  vs. FD,  $8.651 \pm 0.193$  mmol  $L^{-1}$ ) and plasma triglyceride concentration ( $P=0.014$ ) (CD,  $0.72$  vs. FD,  $1.04 \pm 0.08$  mmol  $L^{-1}$ ). With respect to plasma NEFA and uric acid a significant interaction was observed with FD females only, having significantly higher plasma ( $P=0.05$ ) NEFA (e.g. CD,  $0.55 \pm 0.04$  vs. FD,  $0.67 \pm 0.04$  mmol  $L^{-1}$ ) and uric acid ( $P<0.004$ ) concentrations (e.g. CD,  $30.9$  vs. FD,  $66.2 \pm 7.42$   $\mu$ mol  $L^{-1}$ ) compared to all other groups (Table 6).

### 3.3.3 PRE-GESTATIONAL URINARY METABOLITES & OUTPUT

Urine output was monitored and samples taken from animals housed in metabolic crates for 3 days, as previously described in the *Methods* (2.5.3). Urine output in non-pregnant animals was significantly increased ( $P<0.001$ ) by intake of salt and of fructose in SD, FD and in combination a significant interaction was observed ( $P<0.001$ ) with further exacerbated urine output in FSD animals vs. all other groups (Table 7). As expected, on the basis of fluid intake, urine output increased progressively across all groups (CD,  $8 \pm 1$ , SD,  $21 \pm 1.5$ , FD,  $34 \pm 2.5$  and FSD,  $56 \pm 6$  ml  $day^{-1}$ ). The results for urine output are clearly linked physiologically to water intake and similar results were found i.e. individuals effects of fructose and salt on urine output (both increasing it) with a synergistic effect of combining fructose and salt (further increase). In addition, both fructose and salt intake reduced urinary albumin excretion

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( $P < 0.001$ <sub>both cases</sub>), urinary creatinine concentrations ( $P < 0.008$ <sub>fructose</sub>), ( $P < 0.001$ <sub>salt</sub>) and urinary urea concentration ( $P < 0.001$ <sub>in both cases</sub>). For urea, there was a significant interaction ( $P = 0.01$ ) in that urinary urea concentration was further reduced in FSD (Table 7). Increased intake of salt alone, however, significantly increased the urinary albumin:creatinine ratio ( $P < 0.001$ ; Table 7).

**Table 6. Effect of fructose±salt on pre-gestational dam plasma biochemistry at Day 14**

*Effect of fructose±salt on pre-gestational dam plasma biochemistry at Day 14*

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
<b>Glucose</b>	<b>-ve</b>	8.128	8.2				
<b>(mmol L<sup>-1</sup>)</b>	<b>+ve</b>	8.651	8.616	0.198	<0.04	NS	NS
<b>NEFA</b>	<b>-ve</b>	0.55	0.57				
<b>(mmol L<sup>-1</sup>)</b>	<b>+ve</b>	0.67	0.50	0.04	NS	NS	<0.05
<b>Triglyceride</b>	<b>-ve</b>	0.72	0.60				
<b>(mmol L<sup>-1</sup>)</b>	<b>+ve</b>	1.06	0.91	0.08	<0.014	NS	NS
<b>Uric acid</b>	<b>-ve</b>	30.9	31.2				
<b>(µmol L<sup>-1</sup>)</b>	<b>+ve</b>	66.2	32.1	3.71	NS	NS	<0.004

**Table6.** Data represents adult female pre-gestational plasma concentrations at day 14 of habituation. CD (n=8); FD (n=8); SD (n=8); FSD (n=8). All data was analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance) using Genstat v11 (VSNi, UK). The data are shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

**Table 7. Effect of fructose±salt on pre-gestational dam urine biochemistry at Day 14**

*Effect of fructose±salt on pre-gestational dam urine biochemistry at Day 14*

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
<b>Urine output (ml day<sup>-1</sup>)</b>	-ve	8	21	3	0.001	0.001	0.001
	+ve	34	56				
<b>Creatinine (µmol L<sup>-1</sup>)</b>	-ve	6023	2650	140	<0.008	<0.001	NS
	+ve	2715	620.1				
<b>Albumin (g L<sup>-1</sup>)</b>	-ve	0.411	0.325	0.05	<0.001	<0.001	NS
	+ve	0.26	0.141				
<b>Urea (mmol L<sup>-1</sup>)</b>	-ve	1081	458	80	<0.001	<0.001	<0.01
	+ve	364	90				
<b>Creatinine/Albumin ratio</b>	-ve	83.6	108.8	19.8	NS	<0.001	NS
	+ve	152.2	150.8				

**Table7.** Data represents adult female pre-gestational urinary output and metabolite urine profile at day 14 of habituation. CD (n=8); FD (n=8); SD (n=8); FSD (n=8). All data was analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance) using Genstat v11 (VSNi, UK). The data are shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

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### 3.3.4 MATERNAL GROWTH, FOOD, WATER & CALORIC INTAKE

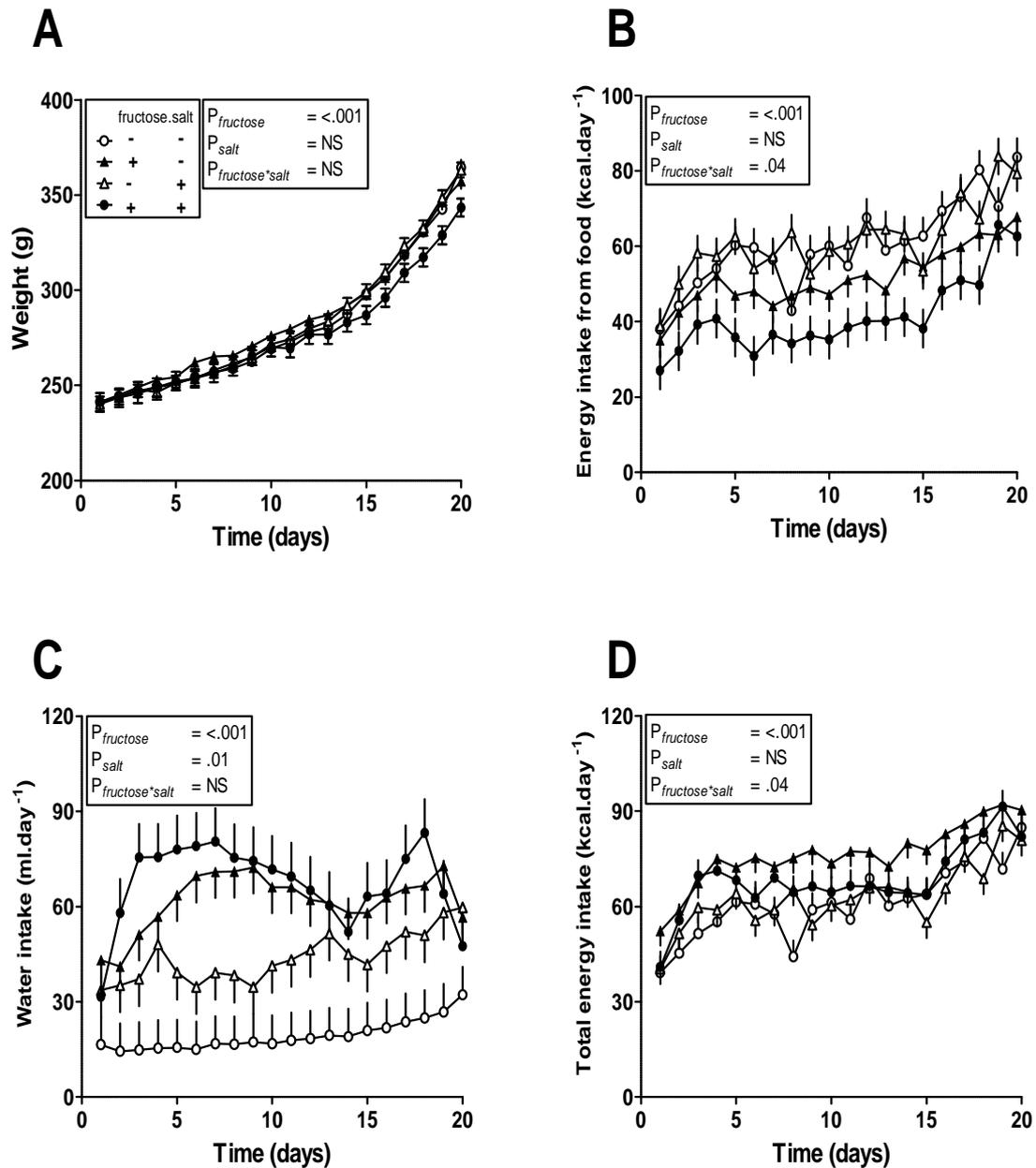
Immediately after the pre-gestational habituation period (1-28 days), all dams were mated with Sprague Dawley stud male rats (300-350g). During the mating period (<10 days) animals were maintained on experimental diets without any significant effect on their weight gain. Due to the lag period involved during mating and with the females approaching maximal adult weight, all groups at conception had similar body weights. During pregnancy, all animals gained weight (i.e. significant effect of time) but, the rate of gain was greater during the last seven days of gestation (i.e. when fetal growth becomes maximal) (Figure 15A). A significant effect of fructose ( $P<0.001$ ) was observed on weight gain in pregnant dams, with the rate of gain being less when fructose was consumed, however this was most apparent in FSD from day 14 gestation onwards (Figure 15A).

During pregnancy, energy intake from food broadly matched the change in weight (Figure 15B) with a consistent intake of calories from chow during the first two weeks of pregnancy, and from day 14, a gradual increase of energy intake from food over the last 7 days. As previously observed during the pre-gestational period, fructose intake led to reduced caloric intake from food ( $P<0.001$ ; Figure 15B) for example, CD and SD consumed  $60.4 \pm 2.2$  vs. FD and FSD,  $51.9 \pm 1.9$  Kcal day<sup>-1</sup>. The salt-fed dams' caloric intake was not different from that of control energy intake. Furthermore, a significant interaction between fructose and salt on energy intake from food was observed, reflecting significantly reduced energy intake from food in FSD females in comparison with all other groups ( $P=0.04$ ).

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Water intake during gestation showed a similar pattern to that observed during pre-gestation (Figure 15C) with all dams' gradually consuming more water as gestation progressed. Also in similarity to pre-gestational results, significant positive effects of fructose and of salt were observed on water intake ( $P < 0.001$ ). Consequently, total energy intake during gestation was significantly increased in dams' consuming fructose ( $P < 0.001$ ; e.g. CD,  $61.4 \pm 2.2$  vs. FD,  $72.79 \pm 1.8 \text{Kcal day}^{-1}$ ), and when consumed with salt in addition a significant ( $P = 0.04$ ) interaction was observed reflecting exacerbated energy intake in FSD animals vs. all other groups. On average, fructose-fed dams tended to consume  $11 \pm 1 \text{Kcal/day}^{-1}$  more calories despite lower weight gain observed in FSD dams'.

Figure 15. Gestational weight gain, food, water and caloric intake in dams



**Figure 15.** Data represents adult female gestational weight gain, food, water and total caloric intake of pregnant rats up to day 20 of pregnancy. Females were fed a gestational fructose  $\pm$  salt diet for 28d prior to mating and during gestation. CD (n=6); FD (n=8); SD (n=7); FSD (n=8). Graph a) represents gestational weight gain (1-20d), graph b) represents gestational energy intake as food (1-20d), graph c) represents gestational water intake (1-20d) and graph d) represents female total energy (Kcal) intake (1-20d). Weight gain, food and water intake was recorded daily for 20d, total energy intake was calculated using the resulting data. All data were analysed as a 2x2 factorial design with fructose\*salt\*time included (repeated measures analysis) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d).

Effects on non-pregnant and pregnant dam

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### 3.3.5 MATERNAL PLASMA HORMONES, METABOLITES & OSMOLALITY

Maternal plasma metabolite analysis revealed plasma fructose and fructosamine were not affected by maternal diet (Table 8). In similarity to the pre-gestation period, fructose intake significantly increased maternal plasma glucose, ( $P=0.029$ ;  $9.84\pm 0.8\text{mmol L}^{-1}$  in FD vs.  $7.31\pm 0.8\text{mmol L}^{-1}$  in CD) and plasma glucose concentrations were on average ( $>2\text{mmol L}^{-1}$ ) greater in all groups when compared to the pre-gestational period. Maternal diet had no effect on plasma insulin levels at day 20, despite increased plasma glucose concentrations. No effect of diet was observed on maternal plasma NEFA, triglyceride or uric acid, but a significant positive effect ( $P=0.021$ ) of fructose on plasma cholesterol was observed, being on average  $2.892\pm 0.2\text{mmol L}^{-1}$  in FD in comparison to  $2.109\pm 0.2\text{mmol L}^{-1}$  in CD. Additionally, a significant interaction effect was observed reflected in the concentration of cholesterol not increasing significantly in FSD in comparison to FD. A significant negative effect of fructose on plasma urea concentrations was also observed ( $P<0.001$ ) and a significant interaction ( $P<0.001$ ) between fructose and salt was observed on plasma creatinine i.e. plasma creatinine was significantly increased in FD alone ( $P=0.02$  for interaction). Plasma albumin concentration was significantly increased ( $P<0.001$ ) by intake of fructose. A significant ( $P<0.004$ ) increase in plasma osmolality was observed in salt fed animals, being on average  $0.368\pm 0.02\text{Osmol.Kg}^{-1}$  in SD in comparison to  $0.329\pm 0.02$  in CD. In addition, a significant interaction effect of fructose and salt was also observed ( $P=0.04$ ) reflected as plasma osmolality not increasing as much in FSD as compared to SD (Table 8).

**Table8. Effect of fructose±salt on Pregnant plasma biochemistry at Day 20***Effect of fructose±salt on Pregnant plasma biochemistry at Day 20*

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
Insulin (ng ml <sup>-1</sup> )	-ve	1.412	1.312	0.07	NS	NS	NS
	+ve	1.329	1.277				
Glucose (mmol L <sup>-1</sup> )	-ve	7.31	7.49	0.79	<0.029	NS	NS
	+ve	9.84	9.26				
Fructose (mmol L <sup>-1</sup> )	-ve	0.224	0.219	0.03	NS	NS	NS
	+ve	0.22	0.306				
Fructosamine (mmol L <sup>-1</sup> )	-ve	23.1	22.8	2.47	NS	NS	NS
	+ve	32	36.5				
NEFA (mmol L <sup>-1</sup> )	-ve	0.614	0.593	0.13	NS	NS	NS
	+ve	0.926	0.558				
Triglyceride (mmol L <sup>-1</sup> )	-ve	2.56	2.030	0.45	NS	NS	NS
	+ve	3.01	2.5				
Chol. (mmol L <sup>-1</sup> )	-ve	2.109	2.577	0.22	<0.021	NS	<0.06
	+ve	2.892	2.555				
Uric acid (µmol L <sup>-1</sup> )	-ve	155	158	23.3	NS	NS	NS
	+ve	170	149				
Creatinine (µmol L <sup>-1</sup> )	-ve	55.48	52.99	1.77	NS	NS	<0.02
	+ve	59.44	51.05				
Albumin (g L <sup>-1</sup> )	-ve	31.78	31.73	1.06	<0.02	NS	NS
	+ve	34.92	33.85				
Urea (mmol L <sup>-1</sup> )	-ve	8.25	6.59	0.49	<0.001	NS	NS
	+ve	6.79	5.77				
Osmolality (Osmol Kg <sup>-1</sup> )	-ve	0.329	0.368	0.02	NS	<0.004	<0.04
	+ve	0.337	0.344				

**Table8.** Data represents adult female gestational plasma metabolite profile at day 20 of pregnancy. CD (n=6); FD (n=8); SD (n=7); FSD (n=8). All data were analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d).

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### 3.3.6 FETAL PLASMA METABOLITES

Fetal plasma glucose concentrations were not significantly different between groups despite maternal hyperglycaemia observed in FD and FSD dams but fetal plasma glucose was significantly lower than that found in maternal plasma of corresponding groups, (e.g, fetal CD,  $2.06 \pm 0.2$  vs. maternal CD,  $7.31 \pm 0.7 \text{mmol L}^{-1}$ ). Fetal plasma fructose and triglyceride concentrations were not different amongst dietary groups. Fetal plasma fructose was on average 8-fold higher than in maternal plasma (e.g.  $1.73 \pm 0.25 \text{mmol L}^{-1}$  in fetal CD in comparison to  $0.22 \pm 0.03 \text{mmol L}^{-1}$  in maternal CD). A significant interaction was observed on fetal plasma NEFA ( $P < 0.01$ ), uric acid ( $P < 0.001$ ), urea ( $P < 0.001$ ) and creatinine ( $P < 0.001$ ); in effect, in all cases, this was due to a greater reduction in their plasma concentrations in FSD in comparison to all other groups. For fetal plasma albumin, a significant main effect of fructose ( $P < 0.001$ ) and salt ( $P < 0.001$ ) was observed in reducing fetal plasma albumin which was exacerbated when fed together, i.e. in FSD, plasma albumin was markedly reduced in comparison to all other groups ( $P < 0.03$  for interaction, Table 9).

**Table 9. Effect of fructose±salt on fetal plasma biochemistry at Day 20***Effect of fructose±salt on fetal plasma biochemistry at Day 20*

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
<b>Glucose (mmol L<sup>-1</sup>)</b>	-ve	2.062	2.029	0.189	NS	NS	NS
	+ve	2.097	1.942				
<b>Fructose (mmol L<sup>-1</sup>)</b>	-ve	1.735	2.184	0.211	NS	NS	NS
	+ve	2.112	1.514				
<b>NEFA (mmol L<sup>-1</sup>)</b>	-ve	0.487	0.568	0.085	NS	NS	<0.01
	+ve	0.540	0.146				
<b>Triglyceride (mmol L<sup>-1</sup>)</b>	-ve	0.874	0.94	0.125	NS	NS	NS
	+ve	0.875	0.532				
<b>Uric acid (µmol L<sup>-1</sup>)</b>	-ve	22.93	26.59	2.16	NS	NS	<0.001
	+ve	25.26	8.55				
<b>Creatinine (µmol L<sup>-1</sup>)</b>	-ve	3.848	5.296	1.916	NS	NS	<0.001
	+ve	7.45	1.868				
<b>Albumin (g L<sup>-1</sup>)</b>	-ve	4.809	2.78	1.102	<0.001	<0.001	<0.03
	+ve	3.796	1.234				
<b>Urea (mmol L<sup>-1</sup>)</b>	-ve	3.748	2.843	0.736	NS	NS	<0.001
	+ve	3.087	0.635				

**Table9.** Data represents fetal gestational plasma metabolite profile at day 20 of pregnancy. Plasma samples were pooled male and female plasma within group,. All data were analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference. For comparison, CD, control diet (n=6 litters); SD, salt diet (n=7 litters); FD, fructose diet (n=8 litters); FSD, fructose & salt diet (n=8 litters).

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### 3.3.7 MATERNAL URINARY METABOLITES & OSMOLALITY

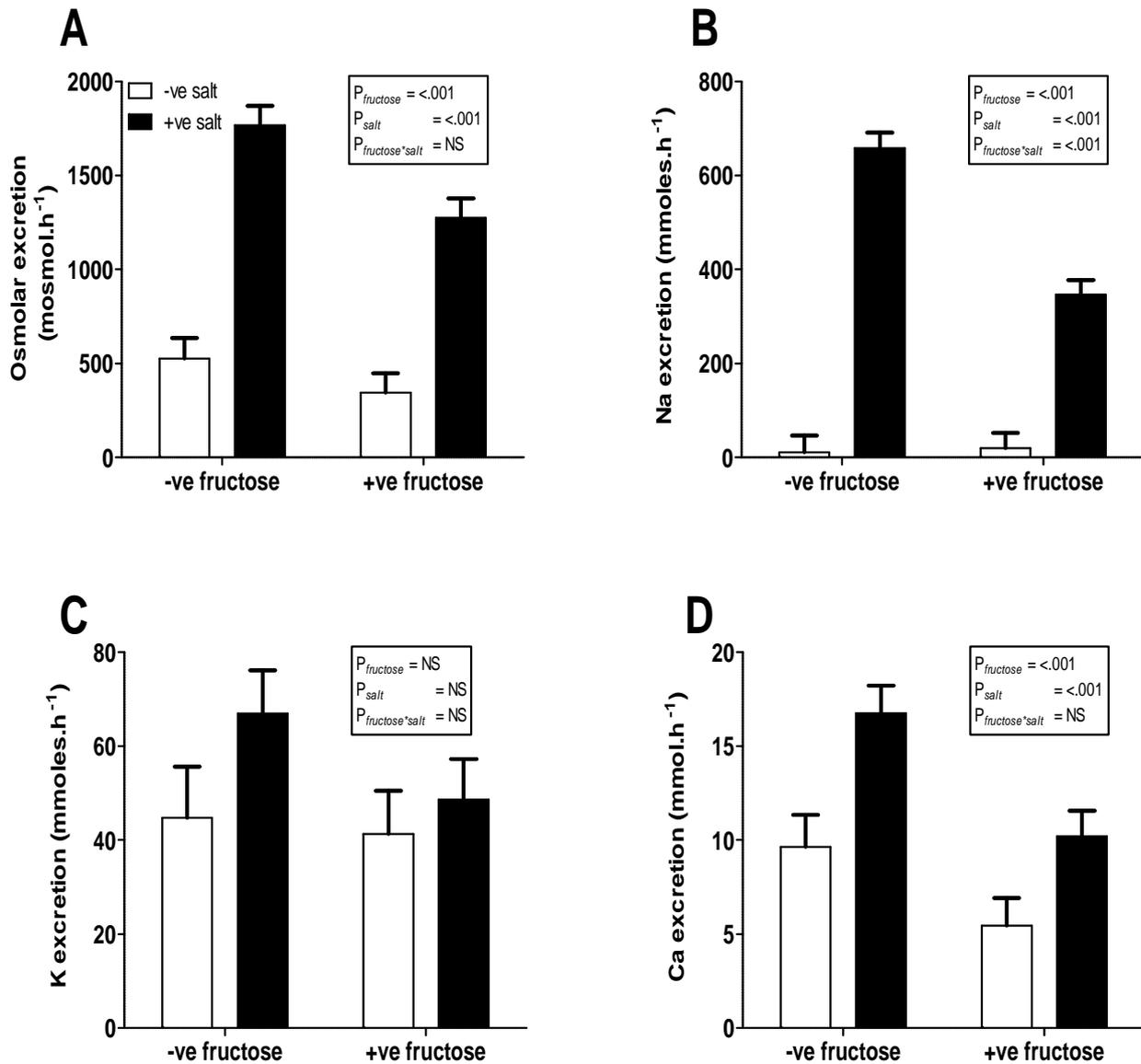
Maternal urine output was monitored at day 19-20 of pregnancy. Urine output was significantly increased by fructose ( $P<0.001$ ) and salt ( $P<0.001$ ) intake and was exacerbated ( $P<0.001$  for interaction) when each were fed together (FSD) i.e. there were large increases in urine output in SD and FSD in comparison to FD and CD. Urinary metabolites at day 20 were relatively unaffected by pregnancy *per se* i.e. when compared to pre-gestation concentrations. Maternal fructose intake significantly reducing excretion of urea ( $P<0.01$ ), for example excretion was on average  $327\pm 0.01\text{ moles/h}^{-1}$  in CD in comparison to  $283\pm 0.01\text{ moles/h}^{-1}$  in FD. Creatinine excretion rate was significantly increased by salt consumption ( $P=0.05$ ), (CD,  $5.74$  vs. SD,  $13.09 \pm 3.33\text{ umol ml min}^{-1}$ ). Osmolar excretion and clearance also increased ( $P<0.001_{\text{both cases}}$ ) with maternal consumption of salt, but osmolar excretion was significantly reduced in those dams consuming fructose ( $P=0.04$ ) (Table 10, Figure 16A). This was paralleled by significantly decreased excretion of  $\text{Na}^+$  and  $\text{Ca}^{++}$  in dams fed fructose ( $P<0.001_{\text{both cases}}$ ). However, as expected,  $\text{Na}^+$  and  $\text{Ca}^{++}$  excretion were significantly increased ( $P<0.001_{\text{both cases}}$ ) in dams consuming salt. For  $\text{Na}^+$ , but not  $\text{Ca}^{++}$ , there was a significant interaction ( $P<0.001$ ) between fructose and salt (Figure 16B,D), reflecting a markedly increased  $\text{Na}^+$  excretion in SD, markedly reduced in FD, with FSD displaying an intermediary level of  $\text{Na}^+$  excretion (Figure 16B). Finally,  $\text{K}^+$  excretion rate was unaffected by maternal diet (Figure 16C).

**Table 10. Urinary biochemistry in pregnant dams at day 20 gestation***Urinary biochemistry in pregnant dams at day 20 gestation*

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
Urine output (ml day <sup>-1</sup> )	-ve	8.2	38.7				
	+ve	22.5	39.7	2.6	<0.001	<0.001	<0.001
Urea excretion (moles h <sup>-1</sup> )	-ve	327	439				
	+ve	283	238	71	0.01	NS	NS
Osmole excretion (mosmoles h <sup>-1</sup> )	-ve	524	1767				
	+ve	343	1273	149	<0.04	<0.001	NS
Creatinine clearance (µmol ml min <sup>-1</sup> )	-ve	5.74	13.09				
	+ve	7.82	10.25	3.33	NS	0.05	NS
Osmolar clearance (mOsmoles ml min <sup>-1</sup> )	-ve	0.028	0.079				
	+ve	0.017	0.061	0.007	NS	<0.001	NS

**Table10.** Data represents adult female gestational urinary output and metabolite urine profile at day 20 of pregnancy. CD (n=6); FD (n=8); SD (n=7); FSD (n=8). All data were analysed as a 2x2 factorial design with fructose\*salt included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

**Figure 16.** Osmolar (A) and electrolyte excretion rates (B,C,D) in pregnant dams at day 20 gestation



**Figure 16.** Osmolar (A) and electrolyte excretion rates (B,C,D) in pregnant dams at day 20 gestation fed 1) control diet and water *ad libitum* (n=8), 2) control diet and 10% fructose in water *ad libitum* (n=8), 3) 4% salt diet and water *ad libitum* (n=8) and 4) 4% salt diet and 10% fructose in water *ad libitum* (n=8). Urine was collected after 24h in a metabolic crate. Superscripts indicate a significant main effect of fructose, salt or an interaction.

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### 3.3.8 MATERNAL TISSUES AT DAY 20

Tissue mass was analysed as absolute wet weight and proportionate weight (i.e. relative to weight at day 20) as dams had a difference in total body weight across dietary groups at this time (Table 11). Maternal wet (g) and relative ( $\text{mg g}^{-1}$ ) liver weights at day 20 were increased (20-25%;  $P < 0.001_{in\ both\ cases}$ ) by fructose, e.g. on average FD liver weight were  $15.22 \pm 0.32\text{g}$  in comparison to  $12.17 \pm 0.32\text{g}$  in CD. In addition, maternal liver in fructose-fed animals had significantly increased ( $P < 0.007$ ) intra-hepatic triglyceride levels and a significant redistribution of adipose tissue; relative to CD dams, gonadal depot (wet) in FD dams was  $4.91 \pm 0.53\text{g}$  vs.  $7.15 \pm 0.59$  in CD ( $P = 0.008$ ) but in the visceral depots mass was increased (both wet & relative weight;  $P < 0.001_{in\ both\ cases}$ ) e.g. in FD visceral fat wet weight was  $2.96 \pm 0.11\text{g}$  in comparison to  $1.98 \pm 0.11\text{g}$  in CD. In salt-fed dams, kidney wet and relative weight were increased ( $P < 0.001_{in\ both\ cases}$ ), e.g. in SD on average kidney weights were  $0.98 \pm 0.02\text{g}$  vs.  $0.76 \pm 0.02$  in CD. Furthermore, the wet and relative weight of the heart was significantly increased by consumption of salt ( $P < 0.001_{in\ both\ cases}$ ), e.g. on average weights were  $0.98 \pm 0.02$  in CD in comparison to  $1.21 \pm 0.02\text{g}$  in SD. Overall, adjusting for dam body weight did not remove any of the significant effects of salt or fructose intake on maternal organ (liver, kidney, heart and visceral adipose tissues) weights.

**Table 11. Effect of fructose±salt on the dams absolute and relative organ weight at Day 20**

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
Liver (g)	-ve	12.1	12.4	0.11	<0.001	NS	NS
	+ve	15.2	14.6				
visceral fat (g)	-ve	1.98	1.45	0.12	<0.001	NS	NS
	+ve	2.96	1.85				
Gonadal fat (g)	-ve	7.15	6.52	0.53	(0.008)	NS	NS
	+ve	4.91	5.81				
Kidney (g)	-ve	0.76	0.98	0.02	NS	<0.001	NS
	+ve	0.86	0.93				
Heart (g)	-ve	0.98	1.21	0.02	NS	<0.001	NS
	+ve	0.99	1.06				
Liver (mg g <sup>-1</sup> )	-ve	33.3	33.5	1.37	<0.001	NS	NS
	+ve	43.2	43.2				
Visceral fat (mg g <sup>-1</sup> )	-ve	5.44	3.901	0.49	<0.001	NS	NS
	+ve	8.41	5.50				
Gonadal fat (mg g <sup>-1</sup> )	-ve	19.6	17.6	2.45	NS	NS	NS
	+ve	14.2	17.2				
Kidney (mg g <sup>-1</sup> )	-ve	4.18	5.32	0.17	NS	<0.001	NS
	+ve	4.95	5.51				
Heart (mg g <sup>-1</sup> )	-ve	2.70	3.26	0.11	NS	<0.001	NS
	+ve	2.84	3.14				
Hepatic triglyceride (mg g <sup>-1</sup> )	-ve	0.843	0.868	0.19	0.007	NS	NS
	+ve	1.781	2.094				

**Table11.** Data represents adult female offspring absolute and relative organ weights at day 20 of gestation. Female rats were fed a maternal fructose ± salt diet for 28d prior to mating and subsequently maintained on a fructose ± salt diet during gestation up to day 20. CD (n=6); FD (n=8); SD (n=7); FSD (n=8). All data was analysed as a 2x2 factorial design with fructose\*salt\*litter size included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

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### 3.4 Discussion

#### *Summary of main findings*

Research into the effects of maternal fructose and/or salt diet upon general physiology and upon plasma and urinary metabolite profiles have been shown previously, but none however have considered and reported any interaction between a combined fructose and salt diet on the pregnant dam or her offspring. Data in this chapter uniquely considers whether a diet moderately high in fructose (10%) and salt (4%) has adverse effects on the non-pregnant and pregnant dam and fetal metabolic profile with consequential changes in organ or tissue masses, plasma and urinary profiles and renal function. During the current research, a model for the evaluation of independent effects of increased NaCl and energy intake from fructose currently seen in the contemporary western diet was developed. Using purified chow diets ( $\pm$  4% salt) and fructose added to drinking water (10%), the study aims to reduce the possible confounding effects and increase reproducibility relative to non-purified diets (Warden & Fislser, 2008). Fructose was added to water, rather than chow, to represent the large increase in dietary fructose from soft drinks and juices seen over the past 20 years. The significance of maternal metabolic status and fetal nutrient supply has been demonstrated within this chapter. For the first time we report the impact of a relatively comparative western diet upon the pre-pregnant and pregnant rat in terms of water, food and energy intake, body condition and metabolic phenotype, together with indices of fetal metabolic status and consequential tissue physiology in the dam.

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***Summary of findings in pre-gestational and pregnant dams***

- Dams consuming fructose prior to and during gestation had increased overall caloric intake despite decreased energy intake from food. Increases in plasma glucose (without an effect on insulin), triglyceride, non-esterified fatty acids, cholesterol and uric acid were all increased by the consumption of fructose in pre-gestational females.
- Dams receiving salt prior to and during gestation elicited an increase in cardiac and kidney tissue mass. All females consuming 4% salt within their diet also presented an increase in plasma osmolality, primarily as a consequence of increased plasma Na<sup>+</sup>.
- During pregnancy, fructose intake resulted in increased plasma glucose, cholesterol and albumin but all other pre-gestational changes as a result of fructose intake were blunted with the onset of pregnancy.
- Fructose-fed dams also displayed a significant redistribution of regional fat depots i.e. visceral fat increased whilst gonadal fat decreased. In addition, fructose intake led to significantly increased liver weight with more intra-hepatic triglyceride levels in the dam.

***Summary of findings in the fetus at day 20***

- At day 20 of gestation fetal plasma profiles and tissues were relatively unchanged and the fetus appears to be relatively protected at this stage.

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It is clearly shown that increased pre-gestation and gestational consumption of salt and fructose has predictable effects on the dam i.e. enlarged kidneys and heart in salt-fed dams and decreased energy intake from food, enlarged livers with ectopic fat deposition and marked regional fat redistribution in fructose-fed dams, all of which can be regularly observed clinically in individuals with a high sugar sweetened beverage consumption (He et al. 2008; Hu et al. 2010; Stanhope & Havel 2010). Salt loading and a long term rise in blood pressure causes the heart, vascular tissue and kidneys to adapt and this is preceded by altered gene expression causing remodelling of tissues usually initiated by local RAAS stimulation (Guyton-Hall, 2001a). This can lead to an increase in heart tissue, usually reflected as an increase in left ventricular mass. It is also observed that a marked increase in heart and kidney tissue might occur, although other similar studies have shown no increase in tissue weight when feeding 4% NaCl and fructose. However, the study looked at non-pregnant animals and Wistar-Koyoto rats were used (Vasdev *et al.*, 2007).

One of the major aims of the work presented in this chapter was to analyse differences in metabolism and consequential changes in plasma profile, tissue and organ changes. The main hypothesis at this stage being that females consuming a moderately high fructose diet will develop or be at risk of developing NAFLD as shown previously by Fan and colleagues (2008). Previous studies of maternal nutrition, particularly moderate increases in NaCl or fructose, and effects upon energy intake from food, weight gain, serum hormones and metabolites in the mother and offspring, have shown alterations in metabolism, plasma profile, organ growth and function (Schulze *et al.*, 2004; Johnson *et al.*, 2009). Studies have

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shown significant weight gain in fructose fed rats (Rawana, 1993) whereas we have reported no greater weight gain and even a reduced weight gain in FSD females despite having relatively a higher caloric intake than other dietary groups. Reduced energy intake from food and increased consumption of fructose sweetened water is consistent with existing studies showing an increased preference to fructose over chow, which can be largely attributed to sweetness and the increased palatability of the fructose solution compared to chow (Rawana *et al.*, 1993; Dai & McNeill, 1995; Srinivasan *et al.*, 2003). Dai and McNeill reported that rats consuming 10% fructose solution displayed larger weight gain and metabolic disturbance when compared to rats receiving 20% fructose solution, also observing a concentration dependent plasma glucose increase in response to fructose feeding (Dai & McNeill, 1995). Similarly, FD & FSD females displayed an increase in pre-gestational and gestational plasma glucose without an effect upon circulating insulin. This apparent hyperglycaemia was increased by fructose consumption by an average  $2.5\text{mmol L}^{-1}$ . However, it is arguable that this can be defined as hyperglycaemia. Indeed, the total concentrations of glucose are well within healthy physiological ranges ( $7\text{-}13\text{mmol L}^{-1}$ , pre-diabetic  $> 15\text{mmol L}^{-1}$ , diabetic  $> 20\text{mmol L}^{-1}$ ) with no noted effect upon insulin concentration. Moreover, fetal plasma glucose was not different between groups and at a much lower level than that seen in the mother. Indeed, hyperglycaemia must be regulated in the mother in order to protect the fetal environment, and placental mediation of glucose transport has been observed to be tightly regulated between the maternal-fetal supply chain (Creasy R. K, 2003) consistent with results presented here. Further studies have shown

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increased plasma glucose in fructose-fed rats (Kazumi *et al.*, 1986), in contrast to no increase (Hwang *et al.*, 1987; Dai & McNeill, 1995; Singh *et al.*, 2008) and modest changes (Singh *et al.*, 2008). The range of conflicting data may be a result of using different strains of rat, but likely reflects the fructose (in excess) being metabolised in preference to glucose within the liver (Zavaroni *et al.*, 1982). This preferential metabolism of fructose could play a role in interfering with the kinetics of glucose metabolism and the dynamic equilibrium of glucose and glucose-6-phosphate needed to supply gluconeogenesis and glycolysis within hepatocytes, leading to a physiological, not pathological, increase in serum glucose (Miller *et al.*, 1977) and a reduction in plasma fructose as the preferred substrate. In the current study we went on to demonstrate that NEFA, TAG and total cholesterol were increased by fructose, indicating increased *de novo* lipogenesis, usually signified by an excess of energy-yielding substrates, in this case fructose (Karpe *et al.* 2007). It has been shown that a high carbohydrate diet, but low in fat can cause rapid increases in lipogenesis, usually characterised by a rise in serum NEFA, TAG and total cholesterol (Frayn, 1996). The hepatic metabolism of fructose is independent of energy status, resulting in unregulated hepatic fructose uptake and elevated lipogenesis, which results in decreased apoB degradation and thus increased production of VLDL-TG, mainly as TG-rich VLDL. Furthermore, increases in total cholesterol would also be expected and indicative of increased production of CM, HDL LDL and VLDL to facilitate transport of insoluble TAG (Karpe *et al.*, 2007). Increased lipogenesis, elevated intermediary substrates such as TAG, NEFA and cholesterol, as well as uric acid as a consequence of excessive dietary fructose, are well established and the current

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data has recapitulated effects seen by previous studies of fructose feeding in humans (Fung *et al.*, 2009; Axelsen *et al.*, 2010; Jalal *et al.*, 2010) and animal models (Hwang *et al.* 1987; Thorburn *et al.* 1989; Jalal *et al.* 2010).

Significant redistribution of adipose depots was also observed in fructose-fed dams. As fructose is predominantly metabolised by the liver, distribution of fat may also be favourably deposited centrally with direct connection to the liver through the portal circulation and the higher localised metabolic activity (Bjorntorp, 1990). In humans, increased central or visceral fat deposition has been associated with insulin resistance and the visceral depot is indicated to have more of a deleterious metabolic effect than subcutaneous or peripheral adipose depots as shown in mice (Tran *et al.*, 2008). However, consideration must be given to a rise in lipogenic profile observed in pre-gestational dams as an artefact of restraint stress whilst taking tail vein blood samples. Restraint stress was kept to a minimum and we are confident that this is not the answer, although a very small proportion of lipogenesis may well be due to restraint stress. The overriding factor in the increase in intra-hepatic lipid deposition is likely due to chronic exposure to elevated TG, NEFA and cholesterol (Vuppalanchi *et al.* 2009; Fan 2008) as observed here with increased fructose intake. No clear evidence of increased sympathetic outflow was observed in cardiovascular results (Chapter 5) and it therefore appears unlikely that the increased lipogenic plasma profile in pre-gestational dams was a consequence of elevated sympathetic outflow due to restraint stress.

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We also recapitulate the clinical physiological phenotype in the dam, characterised as enlargement of the liver and increased intra-hepatic TAG deposition. The increased intra-hepatic TG accumulation and enlarged livers observed in fructose fed animals agrees with much of the current published literature where models of high fructose feeding (66-80%) had increased liver weight and hepatic TAG content compared to control or glucose fed animals, as observed in further studies in mice (Jurgens *et al.*, 2005), rat (Stanhope & Havel, 2008) or humans (Stanhope & Havel, 2009). Interestingly, pregnant females which were fed salt alone had the greatest increase in plasma osmolality and since fetal osmolality tends to reflect maternal osmolality (Ladella *et al.*, 2003a; Boyce *et al.*, 2008), it would be expected that the fetal environment of salt-fed dams would have high osmolality. However, due to a lack of fetal plasma, we were unable to measure fetal plasma osmolality, and future work clarifying the effect of this environment on fetal osmolar regulation is warranted and currently underway. As expected, additional dietary salt fed over approximately six weeks resulted in a marked increase in urine output and sodium/potassium urinary excretion prior to and during gestation. Fructose also increased urine output and in both cases was correlated with water intake. Urinary sodium was elevated by salt feeding and as expected, potassium followed the trend of sodium excretion. Additionally, osmole excretion and osmolar clearance were observed to increase in salt- and fructose-fed animals due to the osmolar effect exerted by the major urinary osmolytes Na<sup>+</sup> and K<sup>+</sup>. Creatinine clearance was higher in salt-fed animals, as there is little tubular reabsorption of creatinine; hence, using creatinine clearance as a measure of GFR. We see a significant effect of salt and a non-

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significant trend (0.09) in fructose fed females for altered renal function in the dam. However, creatinine clearance as a diagnostic of kidney function and GFR can only be taken as an approximation, as creatinine clearance can be readily over-estimated because of active secretion of creatinine into the tubular lumen. Whilst giving an indication, perhaps the BUN (Liang & Barnes, 1995) or the use of the novel biomarker Cystatin C (Ylinen *et al.*, 1999) may have been a more appropriate marker of kidney function given more time.

As greater metabolic demand is placed upon the dam during pregnancy, especially the late stages of pregnancy, there is a consequential increase in basal metabolic rate which can be increased as much as 15% during pregnancy. Increases in placental transfer of FFA's, including NEFA, TAG for fetal brain development and maximal fetal weight gain (Creasy R. K, 2003) during late gestation may explain the apparent 'buffering' effect of metabolic status during pregnancy when compared to pre-gestation. Given the marked changes in dam plasma metabolite profile at day 20 and during the peri-conceptual period, fetal metabolic status appears to be largely unaffected at this stage.

### **Conclusion**

Data in this chapter suggests an effect of fructose and/or salt prior to and during pregnancy impacts significantly upon the dams' metabolism. These alterations lead to significant increases in fat deposition and fat redistribution, her lipogenic profile and increased intra-hepatic fat accumulation in fructose-fed dams and increases in renal and cardiac tissue mass

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in the salt-fed dams. At this stage, despite these marked changes in the dam the developing fetus seems to be largely protected with little effect on fetal metabolite profiles.

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## 4 Dietary effects on the fetus and neonate

### 4.1 Introduction

No other example of nutrient supply and metabolic demand could be of greater biological importance than nutrition and metabolism of an expectant mother. At no other time is the well being and subsequent health of one or more individuals so unequivocally dependent upon the health of another. This absolute dependence and the fragile relationship of mother and fetal intra-uterine milieu led to increased recognition of the unique needs of a woman and the developing fetus during pregnancy. However, the role that nutrition plays during pregnancy has not always been appreciated, and the relationship between diet during gestation, nutrient transfer, placental viability and subsequent fetal health or pregnancy outcome are not fully understood.

It has been suggested that once the placenta is fully established, fetal development is dependent on the integrity of the maternal-placental unit (SACN 2010). Additionally, severely disrupted placental growth or function can impair the delivery of nutrients to the fetus and constrain fetal growth (Pardi & Cetin, 2006). Although the cell types and regions of placentae have different names, the murine and human placenta have analogous functions since both are haemochorial (Georgiades *et al.*, 2002). Researchers have shown in humans that many pregnancy losses and complications are largely due to problems in placental

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formation and function (Rossant & Cross, 2001). Seemingly minor abnormalities in placentation, such as inappropriate trophoblast invasion of maternal spiral arteries, are associated with severe pregnancy-associated conditions such as pre-eclampsia (Rossant & Cross, 2001).

This chapter progresses Chapter 3 by characterising the acute effects of a diet moderately high in salt and fructose upon placental size and architecture, and subsequent knock-on effects on fetal and neonatal growth and development, litter size and the secondary sex ratio of litters. Further chronic effects of the diets upon the adult offspring are presented in Chapter 5.

## 4.2 Results

### 4.2.1 BODY WEIGHT & TISSUE MASS OF FETUS AT DAY 20

As expected, a gender effect was observed on fetal body weight at day 20, with males being larger overall  $\sim 3.53 \pm 0.02\text{g}$  in comparison to females,  $\sim 3.43 \pm 0.02\text{g}$  ( $P < 0.001$ ). At day 20 of gestation, there was no effect of maternal diet on fetal body weight at this stage of development (Table 12).

**Table 12. Effect of gender, fructose $\pm$ salt on fetal body weight at day 20**  
**Effect of gender, fructose $\pm$ salt on fetal body weight at day 20**

Male offspring	$\pm$ fructose	$\pm$ salt		s.e.d	<i>P</i> value		
		-ve	+ve		Fructose	Salt	Fr*S
Body weight (g)	-ve	3.40	3.49	0.1	NS	NS	NS
	+ve	3.46	3.76				
<b>Female offspring</b>							
Body weight (g)	-ve	3.35	3.35	0.1	NS	NS	NS
	+ve	3.48	3.54				

**Table12.** Data represents male and female fetal body weight at day 20 of gestation. Female rats were fed a maternal fructose  $\pm$  salt diet for 28d prior to mating and subsequently maintained on a fructose  $\pm$  salt diet during gestation up to day 20. CD (n=6); FD (n=8); SD (n=7); FSD (n=8). All data were analysed as a 2x2 factorial design with fructose\*salt\*litter size included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

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### 4.2.2 PLACENTAL MASS

Placental wet weight at day 20 was not affected by maternal diet. However, there was a trend for placental weight to be greater ( $P=0.07$ ) in salt fed dams (e.g. on average  $0.49\pm 0.02\text{g}$  in CD in comparison to  $0.55\pm 0.02\text{g}$  in SD). Subsequently, placentas were freeze dried and placental dry weight displayed a trend for an interaction ( $P=0.06$ ) between fructose and salt, that is, an increase in mass of FSD dam placentas when compared to all other groups. Nevertheless, the percentage water content and fetal:placental ratio (g.fetus/mg.placenta) were not different between groups (data not shown).

### 4.2.3 PLACENTAL ARCHITECTURE

Placental micrographs (e.g. Figure 17) illustrated a significant negative effect of fructose ( $P=0.03$ ) on the width of the placental trophoblast, and a significant positive effect of fructose on the proportion of placental labyrinthine ( $P=0.03$ ; Table 13). In contrast, in salt-fed animals there was a significant decrease in the proportion of labyrinthine zone and significant increase in the proportion of trophoblast tissue ( $P<0.007$ ). Observational, descriptive data suggest that differences were also apparent in the colour and integrity of the placental decidua from dams consuming 4% salt diet (SD, FSD), which were observed to have an opaque appearance and tended to lack membrane integrity in comparison to CD and FD placentas. Additionally, placental decidua were observed to dissociate from the spongio-trophoblast layers much more readily in placentas from SD and FSD than those collected from CD and FD dams.

Figure 17. Overview of placental morphology

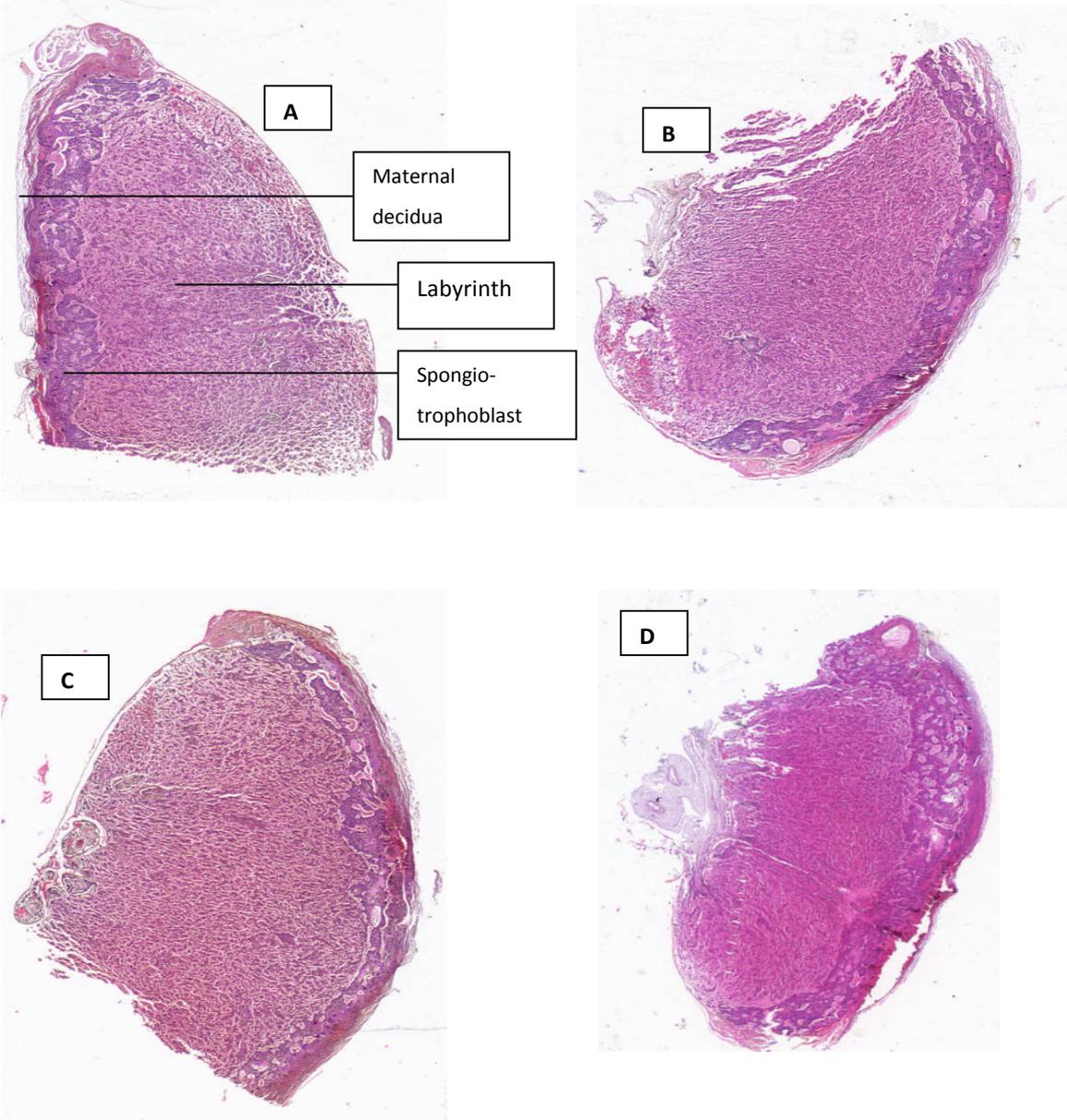


Figure17. Photomicrographs of rat placenta sections at day 20, stained with H&E at X5 magnification. One section from each dietary group is shown and the main areas labelled. A) CD placenta, B) FSD placenta, C) FD placenta, D) SD placenta.

**Table 13. Placental proportion of labyrinthine & trophoblast layers in dams fed salt  $\pm$  fructose**

<i>Placental proportion of labyrinthine &amp; trophoblast layers in dams fed salt <math>\pm</math> fructose</i>							
% of placenta	$\pm$ fructose	$\pm$ salt			P value		
		-ve	+ve	s.e.d.	Fructose	Salt	Fr*S
<b>Labrynthine area (%)</b>	<b>-ve</b>	81.27	74.38	1.87	0.03	0.007	NS
	<b>+ve</b>	83.08	79.74				
<b>Trophoblast layer (%)</b>	<b>-ve</b>	18.73	25.62	1.87	0.03	0.007	NS
	<b>+ve</b>	16.92	20.26				

**Table13.** Data represents proportion of placental area taken from placentas from mothers at day 20 of gestation, fed a maternal fructose  $\pm$  salt diet. Graph a) represents placental proportion of labyrinthine area. graph b) represents placental percentage of trophoblast layer & Labyrinthine area relative to total placental area. Data were analysed using a two-way ANOVA, blocked by animal to allow for the repeated measurements (x5) of slides from the same placenta (on average 10 determinations per placenta for width and 5 for area) using Genstat v11 (VSNi, UK).

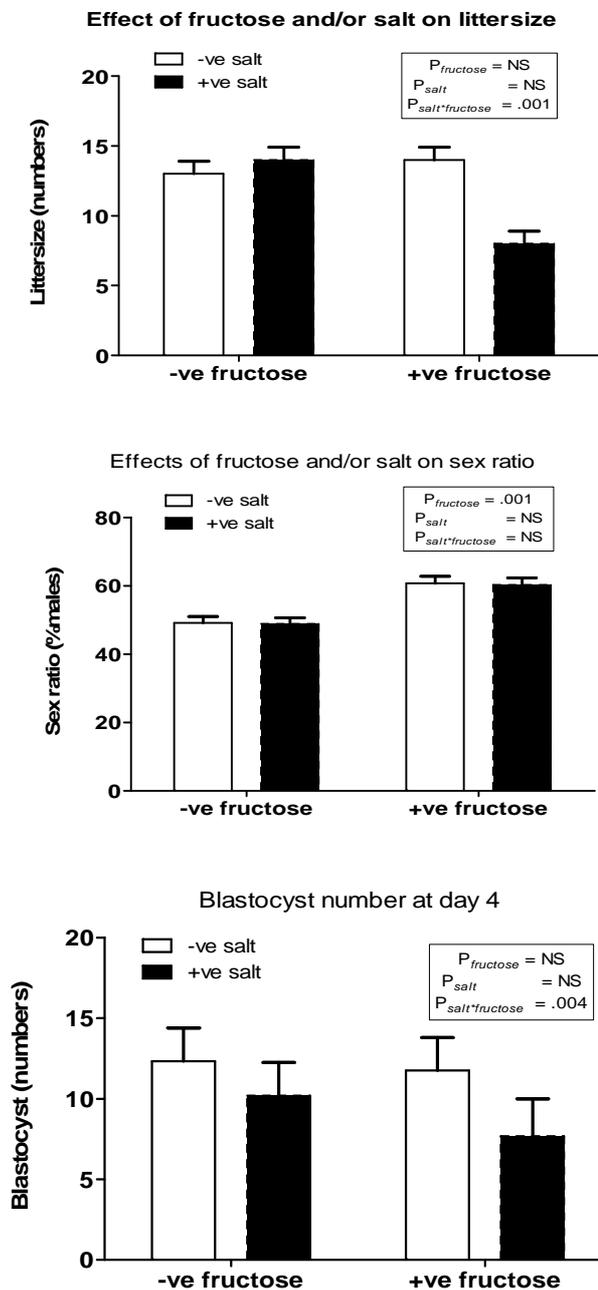
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#### 4.2.4 LITTER SIZE & SEX RATIO OF PUPS

Study one litter size at day 20 and study two litter size at term displayed an interaction of fructose and salt ( $P < 0.001$ ) in their effects on litter size, i.e. FSD alone had significantly reduced litter size in comparison to all other groups (e.g. on average, there were  $14 \pm 1$  pups/litter in CD, SD and FD in comparison to  $7.77 \pm 1$  pup/litter in FSD; Figure 18). Therefore, total pup count across all litters in study 1 was significantly reduced in total, being only 52 in FSD in comparison to 83 in CD, 117 in FD and 94 in SD. Observational data also suggested that the number of still births throughout the course of experiments was greater in FSD ( $n=3$ ) vs. other groups ( $n=0$ ).

Interestingly, a significant effect of fructose ( $P < 0.001$ ) on sex ratio within litters was observed; sex ratio in study 1 and study 2 were skewed on average 60:40 in favour of male offspring (Figure 18B). In addition, non-parametric analysis indicated a significant interaction between fructose and salt in reducing pre-implantational blastocysts ( $P=0.004$ ), reflecting a greatly reduced total number of blastocysts collected from the reproductive tract of FSD dams at day 4 of gestation vs. other groups (e.g. on average  $12.3 \pm 2$  blastocysts in CD, SD and FD in comparison to  $7 \pm 2.3$  in FSD pregnant females; Figure 18).

**Figure 18. Effect of fructose±salt on litter size, sex ratio & blastocyst number**



**Figure18.** Data represents Litter size (A), sex ratio (B) at term & blastocyst number at day 4 (C). Female rats were fed a maternal fructose ± salt diet for 28d prior to mating and subsequently maintained on a fructose ± salt diet during gestation up to day 20. Data were analysed as a 2x2 factorial design with fructose\*salt\*littersize included (general analysis of variance) (graph A&B), blastocyst data was analysed using a non-parametric one-way anova (Mann-Whitney U test) all data analysed using Genstat v11 (VSNi, UK). The data are shown as predicted means.

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#### 4.2.5 BIRTH WEIGHT & POSTNATAL GROWTH OF NEONATES AND ADULT OFFSPRING

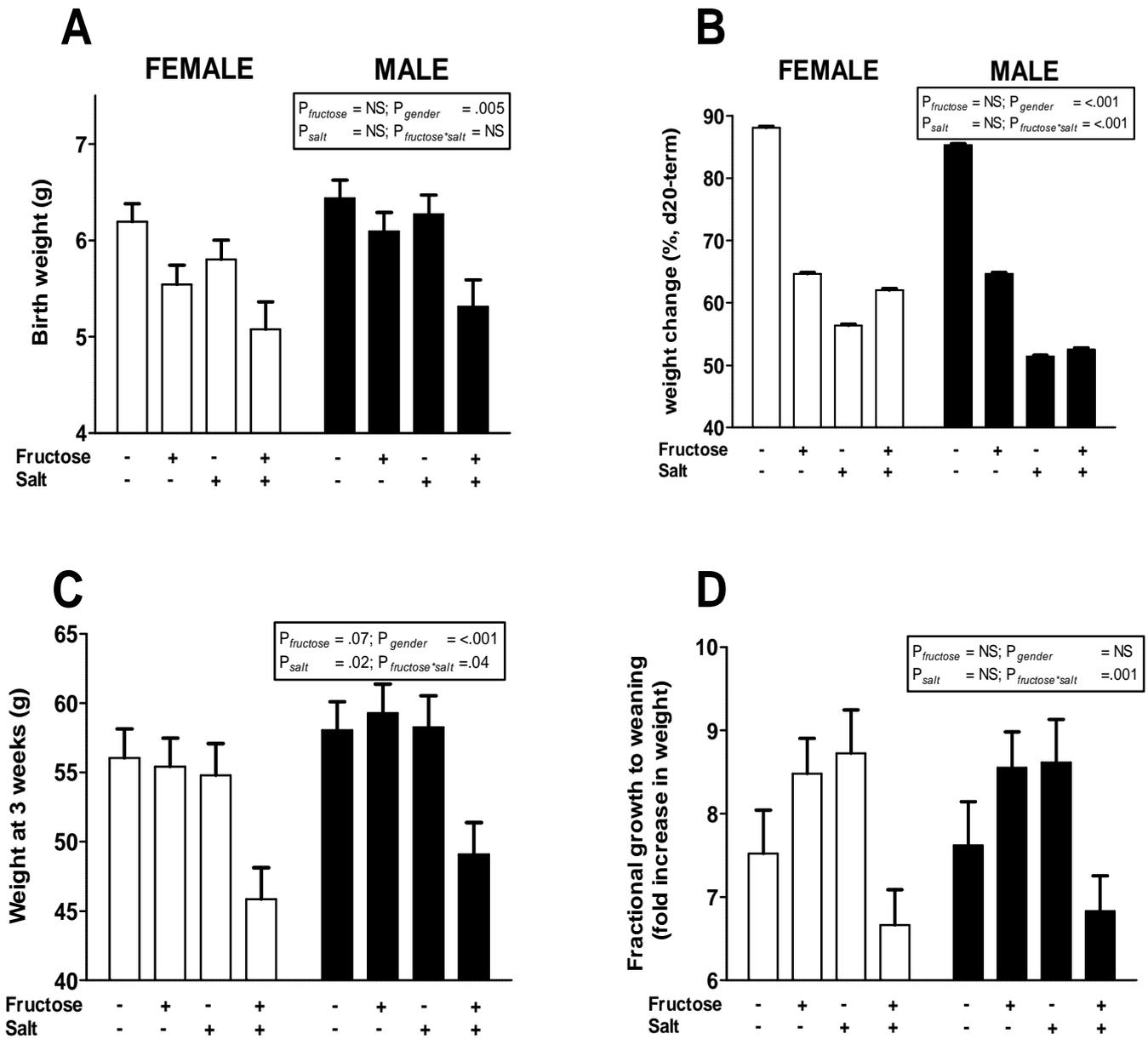
Since litter size was significantly different amongst groups, all subsequent values were adjusted for litter size. Gestational age at delivery for all dams was not different between groups and was  $21.5 \pm 0.5$  days with average pup birth weight being unaffected by maternal diet or litter size. At birth, only gender ( $P < 0.005$ ) had an effect on average birth weight, with males on average weighing more than females at term (Figure 19). When examined cross-sectionally within groups i.e. average group weight at d20 vs. average group weights at term, and therefore different dams, then the rat pups increased body weight by up to 85% from day 20 to term (Figure 19B). For this data, males on average gained more weight than females from day 20 to term ( $P < 0.005$ ) and a significant interaction between fructose and salt ( $P < 0.001$ ) was also observed. This reflects fructose reducing weight gain in comparison to controls, but does not reduce weight gain to any further extent when combined with salt (Figure 19).

At weaning, as expected, males were heavier than females ( $\sim 56.2$  vs.  $52.9 \pm 0.03$ g). However, FSD male and female weanlings were lighter, on average by 10g, in comparison to all other groups due to reduced weight gain through lactation ( $\sim 0.44 \text{g} \cdot \text{day}^{-1}$ ; Figure 19C). This reflects the significant reduction observed in FSD animals when compared to all other groups. Additionally, there was a significant interaction between the effects of fructose and salt on fractional growth to weaning in both males and females (i.e.  $\text{g} \cdot \text{day}^{-1} / \text{g}^{-1}$  birth weight ( $P < 0.001$ ) Figure 19D)). This reflects the slower growth of FSD offspring in comparison to all

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other groups. As adults and as expected, males weighed more than females at 15 weeks of age with no residual effects of maternal diet (Data not shown).

**Figure 19. Birth weight (A), late fetal growth (B), weaning weight (C) and early postnatal growth (D) of rat pups**



**Figure 19.** Data represents % increase in late gestational growth (day 20 - term) (A), growth from birth to weaning (day 21) (B), total body weight at weaning (day 21) (C) and fractional growth to weaning (D). All data was analysed as a 2x2 factorial design with fructose\*salt\*litter size included (general analysis of variance) using Genstat v11 (VSNi, UK). The data are shown as predicted means.

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### 4.3 Discussion

#### *Summary of main findings*

The lifespan of an individual is usually accepted as the time from birth to the time of death. However, the gestational period preceding birth could be defined as one of the most critical periods of a person's life. Nutrition during this critical period of growth and development could have, and has been shown to have, profound effects upon the remaining lifespan (Barker 1989; Lucas 1991). One of the main aims of the thesis was to assess differences in maternal metabolic and physiological profile and what effects if any these perturbations would have on the developing fetus. Data presented in this chapter has demonstrated some less predictable metabolic perturbations and physiological effects of nutrition on litter size, sex ratio and fetal growth with evidence of an unfavourable fetal environment and the possible effects of a deleterious maternal metabolic phenotype up on ovulation, blastocyst development and placental integrity in response to a maternal fructose±salt diet. Ultimately, these perturbations in maternal metabolic phenotype will have an effect on the intra-uterine milieu presenting differences in fetal environments between diets resulting in subtle changes in fetal metabolic profile, development and growth trajectory. Moreover, it can be argued that the months leading up to conception can be just as important, in terms of fertility, pregnancy outcome and fetal health (Sinclair et al 2007). In addition, nutrition has also been hypothesised to have an effect on sex allocation (Rosenfeld & Roberts, 2004).

***Summary of findings in the fetus and neonate***

- Overall rats increased in weight by 85% from day 20 to term, increase was less in response to fructose and, to a greater extent, salt. Combining fructose and salt did not reduce growth any more than feeding salt alone.
- At weaning, FSD male and female rats were significantly lighter than all other groups. This reflected a reduction in growth rate in this group during lactation.
- Fructose affected sex ratio. On average, skewing sex ratio 60:40 in favor of males to females. Dams consuming fructose and salt (FSD) displayed a severe reduction in litter size compared to all other diets.

***Summary of findings in placental phenotype***

- Placentas from dams fed 4% salt showed a reduction in proportion of labyrinthine area and an increase in trophoblast relative to total placental area.
- Placentas from dams fed 10% fructose (FD) showed a reduction in proportion of trophoblast layer and an increase in labyrinthine area relative to total placental area.

One of the main aims presented in this study was to assess differences in fetal growth, tissue weights and placental morphology. In search of a mechanistic insight into previously described programmed phenotypes a number of factors previously considered important can be ruled out. The offspring of dams that received salt diet alone were similar to control offspring in terms of fetal and adult offspring plasma metabolite status, birth weight and

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postnatal growth. Nevertheless, a subtle retardation of growth *per se*, from day 20 to term was observed in salt fed offspring. However, late gestation growth retardation did not have an effect on birth weight. Consideration must be given to the analysis of late gestational growth. Data was collected from both study one (Day 20) and study two (Term, weaning and adult growth) and a cross-sectional analysis was performed at different time points of *in utero* and neonate development on multiple offspring across two separate studies to give a representation of late gestational growth. 'Catch-up growth' was similarly increased in offspring from dams fed fructose alone and both FD and FSD groups went on to exhibit an 'intermediate' phenotype later in life relative to controls.

The labyrinth is where 80% of nutrient transfer occurs in the rodent placenta (Hernandezverdun & Legrand, 1975). Given that a difference was observed in relative placental labyrinthine and trophoblast layers in placentas from SD, whose pups display relative elevated blood pressure in later-life. This may indicate a possible mechanistic explanation (i.e. restriction of placental nutrient transfer or reduced placental angiogenesis leading to less fetal/maternal spiral arteries within the labyrinthine area) to a maternal high salt diet and their subsequent offspring cardiovascular health (Chapter 5). The difference in placental architecture presents a possible phenotypic trait in both male and female SD offspring that could offer a link between maternal nutrition, placental phenotype, fetal development and later-life metabolic and cardiovascular health. There is currently no literature that has investigated the relationship between proportional structure of the

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placental tissue and subsequent fetal development in mothers fed maternal fructose and salt. Studies by Veras *et al* suggest that exposure to pollution may reduce maternal blood supply and this is associated with low birth weight. They also reported that placental size and proportionality were unaffected (Veras et al, 2008). Consideration must be given to the low  $n$  involved in collecting this preliminary data from placental tissue and to the mix of sexes in each group (Section 4.4.3). Further work is currently underway and includes sectioning of additional placentas of which three per gender per litter will be analysed. This will enable determination of diet effects and any gender differences, although current work does not suggest an apparent gender difference within groups. Completion of the analysis must be concluded before any definite conclusions can be made.

Interestingly, during the course of study one and study two an effect of fructose was observed upon sex ratio, producing a shift of 60:40 in favour of male offspring. This data supports the original sex-allocation hypothesis proposed by Trivers and Willard (1973), one of two generally accepted sex allocation hypotheses, the other being the 'local mate theory' (Hamilton, 1967). The sex-allocation hypothesis of Trivers & Willard predicts females in the best body condition would tend to produce male offspring, as males have greater potential to distribute genes to a larger population. Males from 'over nourished' mothers would therefore benefit from increased parental investment and most likely, as adults, join the upper echelons of breeding males. Consequently, mothers are more likely to pass on their genes to future generations (Trivers & Willard, 1973). In similarity to this study, a number of

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animal models have shown results consistent with Trivers and Willard's hypothesis and its various extensions.

Data in this chapter demonstrates for the first time in the rat it has been shown that there is a shift in sex ratio in favour of male offspring in dams fed maternal fructose. The exact mechanism by which maternal nutrition affects sex ratio is yet to be elucidated. However current knowledge has highlighted four primary proposed pre-implantation mechanisms by which sex ratio may be altered (differential sperm motility, selective fertilisation, gender pre-implantation rate and gender specific developmental arrest) (Rosenfeld & Roberts 2004). Harvested blastocysts at day 4 (prior to implantation) will be analysed further to determine primary sex ratio within litters to further elucidate at which stage the sex ratio shift is occurring. The changes that allow one sex to prevail over the other could occur pre- or post-implantation (preferential fertilisation). A recent study shows that a skew in sex ratio is unlikely to be due to post-implantation events such as sex-selective reabsorption of embryos (Kruger et al 2005), indicating the primary sex ratio as the regimenting factor of sex ratio. Consideration must also be given to other related factors which may contribute to primary sex ratio alteration e.g. genetic factors, hormonal concentrations, endocrine disruptors and environment (Pergament *et al.*, 2007). In conclusion alterations in sex ratio can occur in a number of mammals and these could be adaptive to maternal diet. Our studies in the rat indicate that maternal diet and consequential caloric intake, more specifically in the form of fructose, can play a direct role in sex allocation. More importantly, the maternal diet, both pre and post-conceptual, needs to be considered as the main

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causative factors in the sex ratio shift observed in the current studies. In addition to sex ratio, a significant interaction between fructose and salt was observed on the apparent reduction of blastocysts at day 4 in FSD pregnant females may indicate a reduced ovulation rate in dams with reduced litter size, and not a result of implantation and resorption of early embryos as originally hypothesised. Consideration must be given to the original sample size (n=6) with only three FSD dams becoming pregnant. However, the apparent lower conception rate and the reduction in FSD blastocyst numbers imply that the FSD maternal diet may be having an effect upon fertility *per se*. Reduced weight gain in FSD females prior to pregnancy suggests dams were receiving a lower plain of nutrition than other groups. Studies on spiny mice have shown that the uterus and ovaries were reduced in size and weight in mice fed 2.5%, 3.5% and 5% salt diets with associated reduction in fertility (Shanas & Haim, 2004). However, without an effect observed in SD dam litter size or blastocyst number, the level of salt seems to be an unlikely causal factor in the current study. Although, Bird and Contreras observed an inverse relationship between maternal NaCl and sex ratio (Bird & Contreras, 1986); they observed a reduction in male offspring when feeding a maternal high salt diet. Similar to the current study, they also report no change in litter size and birth weight in salt-fed animals. Currently, there are substantial gaps in the literature regarding nutrition and associated placental development, sex ratio and litter size and the current data clearly highlights the need for further research into maternal nutrition and effects upon sex ratio, litter size, placental development and subsequent fetal health. Few

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studies have examined the effects of salt and no studies to our knowledge have considered the effect fructose may have on sex ratio in the rat.

***Conclusion***

Data in this chapter suggests an effect of fructose and/or salt (particularly fructose and salt (FSD)) during gestation upon the developing placenta and fetus. Marked alterations in litter size, blastocyst number and fetal growth suggest the interaction of fructose and salt is severely impacting the maternal-fetal environment. Sex ratio also appears to be significantly affected by the consumption of fructose but it is clear further work must be carried out to elucidate possible mechanisms of this change in ratio.

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## 5 Dietary effects on offspring cardiorenal function

### 5.1 Introduction

Hypertension is an important modifiable risk factor for stroke and related cardiovascular diseases. However, the exact origins or cause of hypertension are not fully understood. Modern diets superimposed on a genotype shaped by Paleolithic nutrition is suspected to largely account for the increased incidence of non-communicable disease we see today, estimated to account for 60% of all deaths worldwide (Daar *et al.*, 2007). The potential economic impact is startling; the combined cost of heart disease, stroke and diabetes in China, India and the UK to 2017 is estimated at \$558, \$237 and \$33 billion, respectively (WHO, 2005). Diabetes and hypertension alone are estimated to cost the United States \$750 billion annually (Narayan *et al.*). Early prevention is key, since adverse childhood risk factors translate into greater susceptibility to non-communicable adult disease (Franks *et al.*, 2010). Short-term consumption of a 'Paleolithic' diet results in favorable reductions in blood pressure, cholesterol, triglyceride and insulin resistance in humans (Lindeberg *et al.*, 2007; Frassetto *et al.*, 2009; Jonsson *et al.*, 2009) and pigs (Jonsson *et al.*, 2006). Translational of these benefits to current human populations will have a significant economic impact; indeed it has recently been proposed that an achievable reduction in salt intake (to 3g/day) will reduce all-cause mortality in the United States by 44,000 to 92,000 saving \$10 billion to \$24 billion in annual health care costs (Bibbins-Domingo *et al.*, 2010b). In addition to environmental, social and genetic factors, intra-uterine conditions are now recognized as an

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important factor in the progression or susceptibility of these pathologies. The disturbance of the growth process in the short term, in response to nutrient restriction or excessive nutrient availability, may involve adaptation in the fetal milieu which serves to maintain functionality necessary for development. Blood flow, nutrients, hormones and O<sub>2</sub> within the blood, are diverted preferentially to the brain and heart which may compromise optimal development and growth of peripheral organs (Rudolph 1984). Consequently this may lead to decreased function of said organs and when presented with a further challenging nutritional environment may manifest or increase susceptibility to disease earlier in later life. However, the mechanisms involved in the progression of fetal insults leading to the manifestation of adult disease are poorly understood.

Kermack *et al* (1934) first reported a relationship between living conditions, socioeconomic status and subsequent death rates from all causes. Forsdahl (1977) and colleagues have since described a correlation between infant mortality and CHD-related deaths. Following these early findings, many more perinatal epidemiological studies have accumulated a large amount of evidence relating to the developmental programming (fetal origins) of adult disease hypothesis, and suggested it to be more than just a consequence of adult environment and other such confounding factors e.g. socioeconomic factors. The suggestion of a stimulus or insult acting within the gestational environment having such a profound effect upon the fetus during development and health and disease in subsequent later life has changed how we address the role of nutrition during pregnancy.

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Epidemiological studies supported by a large amount of small and large animal data have identified the existence of metabolic or 'fetal' programming; in particular, fetal nutrition and later life cardiovascular health. Taking into account the large amount of data available, very few studies have investigated the role in which fructose and/or salt may play in the development of predisposition to disease during later life. As discussed (Chapter 3), excessive fructose has been shown to exert detrimental effects upon the fetus *in utero*. Fructose can produce differential metabolic effects than that of glucose or sucrose and lead to hypertriglyceridemia and hyperglycaemia in dams during gestation (Chapter 3). Pups born to fructose fed dams have also been shown to be hyperglycaemic (Jen *et al.*, 1991). Therefore, excessive fructose during pregnancy has acute adverse effects in the mother, which may be inherited by the newborn. The nutritional model in the present study illustrates that maternal diets moderately high in fructose can induce physiological differences in maternal plasma metabolites, urine metabolite excretion (Gray, 2009) electrolytes and osmolality and, as a consequence, are in some cases transferred to the fetus (Chapter 3). Studies have also shown evidence of programmed hypertension in adult offspring from mothers fed salt diets at varying levels of additional dietary NaCl (Contreras & Oparil, 1992; Contreras *et al.*, 2000; Swenson *et al.*, 2004). Researchers have shown that 2% NaCl in drinking water was sufficient to induce hypertension in adult offspring (Grollman & Grollman, 1962). Other studies have shown 1.5% and 4% NaCl added to the diet (in chow) did not promote a rise in resting pressure, although King and colleagues did report a hyper-responsiveness to stress in adult female offspring fed 4% NaCl prenatally, whereas a 2.3%

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NaCl maternal diet (in chow) did not cause hypertension (Myers *et al.*, 1985). Of course, timing of the prenatal NaCl can also affect the progression of hypertension in adult offspring. Studies feeding a 3% NaCl diet throughout pregnancy and lactation, until 10 days post weaning (Contreras *et al.*, 2000) reported hypertensive offspring at 12 weeks of age. Despite the large number of studies, the mechanisms involved in the progression of fetal insults leading to the manifestation of adult cardiovascular disease are poorly understood. Many mechanisms have been proposed as key factors underpinning the fetal origins hypothesis such as altered kidney development (LangleyEvans, 1996) – specifically nephron deficit, over exposure to glucocorticoids (Gardner *et al.*, 1997), perturbed angiogenesis and/or altered vascular function (Hemmings *et al.*, 2005; Pladys *et al.*, 2005).

This chapter characterises the longer-term physiological effects of a diet moderately high in salt and/or fructose upon adult male and female offspring in terms of plasma metabolites, urinary metabolic profiles, plasma and urinary electrolytes and osmolality and resting and stimulated cardiovascular responses were each assessed for potential effects on offspring metabolism, renal and cardiovascular function.

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**5.2 Protocol**

During study 2, dams were fed purified diets containing either additional 4% salt or control chow with or without 10% fructose solution prior to and during gestation and lactation (as described in study 1). Male and female weanlings were fed purified control diet up to 10 weeks of age when they were surgically implanted with radiotelemetric probes (CD, n=6, FD, n=5, SD, n=5, FSD, n=5 per gender per dietary group) and basal cardiovascular pressures and cardiovascular responses to pharmacological and dietary challenges were measured. 'Control' uninstrumented offspring (litter mates) (n=5/gender/dietary group, unless stated otherwise) were housed under control conditions up to 14-15 weeks of age and euthanised for a control tissue collection. Cardiovascular data, hormones, metabolites, electrolytes and osmolalities were conducted and assayed according to previously described methodology in the relevant sections of *Methods* (Section 2.5,2.8,2.9,2.10).

## 5.3 Results

### 5.3.1 ADULT OFFSPRING FOOD AND WATER INTAKE

Energy intake from food was significantly ( $P < 0.001$ ) higher in males compared to females, but not different between dietary groups (data not shown). Water intake was not different between groups in adult male and female adult offspring (Table 14).

**Table 14. Baseline food and water intake in 14 week-old offspring of dams fed fructose $\pm$ salt**

Baseline food and water intake in 14 week-old offspring of dams fed fructose $\pm$ salt							
Male offspring	$\pm$ fructose	$\pm$ salt		s.e.d	P value		
		-ve	+ve		Fructose	Salt	Fr*S
Food intake (g/24h)	-ve	25.8	29.3	2.6	NS	NS	NS
	+ve	25.6	25.6				
Water intake (ml/24h)	-ve	27.9	30.3	2.9	NS	NS	NS
	+ve	30.1	32.1				
<b>Female offspring</b>							
Food intake (g/24h)	-ve	21.4	16.8	2.6	NS	NS	NS
	+ve	20.1	20.9				
Water intake (ml/24h)	-ve	26.0	23.2	2.9	NS	NS	NS
	+ve	28.7	21.6				

**Table 14.** Baseline food and water intake were calculated and averaged over a 3-day period. Data are from n=5-6 male or females per dietary group (n=5-6 dams per dietary group) and were analysed by General Linear Mixed Model (Genstat v13). s.e.d., standard error of the difference. Fr\*S; interaction of fructose\*salt. There was a significant effect of sex on food intake ( $P < 0.0001$ ), but no effect of prenatal diet on any outcome.

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### 5.3.2 ADULT OFFSPRING PLASMA AND URINARY METABOLITES

At 14 weeks of age in male offspring of dams fed maternal salt  $\pm$  fructose, plasma glucose, NEFA, triglycerides, uric acid, albumin, urea and creatinine were not affected by prenatal diet (Table 15).

Plasma osmolality was significantly higher in males compared females ( $P=0.04$ ) *per se*. There was a significant positive effect of prenatal salt ( $P=0.006$ ) on plasma osmolality, but male and female salt-exposed offspring had increased plasma osmolality far greater in SD than in FSD, reflecting a significant interaction between fructose and salt ( $P=0.03$  for interaction; Figure 20A,B). In the baseline state, osmolar excretion was significantly higher in females in comparison to males ( $P<0.001$ ) but was also significantly increased in salt-exposed offspring ( $P=0.02$ ; Figure 20C,D). Osmolar clearance (which takes into account plasma concentrations) was significantly less in females in comparison to males ( $P<0.001$ ) but was otherwise unaffected by prenatal diet (Figure 20E,F). At this time, baseline urine output was significantly greater in prenatal fructose-exposed offspring ( $P=0.01$ ), being on average  $9.48\pm 1.30\text{ml/day}$  in FD in comparison to  $12.28\pm 1.30\text{ml/day}^{-1}$  in CD adult offspring (Table 16). All other urinary biochemistry in the adult offspring was unaffected by prenatal diet at this age (Table 16).

Analysis of urinary electrolyte concentration revealed that excretion of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  were all affected by gender ( $P=0.03$ ), ( $P=0.06$ ) and ( $P=0.008$ ) respectively, with males excreting

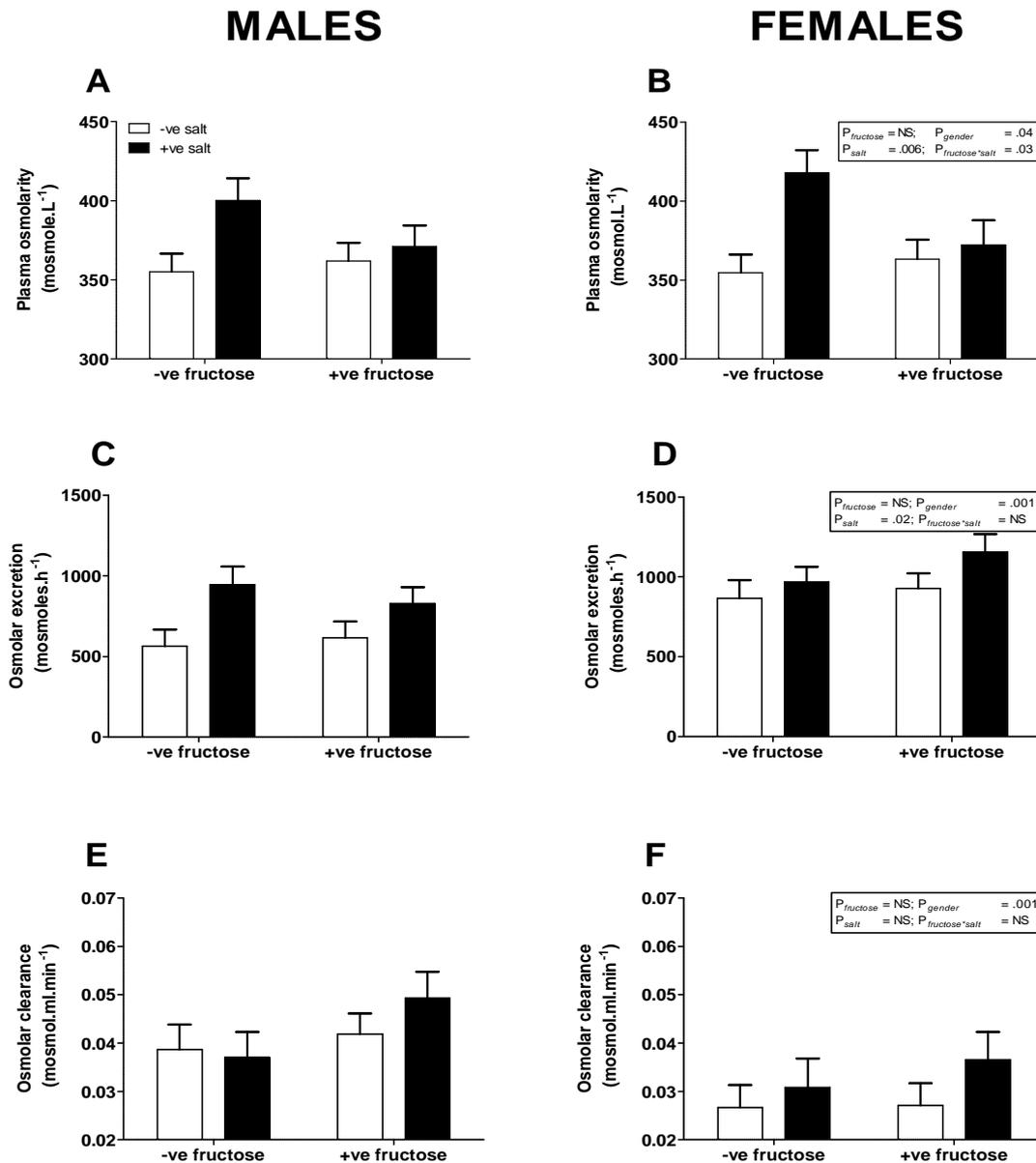
more electrolytes on average than female adult offspring. Furthermore, significant effects of maternal salt-intake were observed, with greater Na<sup>+</sup> (P<0.002) and K<sup>+</sup> (P<0.004) excretion in salt-exposed offspring vs. other groups (Figures 21A,B,C and D). Urinary excretion of Ca<sup>++</sup> was unaffected by maternal diet or gender (Figure 21E,F).

**Table 15. Plasma metabolites in offspring at 14 weeks of age**

	<i>Plasma metabolites in offspring at 14 weeks of age</i>				<i>P value</i>		
	$\pm$ fructose	$\pm$ salt		s.e.d.	Fructose	Salt	Fr*S
		- ve	+ ve				
<b>Glucose (mmol L<sup>-1</sup>)</b>	-ve	7.51	7.91	2.2	NS	NS	NS
	+ve	8.85	7.57				
<b>NEFA (mmol L<sup>-1</sup>)</b>	-ve	0.28	0.24	0.07	NS	NS	NS
	+ve	0.29	0.34				
<b>TG (mmol L<sup>-1</sup>)</b>	-ve	0.62	0.65	0.23	NS	NS	NS
	+ve	0.58	0.84				
<b>Cholesterol (mmol L<sup>-1</sup>)</b>	-ve	1.51	2.24	0.66	NS	NS	NS
	+ve	2.73	2.51				
<b>Uric acid (<math>\mu</math>mol L<sup>-1</sup>)</b>	-ve	30.7	40.0	15.2	NS	NS	NS
	+ve	35.2	50.1				

**Table15.** Plasma was collected from individual pups by cardiac puncture and metabolites measured by auto-analyser (see Methods). Data represent at least n=5 males/females/ dam (n=6 for CD; n=5 for FD, SD and FSD) for each analyte and were analysed by General Linear Mixed Model, with dam included as a random term to account for the reduced variance of littermates (Genstat v13). s.e.d., standard error of the difference. Fr\*S; interaction of fructose\*salt.

Figure 20. Plasma osmolality (A,B), osmolar excretion (C,D) and clearance (E,F) in 14 week old male and female offspring



**Figure 20.** Plasma osmolality (A,B), osmolar excretion (C,D) and clearance (E,F) in 14 week old male and female offspring from dams fed 1) control diet and water *ad libitum* (n=6 males/females), 2) control diet and 10% fructose in water *ad libitum* (n=5 males/females), 3) 4% salt diet and water *ad libitum* (n=5 males/females) and 4) 4% salt diet and 10% fructose in water *ad libitum* (n=5 males/females). All data analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

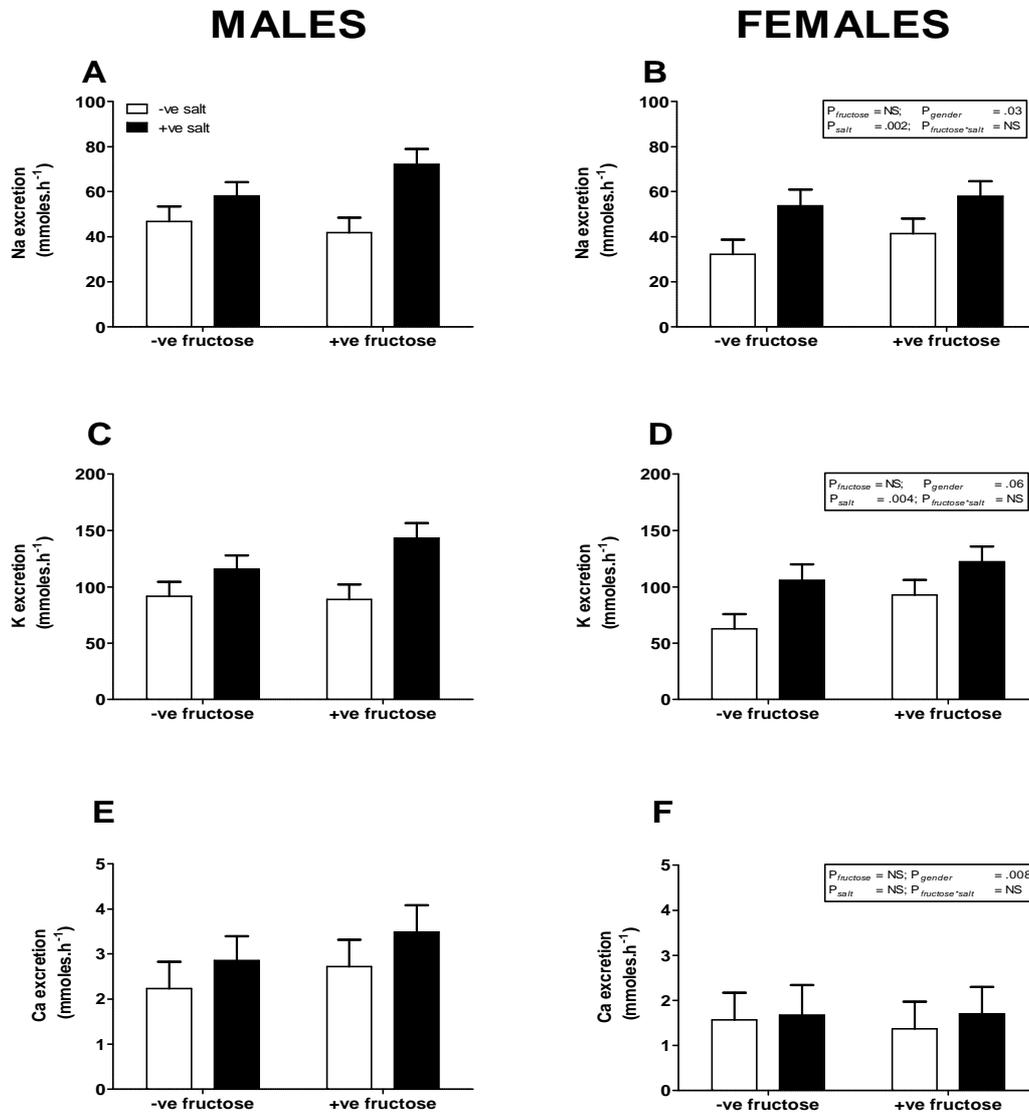
Table 16. Urinary biochemistry in adult offspring at 14 weeks of age

*Urinary biochemistry in adult offspring at 14 weeks of age*

	$\pm$ fructose	$\pm$ salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
Urine output (ml day <sup>-1</sup> )	-ve	9.48	9.97	1.30	0.01	NS	NS
	+ve	12.28	11.90				
Plasma urea (mmol L <sup>-1</sup> )	-ve	4.88	5.90	1.36	NS	NS	NS
	+ve	5.79	6.20				
Urea excretion (mmoles h <sup>-1</sup> )	-ve	349	433	62	NS	NS	NS
	+ve	492	482				
Plasma albumin (g L <sup>-1</sup> )	-ve	25.6	33.0	4.9	NS	NS	NS
	+ve	31.4	29.6				
Urine albumin:creatinine (mg $\mu$ mol <sup>-1</sup> )	-ve	0.18	0.14	0.05	NS	NS	NS
	+ve	0.19	1.14				
Creatinine clearance ( $\mu$ mol ml min <sup>-1</sup> )	-ve	0.96	0.69	0.26	NS	NS	NS
	+ve	0.65	1.16				

**Table 16.** Data represents adult male & female offspring urine biochemistry at 14 weeks of age. Rats weaned from mothers fed a maternal fructose  $\pm$  salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). All data were analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

**Figure 21.** Renal excretion of Na (A,B), K (C,D) and Ca (E,F) in 14 week old male and female offspring



**Figure 21.** Renal excretion of Na (A,B), K (C,D) and Ca (E,F) in 14 week old male and female offspring from dams fed 1) control diet and water *ad libitum* (n=6 males/females), 2) control diet and 10% fructose in water *ad libitum* (n=5 males/females), 3) 4% salt diet and water *ad libitum* (n=5 males/females) and 4) 4% salt diet and 10% fructose in water *ad libitum* (n=5 males/females). Urinary values were measured from a 24h collection in a metabolic crate. Data analysed by General Linear Mixed Model (Genstat v13).

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### 5.3.3 ADULT OFFSPRING SALT-CHALLENGED FOOD AND WATER INTAKE

As expected energy intake from food was significantly ( $P<0.001$ ) different between male and females but without an effect of diet in either gender during the acute salt challenge. An effect of time ( $P<0.001$ ) was observed as all animals reduced their intake of diet in all groups over the 3 days of the challenge. Water intake was also significantly increased ( $P<0.001$ ) in male and female offspring over the period of the salt challenge. Offspring on average doubled their water intake between day 1 and day 3 of the challenge (Data not shown).

Over the period of acute salt feeding (3 days), as expected and because of increased water intake, urine output increased with time ( $P<0.001$ ) in male and female offspring without a difference between groups. A significant effect of gender ( $P<0.03$ ) was observed, with on average urine output being  $11.7\pm 0.7$ ml/day in males in comparison to  $9.9\pm 0.7$ ml/day in females. Additionally, both male and female offspring at 14 weeks were similarly affected by prenatal fructose ( $P<0.009$ ); adult offspring from mothers fed fructose had an increased urine output when compared to those unexposed to fructose. Urinary metabolites did not change significantly from baseline (presented in Table 16) when offspring were fed salt over 3 days. Urinary albumin, creatinine, urea and the albumin/creatinine ratio were not different amongst all groups in both genders and relative to the control state. Urinary osmolality, urinary sodium and potassium in these animals were also significantly influenced by prenatal salt exposure but not significantly by postnatal salt-exposure, i.e. data were as described previously in the baseline state.

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### 5.3.4 ADULT OFFSPRING TISSUE MASS AT 14 WEEKS

Within gender, there was no effect of prenatal diet on body weights at 14 weeks of age in either males or females across all dietary groups. As expected, at 14 weeks of age males were heavier than females (males were on average  $408 \pm 14$ g in comparison to  $236 \pm 14$ g in females). As expected, organs and fat depots were significantly heavier in male offspring vs. female offspring and tissue weights reflected the greater body size of the male offspring. Therefore, absolute (wet) male/female weights and weights as a proportion of body weight are reported. When expressed relative to body weight, females had relatively more gonadal adipose tissue, being on average  $13.33 \pm 0.93$ mg/g in females in comparison to  $10.97 \pm 0.93$ mg/g in males. Heart mass was also significantly increased in females in comparison to males ( $4.59$  vs.  $4.07 \pm 0.11$  mg/g, respectively). Additionally, females had relatively less visceral adipose tissue, being on average  $7.42 \pm 0.54$ mg/g in comparison to  $8.93 \pm 0.54$ mg/g in males (Table 18).

No significant effects of the diets were seen in absolute liver weight in male and female adult offspring. However, when expressed relative to body weight there was a residual effect of prenatal fructose, i.e. female liver weight was significantly ( $P=0.02$ ) increased relative to non-fructose exposed offspring ( $7.92 \pm 0.49$ mg/g in CD in comparison to  $8.28 \pm 0.49$ mg/g in FD). A similar, albeit non-significant trend ( $P=0.07$ ), was observed in the male offspring from prenatal fructose-exposed offspring, being on average  $13.14 \pm 0.4$ mg/g in CD in comparison to  $15.62 \pm 0.4$ mg/g in FD (Table 17). In adult males, but not females, there was a significant

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effect of prenatal fructose exposure on visceral fat mass (i.e. an increased weight;  $P=0.04$ ), being on average  $3.028\pm 0.48\text{g}$  in CD in comparison to  $3.987\pm 0.48\text{g}$  in FD. Indeed, the effect of fructose on male visceral adipose remained when weights were expressed relative to body weight ( $P<0.005$ ) (Table 17). However, maternal diet had no lasting effect upon male or female gonadal adipose deposition. An effect of prenatal fructose ( $P<0.005$ ) was also evident on male offspring heart tissue, being on average  $1.693\pm 0.06\text{mg/g}$  in CD in comparison to  $1.57\pm 0.06\text{mg/g}$  in FD, which remained when expressed relative to body weight ( $P<0.013$ ). No residual effect of prenatal diet was observed on adult kidney mass.

**Table 17. Effect of prenatal fructose $\pm$ salt on the adult male offspring absolute and relative organ weight**

*Effect of prenatal fructose $\pm$ salt on the adult male offspring absolute and relative organ weight*

	$\pm$ fructose	$\pm$ salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
Liver (g)	-ve	13.14	15.41	0.4	NS	NS	NS
	+ve	14.29	15.62				
Visceral fat (g)	-ve	3.028	3.668	0.48	0.04	NS	NS
	+ve	3.987	3.914				
Gonadal fat (g)	-ve	4.288	4.524	0.59	NS	NS	NS
	+ve	4.455	4.53				
Kidney (g)	-ve	2.655	2.86	0.12	NS	NS	NS
	+ve	2.818	2.792				
Heart (g)	-ve	1.693	1.762	0.06	0.005	NS	NS
	+ve	1.57	1.61				
Liver (mg g <sup>-1</sup> )	-ve	33.63	36.93	2.06	(0.07)	NS	NS
	+ve	34.45	38.33				
Visceral fat (mg g <sup>-1</sup> )	-ve	7.743	8.711	1.38	0.056	NS	NS
	+ve	9.619	9.654				
Gonadal fat (mg g <sup>-1</sup> )	-ve	11.1	10.82	2.03	NS	NS	NS
	+ve	10.78	11.16				
Kidney (mg g <sup>-1</sup> )	-ve	6.731	6.845	0.26	NS	NS	NS
	+ve	6.806	6.847				
Heart (mg g <sup>-1</sup> )	-ve	4.301	4.235	0.22	0.013	NS	NS
	+ve	3.806	3.96				

**Table17.** Data represents adult male offspring absolute and relative organ weights at 14 weeks of age. Male rats weaned from mothers fed a maternal fructose  $\pm$  salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). All data were analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK). Data shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

**Table 18. Effect of prenatal fructose±salt on the adult female offspring absolute and relative organ weight**

*Effect of prenatal fructose±salt on the adult female offspring absolute and relative organ weight*

	±fructose	±salt		s.e.d.	P value		
		- ve	+ ve		Fructose	Salt	Fr*S
Liver (g)	-ve	7.92	7.86	0.99	NS	NS	NS
	+ve	8.41	8.28				
Visceral fat (g)	-ve	1.82	1.986	0.48	NS	NS	NS
	+ve	1.449	1.808				
Gonadal fat (g)	-ve	3.373	4.288	0.59	NS	NS	NS
	+ve	3.576	4.524				
Kidney (g)	-ve	1.6	1.562	0.12	NS	NS	NS
	+ve	1.565	1.594				
Heart (g)	-ve	1.067	1.094	0.06	NS	NS	NS
	+ve	1.132	1.058				
Liver (mg g <sup>-1</sup> )	-ve	32.53	32.64	2.06	0.02	NS	NS
	+ve	36.76	35.27				
Visceral fat (mg g <sup>-1</sup> )	-ve	7.323	8.299	1.38	NS	NS	NS
	+ve	6.379	7.715				
Gonadal fat (mg g <sup>-1</sup> )	-ve	13.67	15.01	2.03	NS	NS	NS
	+ve	12.71	11.93				
Kidney (mg g <sup>-1</sup> )	-ve	6.616	6.515	0.26	NS	NS	NS
	+ve	6.807	6.78				
Heart (mg g <sup>-1</sup> )	-ve	4.435	4.554	0.22	NS	NS	NS
	+ve	4.899	4.501				

**Table18** Data represents adult female offspring absolute and relative organ weights at 14 weeks of age. Female rats weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). All data were analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK). Data was shown as predicted means with the average standard of error of the difference (s.e.d). P, for effect of diet. NS, not significant.

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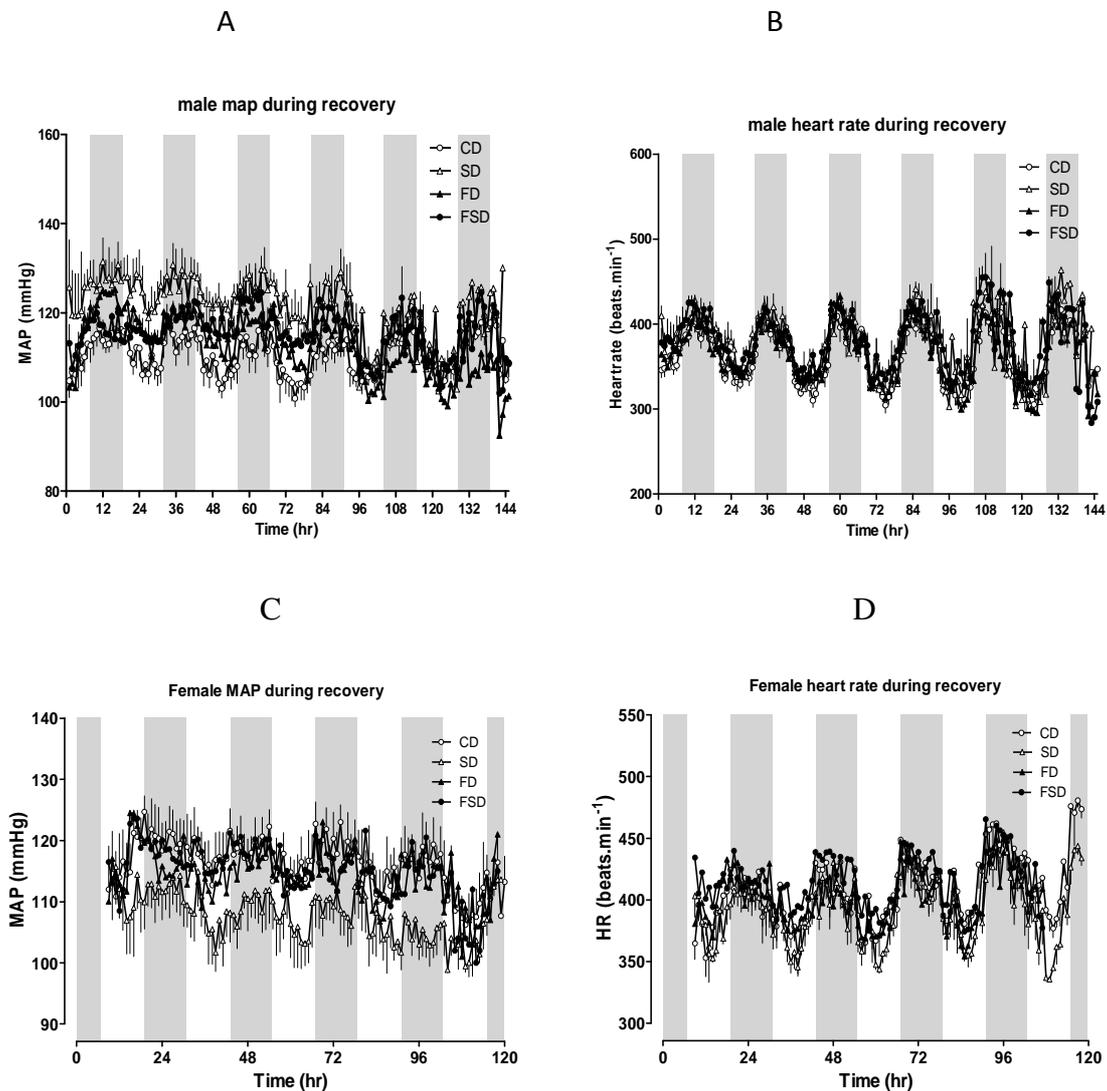
### 5.3.5 ADULT OFFSPRING CARDIOVASCULAR DATA

Blood pressure in telemetered males and females showed a significant diet\*gender interaction and also varied significantly with time of day due to circadian rhythms, therefore results are presented as graphs with time as the x-axis. Cardiovascular data were recorded at 30s intervals for 24h. Data were analysed either by compressing the data to hourly mean (Figure 22) or by fitting separate Fourier curves to all data points (2880/animal/day) within groups (Figure 25). For all results shown except that of the anxiety challenge, telemetered animals had a companion in the same cage and group sizes were CD, male n=6; female n=6, FD, male n=5; female n=5, SD, male n=5; female n=5 and FSD, male n=5; female n=5.

### 5.3.6 POST SURGICAL RECOVERY

A seven day recovery period was allowed post-surgery, when rats were left alone with cage mates and with minimal disturbance from technicians. During this period cardiovascular data were recorded for 30s at 15min intervals. An effect of time on MAP was observed; MAP was significantly ( $P<0.001$ ) higher in all groups and genders at day one (post-surgery) when compared to days 3 and 4 (Figure 22A,C). Heart rate increased over time showing more variation between day and night as the recovery period progressed. On average it took 3 to 4 days for telemetered rats to fully recover from surgery to a consistent (assumed pre-surgical) basal blood pressure and heart rate (Figure 22A,B,C,D). This phenotype was observed in males and females without an effect of maternal diet on recovery rate.

**Figure 22. Blood pressure and heart rate responses in male & female offspring post-surgery**



**Figure 22.** Data represents adult male & female offspring cardiovascular data. Offspring were weaned from mothers fed a maternal fructose  $\pm$  salt diet and subsequently maintained on a postnatal control chow diet. A 7 day period was allowed for recovery and rats were left alone in a sound proofed room on a 12h (07.00am-0700pm) light-dark cycle. Graph a) represents male mean arterial pressure (MAP), graph b) represents male heart rate (HR), graph c) represents female MAP and graph d) represents female HR. Blood pressure was recorded at 15min intervals for 7 days. All data were analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

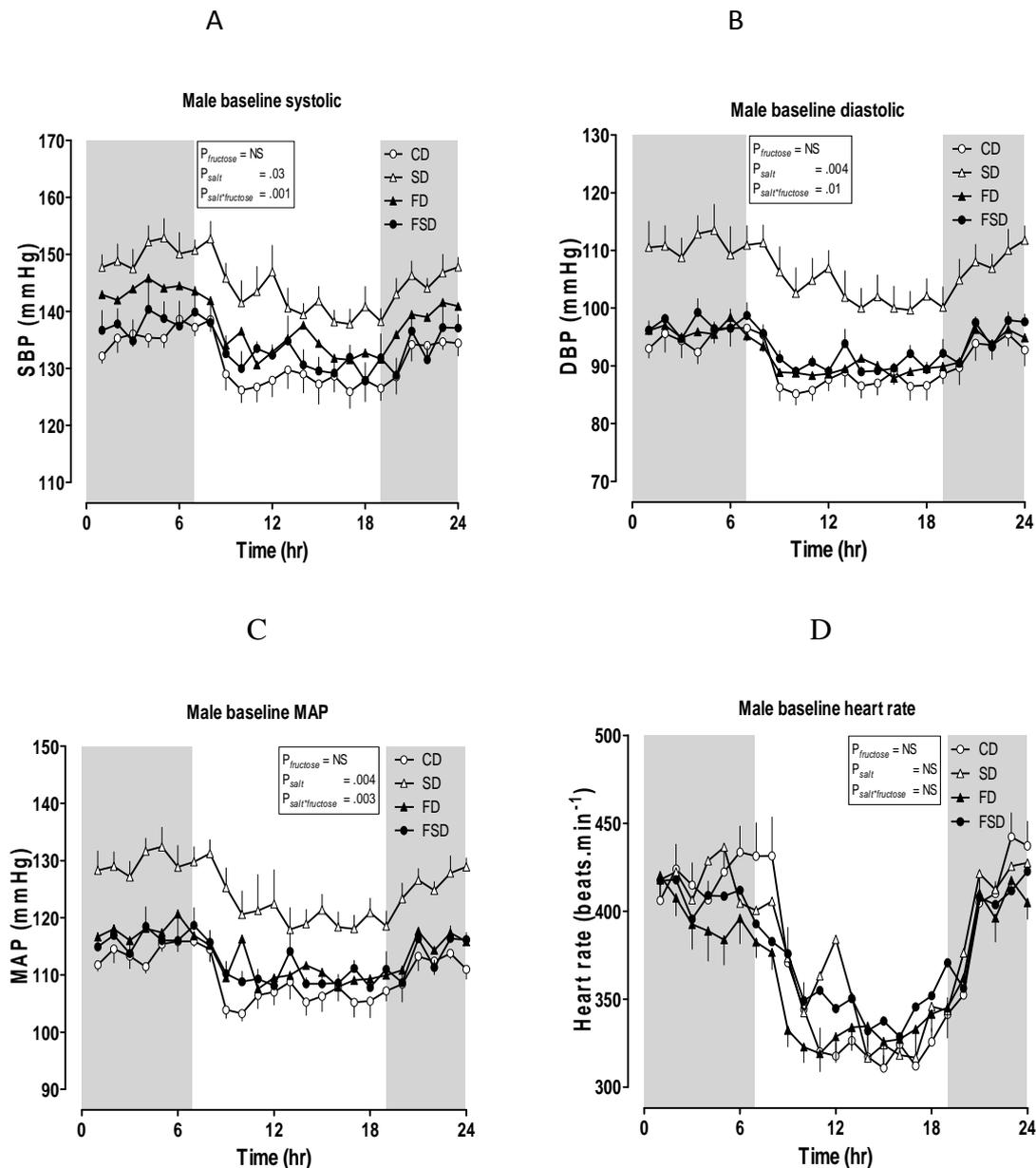
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### 5.3.7 RESTING CARDIOVASCULAR PRESSURES

There was a significant interaction between salt and fructose in their effects on systolic ( $P<0.001$ ), diastolic ( $P=0.01$ ) and MAP ( $P=0.003$ ). This reflects higher values for all 3 variables in SD offspring compared to all other groups. Resting diastolic blood pressure in the male offspring being, on average  $87.9\pm 2.4$ mmHg in CD in comparison to  $103\pm 2.6$ mmHg for SD during the day and  $94.1\pm 2.3$ mmHg in CD compared with  $109.8\pm 2.6$ mmHg in SD during the night (Figure 24B). MAP of male offspring, being on average  $106.8\pm 2.1$ mmHg in CD in comparison to  $121\pm 2.1$ mmHg in SD for day and  $113.21\pm 2.1$  in CD compared with  $128\pm 2.3$ mmHg in SD during the night (Figure 23C). Basal heart rate was not influenced by maternal diet at this stage (Figure 23D).

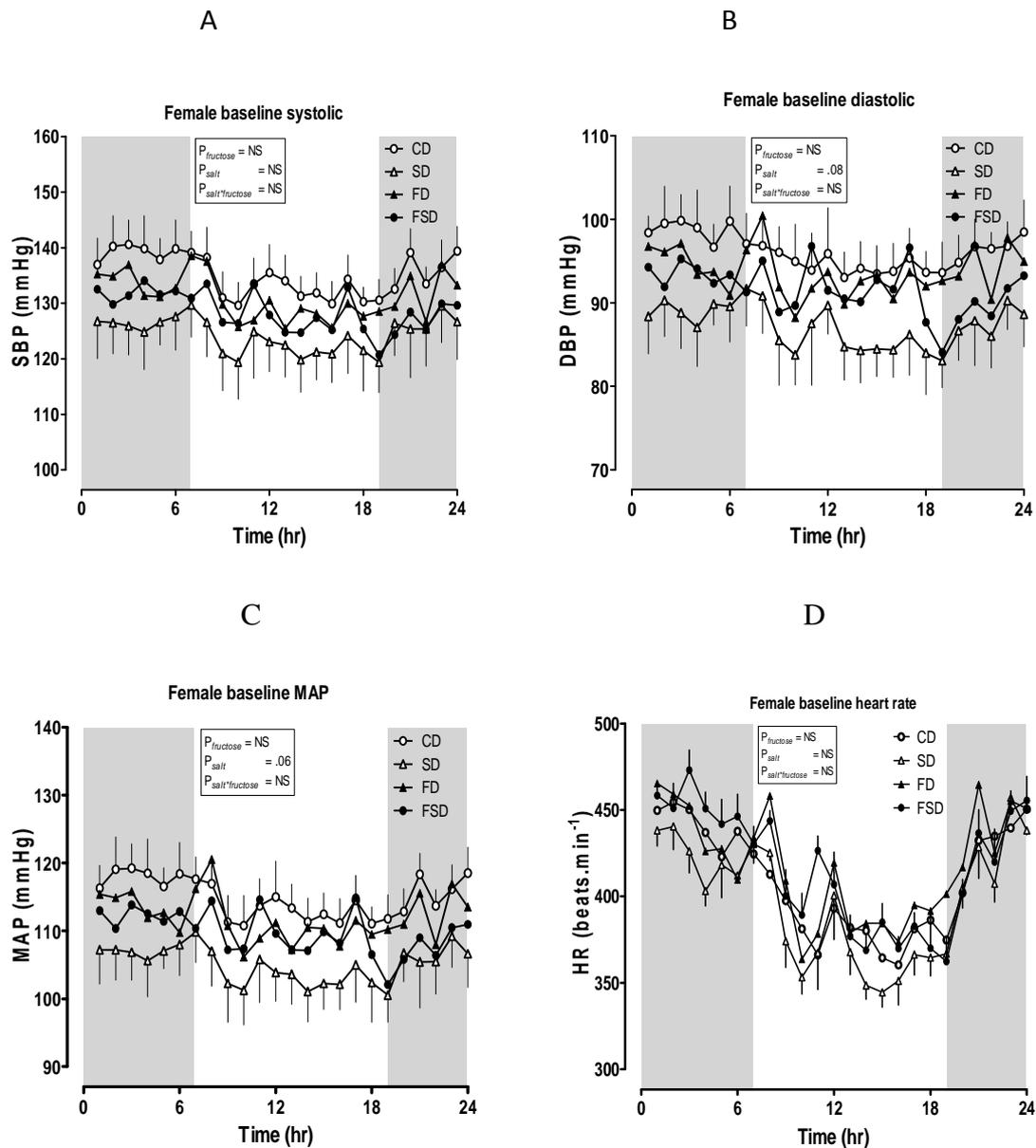
For females, blood pressures tended to be lower in the offspring of salt fed vs. not salt fed dams, for example MAP in females was on average 9mmHg lower ( $P=0.06$ ) and diastolic pressure was 9 mmHg lower ( $P=0.08$ ; Figures 24A B and C). Female offspring of fructose fed dams did not show any differences in baseline systolic and heart rate when compared to all other groups (Figure 24D).

**Figure 23. Effect of prenatal fructose±salt on the male offspring blood pressure and heart rate**



**Figure 23.** Data represents adult male offspring baseline cardiovascular data at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Graph a) represents male baseline systolic pressure (SBP), graph b) represents male baseline diastolic (DBP), graph c) represents male mean arterial pressure and graph d) represents male heart rate. Blood pressure was recorded continuously (30sec. Intervals) for 24h and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 24. Effect of prenatal fructose±salt on the female offspring blood pressure and heart rate**



**Figure 24.** Data represents adult female offspring baseline cardiovascular data at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Graph a) represents female baseline systolic pressure (SBP), graph b) represents female baseline diastolic (DBP), graph c) represents female mean arterial pressure and graph d) represents female heart rate. Blood pressure were recorded continuously (30sec. Intervals) for 24h and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

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### 5.3.8 FOURIER ANALYSIS OF THE CIRCADIAN VARIATION IN BASELINE CV STATUS

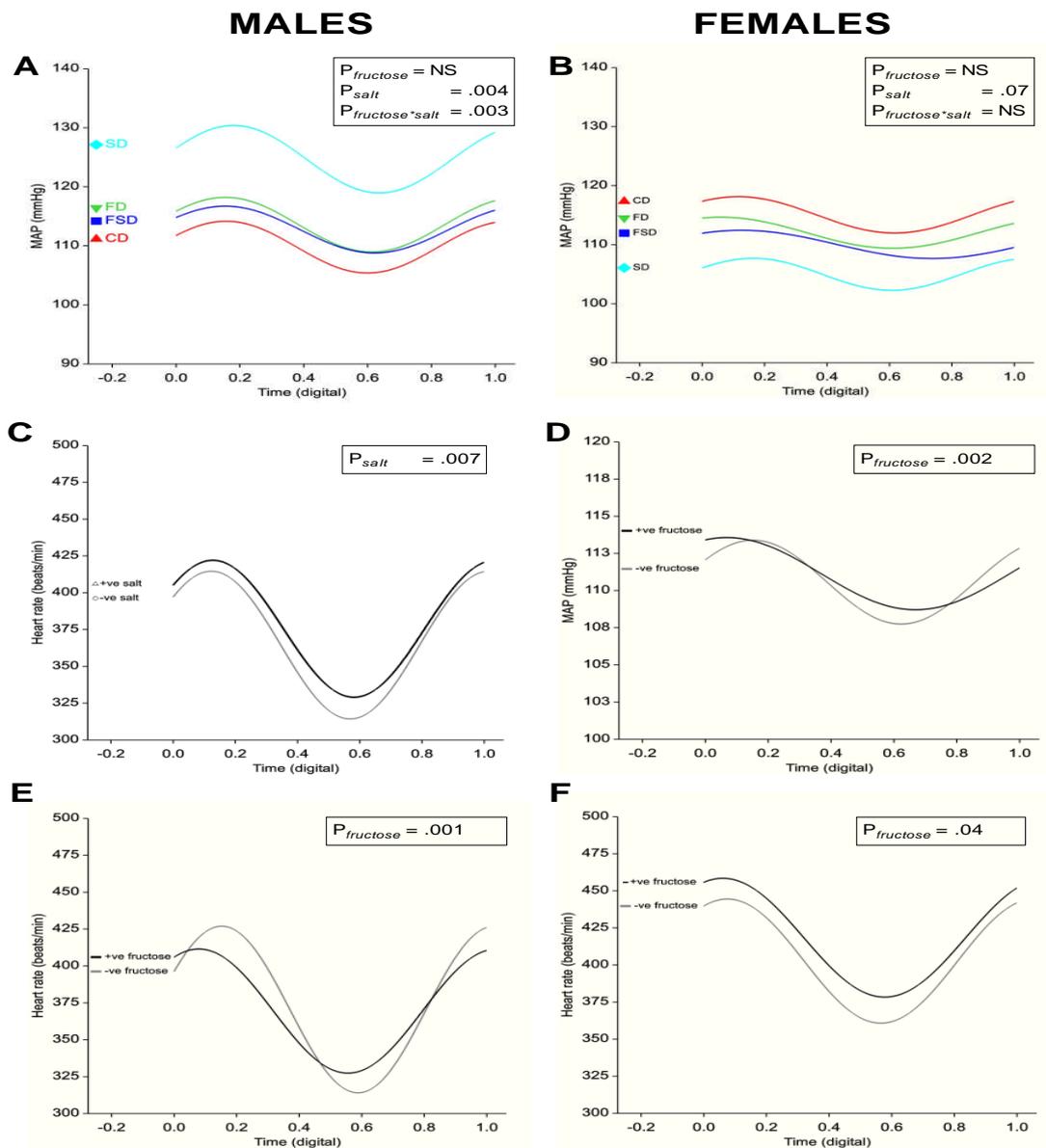
In addition to the rise in basal ambulatory blood pressures observed in male SD offspring, there was a similar interactive effect on the circadian variation (non-dipping) in MAP. This reflects higher values for all 3 variables (systolic, diastolic and MAP) in SD offspring compared to all other groups (Figure 26A and Figure 24A,B,C). Further Fourier analysis indicated that the male offspring of salt-fed dams had significantly higher set-points for heart rate and this effect was greatest during the light (or resting) period; e.g. on average, the set point for circadian variation in heart rate was  $375 \pm 0.30$  beats/min in SD and FSD in comparison to  $364 \pm 0.30$  beats/min in CD and FD ( $P < 0.007$ ; Figure 25C). For the male offspring of dams that received fructose, the amplitude ( $b$ , in the Fourier equation) of the circadian oscillation in heart rate was less relative to those offspring from dams that did not receive fructose, being on average  $42.035$  beats/min in FD and FSD in comparison to  $56.4 \pm 0.35$  beats/min in CD and SD ( $P < 0.001$ ; Figure 25E). There was no effect of prenatal diet on the oscillation wavelength ( $w$ ) of pressure or heart rate.

Similarly to the decrease in diastolic blood pressure and MAP in female offspring exposed to maternal salt diets, there was a tendency in circadian variation in MAP in SD and FSD offspring ( $P = 0.07$ ). Further Fourier analysis indicated that the effect size i.e. the amplitude ( $b$ -coefficient from the equation) around the set-point for MAP was less, on average  $2.42 \pm 0.09$  mmHg in FD and FSD in comparison to  $2.81 \pm 0.09$  mmHg in CD and SD ( $P < 0.002$ ; Figure 25D). Average heart rate of female offspring from fructose-fed dams was significantly elevated relative to unexposed control offspring, being on average  $402 \pm 6.2$  mmHg in CD and

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SD in comparison to  $416 \pm 6.2$  mmHg in FD and FSD ( $P=0.04$ ) but the circadian variation was similar (Figure 25F). There was no effect of prenatal diet on the oscillation wavelength ( $w$ ) of pressure or heart rate.

**Figure 25. Fourier analysis of the circadian variation in mean arterial pressure (MAP; A,B,D) and heart rate (C,E,F) in 14 week old male and female offspring**



**Figure 25.** Fourier analysis of the circadian variation in mean arterial pressure (MAP; A,B,D) and heart rate (C,E,F) in 14 week old male and female offspring (from radio telemetry signals) from dams fed 1) control diet and water *ad libitum* (CD, n=6 males/females), 2) control diet and 10% fructose in water *ad libitum* (FD, n=5 males/females), 3) 4% salt diet and water *ad libitum* (SD, n=5 males/females) and 4) 4% salt diet and 10% fructose in water *ad libitum* (FSD, n=5 males/females). Non-linear regression using a Fourier transform of 2880 recorded data points from each offspring was used to generate a mean curve for each dietary group over a representative resting 24h period (Genstat v13). P values represent significance in amplitude shift, not mean values.

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### 5.3.9 ANXIETY CHALLENGE

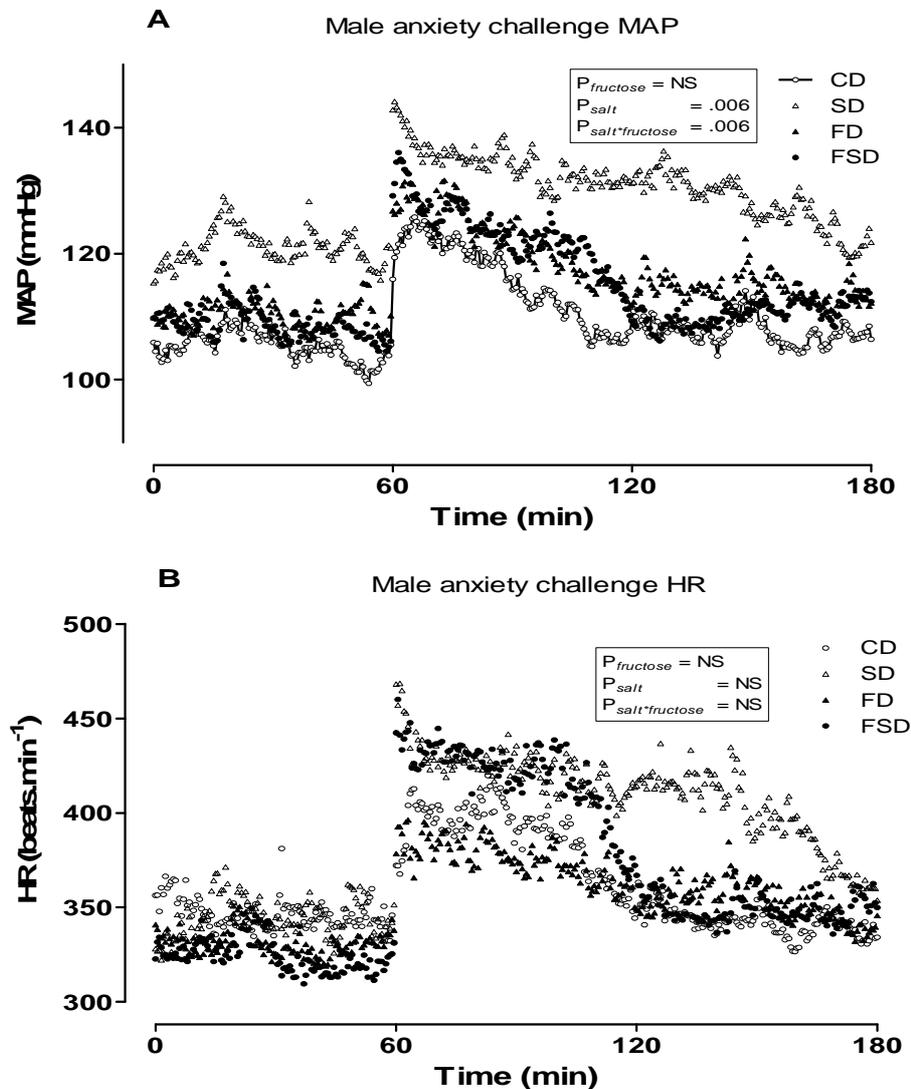
Upon removal of cage mates, single-housed male and female offspring exhibited a robust isolation-induced anxiety response as reflected by increases in mean arterial pressure and heart rate (Figure 26A,B 29A,B). Overall, an effect of salt ( $P>0.006$ ) was observed in male MAP, most likely due to the higher basal blood pressures already present in the salt-fed offspring before initiation of the anxiety challenge. A significant interaction between fructose and salt in their effects on male MAP was observed ( $P<0.006$ ), reflecting the large increase in SD male MAP without a significant increase in male FSD MAP (Figure 26A). SD male offspring MAP and HR also appeared to display a blunted recovery response to the anxiety challenge (i.e. from the  $\Delta$  response until 180 minutes after cage mates were removed), however this effect did not reach statistical significance (Figure 26A).

Female MAP response to anxiety was not affected by prenatal diet. However, in females, a significant interaction between fructose and salt ( $P>0.03$ ) was observed in heart rate (Figure 27B), reflecting an increased response to the anxiety challenge in FSD offspring when compared to all other groups. Over the 24h anxiety period, blood pressures reduced back to basal levels over time (2-3hr) and were back to pre-anxiety levels 24h later i.e. when cage mates had been returned.

In addition to changes in blood pressure and heart rate observed, when the slopes of the relationship between paired values for BP and HR were analysed, the male, but not female, offspring of salt-fed dams exhibited a significantly steeper relationship: i.e. calculated slopes and confidence interval for male offspring were: CD, 3.26 (Confidence interval, 3.02-3.49);

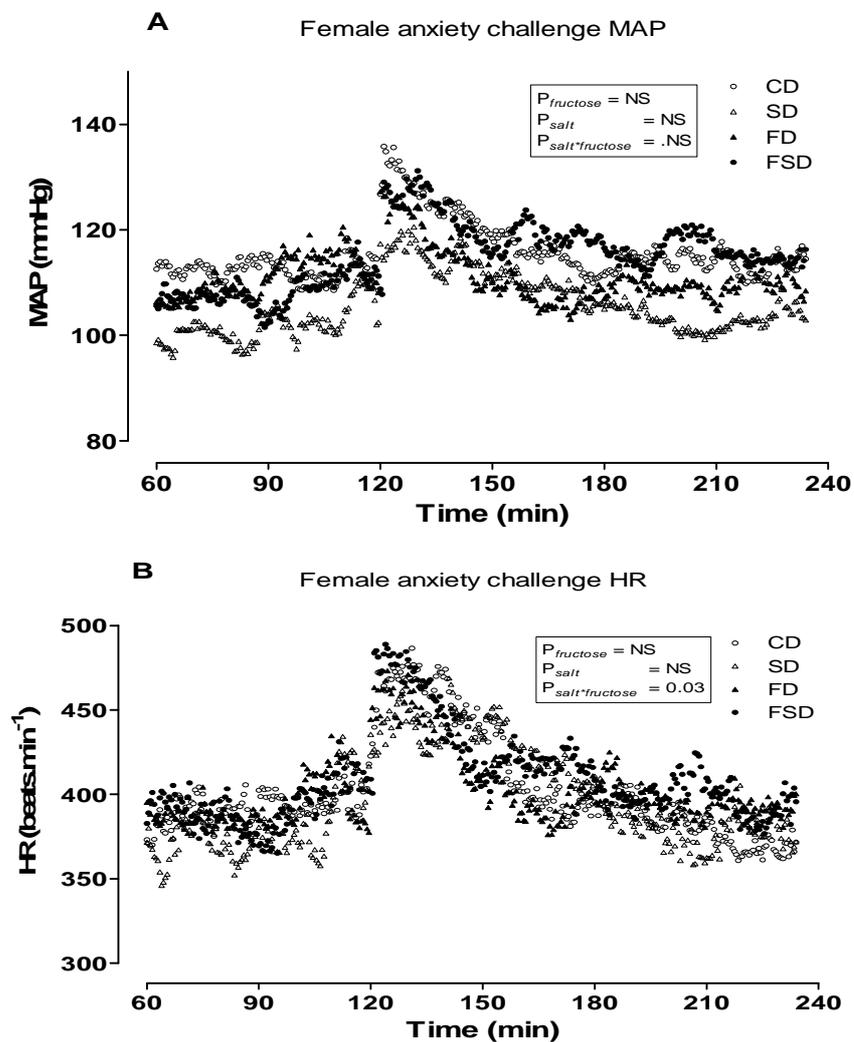
FD, 2.81 (2.63-2.99); SD, 5.36 (5.17-5.55); FSD, 5.38 (5.15-5.60) beats  $\text{min}^{-1}$   $\text{mmHg}^{-1}$  ( $P < 0.001$ ) (Figure 28A,B).

**Figure 26. Effect of acute anxiety challenge and prenatal fructose $\pm$ salt on adult male offspring blood pressures and heart rate**



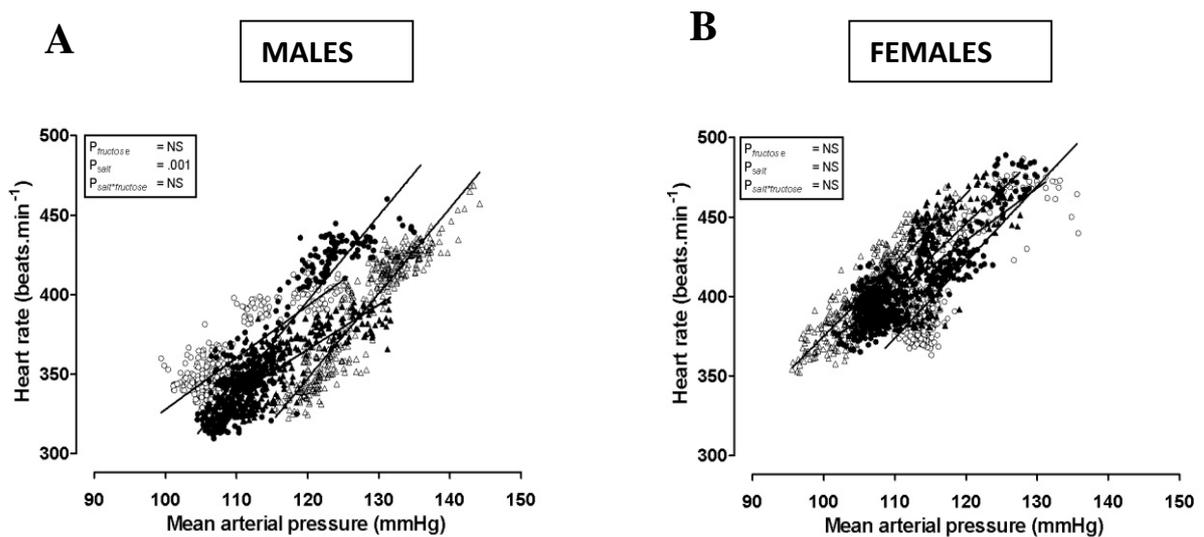
**Figure 26.** Data represents adult female offspring baseline cardiovascular data at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose  $\pm$  salt diet and subsequently maintained on a postnatal control chow diet. CD ( $n=6$ ); FD ( $n=5$ ); SD ( $n=5$ ); FSD ( $n=5$ ). Graph a) represents male MAP response to anxiety; graph b) represents male HR response to anxiety. Blood pressure were recorded continuously (30sec. Intervals) for 4h and individual data means presented. All data analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 27. Effect of acute anxiety challenge and prenatal fructose±salt on adult female offspring blood pressures and heart rate**



**Figure27.** Data represents adult female offspring anxiety cardiovascular data at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Graph a) represents female MAP response to anxiety; graph b) represents female HR response to anxiety. Blood pressure were recorded continuously (30sec. Intervals) for 4h and individual data means presented. All data analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 28.** Mean arterial pressure, heart rate, and slopes of the relationship between mean arterial pressure and heart rate in male and female offspring.



**Figure 28.** Slopes of the relationship between mean arterial pressure and heart rate (A,B) during isolation-induced anxiety in the male and female offspring of dams fed; (○) control diet and water *ad libitum* (n=6), control diet and 10% fructose in water *ad libitum* (n=5), (Δ) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5) for males and females. Data were measured continuously (i.e. sampled at 2 outputs per minute) by telemetry for a 1h baseline period and subsequently for 2hours after removal of their sibling from the cage. Regression lines were generated in Graphpad Prism 5.0.

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### 5.3.10 L-NAME CHALLENGE

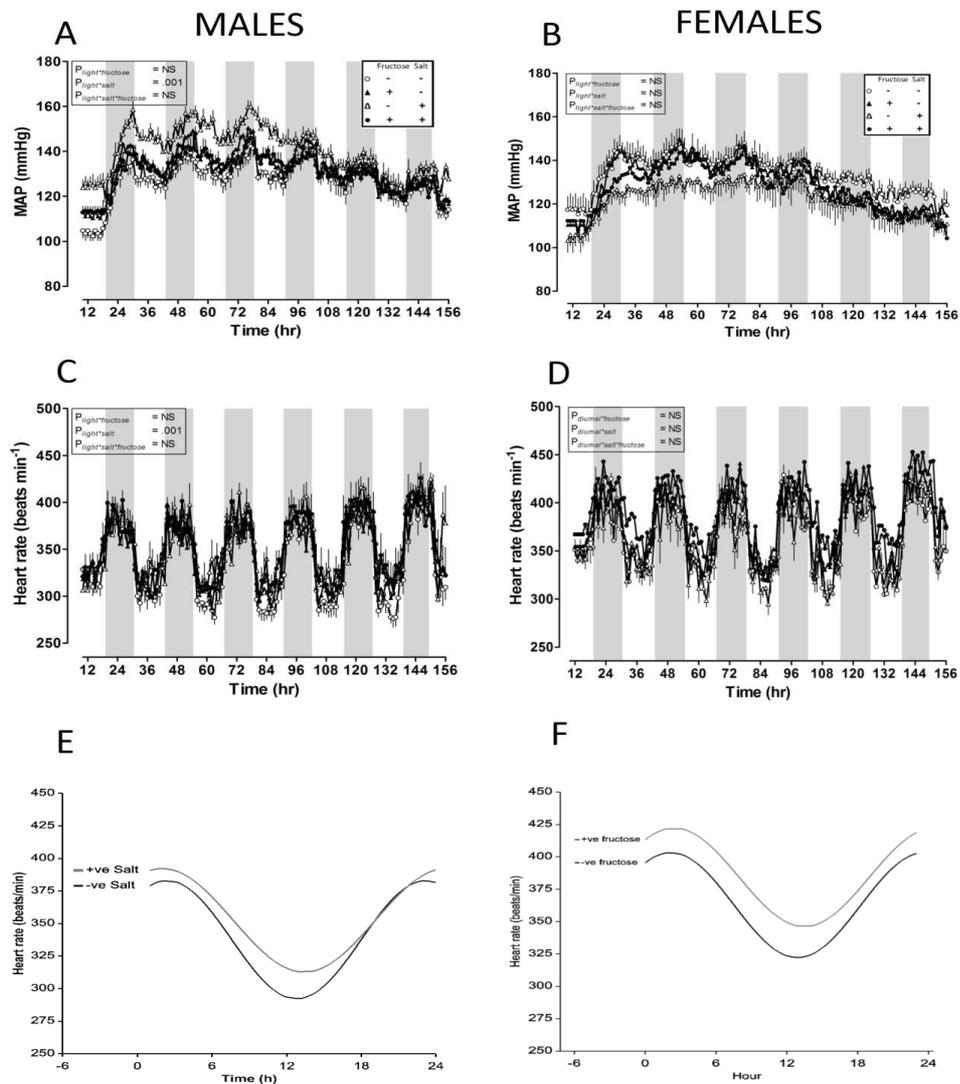
Adult rats were found to drink on average 27ml day<sup>-1</sup> (Table 14), with no effect of prenatal dietary group. Therefore during the challenge the rats were consuming on average 4.12 mg L-NAME day<sup>-1</sup>. Blood pressure rose significantly from baseline in all groups approximately 8h after the L-NAME was made available in the drinking water (i.e. when rats start to drink fluid). During nitric oxide synthase inhibition, diurnal mean arterial pressure increased significantly in both sexes of all dietary groups (Figure 29A,B), with the magnitude of change being similar between all groups and between male and females, being on average a maximal change of 43.3±2.6 mmHg (pooled estimate of delta max change in pressure). On average, the circadian set point for heart rate during the LNAME challenge was higher in salt-exposed male offspring and in fructose exposed female offspring relative to those unexposed to each (P<0.001; Figure 29 E,F).

Overall, during the LNAME challenge, diurnal heart rate was higher in all groups during the night time active period vs. during the day resting period i.e. pooled estimate of ~320 beat/min vs. 302 beats/min. Prenatally salt-exposed male, but not female, offspring demonstrated less variation in diurnal heart rate change (P<0.001). Additionally, there was no decrease in diurnal heart rate observed alongside the increase in blood pressure elicited by L-NAME treatment. However, heart rate was shown to increase with duration of L-NAME treatment in both males and females (Figure 29C,D).  $\beta$ -coefficient (i.e. mean amplitude of deviation from the set-point) from Fourier analysis increased (F=4.6;  $P_{\text{time}}<0.001$ ) with time

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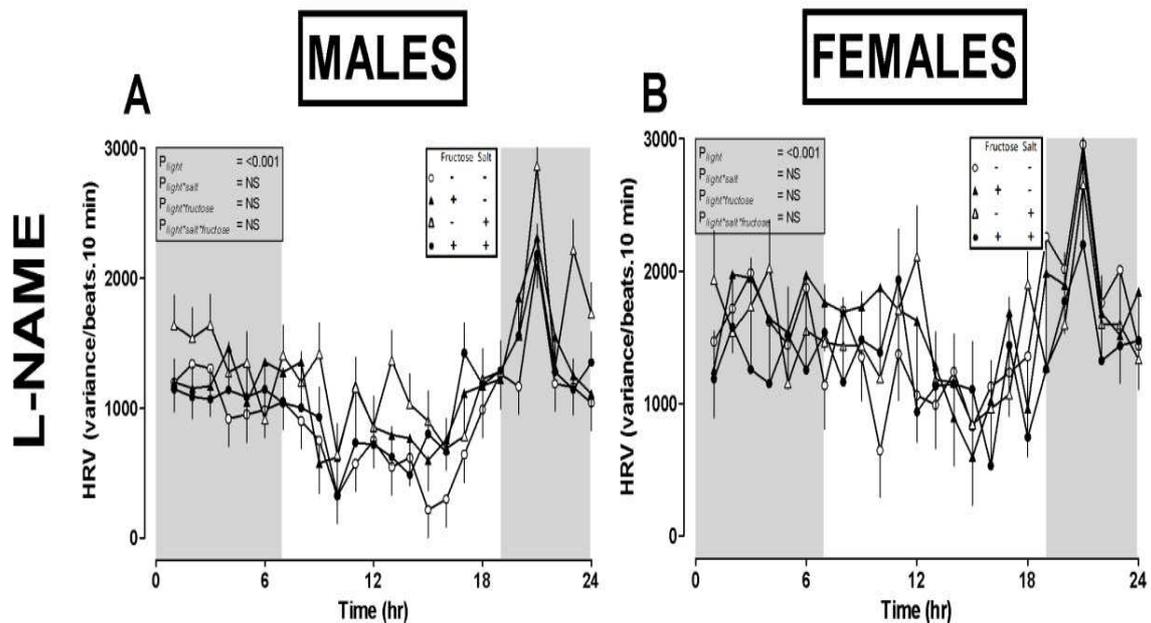
from  $37.1 \pm 3.1$  [day 1-2] to  $50.1 \pm 3.0$  beats.min<sup>-1</sup> [day 5-6] for males and females alike. The set-point around which heart rate oscillated was higher in male, but not female, offspring of salt-fed dams ( $352$  vs.  $337 \pm 2.1$  beats.min<sup>-1</sup>;  $F=78$ ,) ( $P<0.001$ ) (Figure 29E) and higher in female, but not male, offspring of fructose-fed dams ( $384$  vs.  $362 \pm 2.1$  beats.min) (Figure 29F). The circadian pattern of heart rate variability observed under control conditions was also unaffected by L-NAME treatment (Figure 30A,B). Blood pressure was observed returning to basal levels over the seven day 'washout' period subsequent to cessation of L-NAME treatment.

**Figure 29.** Effect of acute L-NAME administration and prenatal fructose±salt on the adult male offspring diurnal blood pressures and heart rate



**Figure 29.** Mean arterial pressure (A,B), heart rate (C,D) and Fourier curves (E,F) for circadian variation in heart rate in response to L-NAME in the drinking water (150 $\mu$ g ml<sup>-1</sup>) in the male and female offspring of dams fed; (○) control diet and water *ad libitum* (n=6), control diet and 10% fructose in water *ad libitum* (n=5), (△) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5) for males and females. Data were measured intermittently (for 30secs every 15mins for 7 days) by telemetry and hourly means calculated as a summary measure of the cardiovascular response. Data were analysed within sex by General Linear Mixed Model (Genstat v13). NS, non-significant.

**Figure 30. Diurnal heart rate variability during L-NAME postnatal challenge in male and female offspring from dams fed fructose and/or salt.**

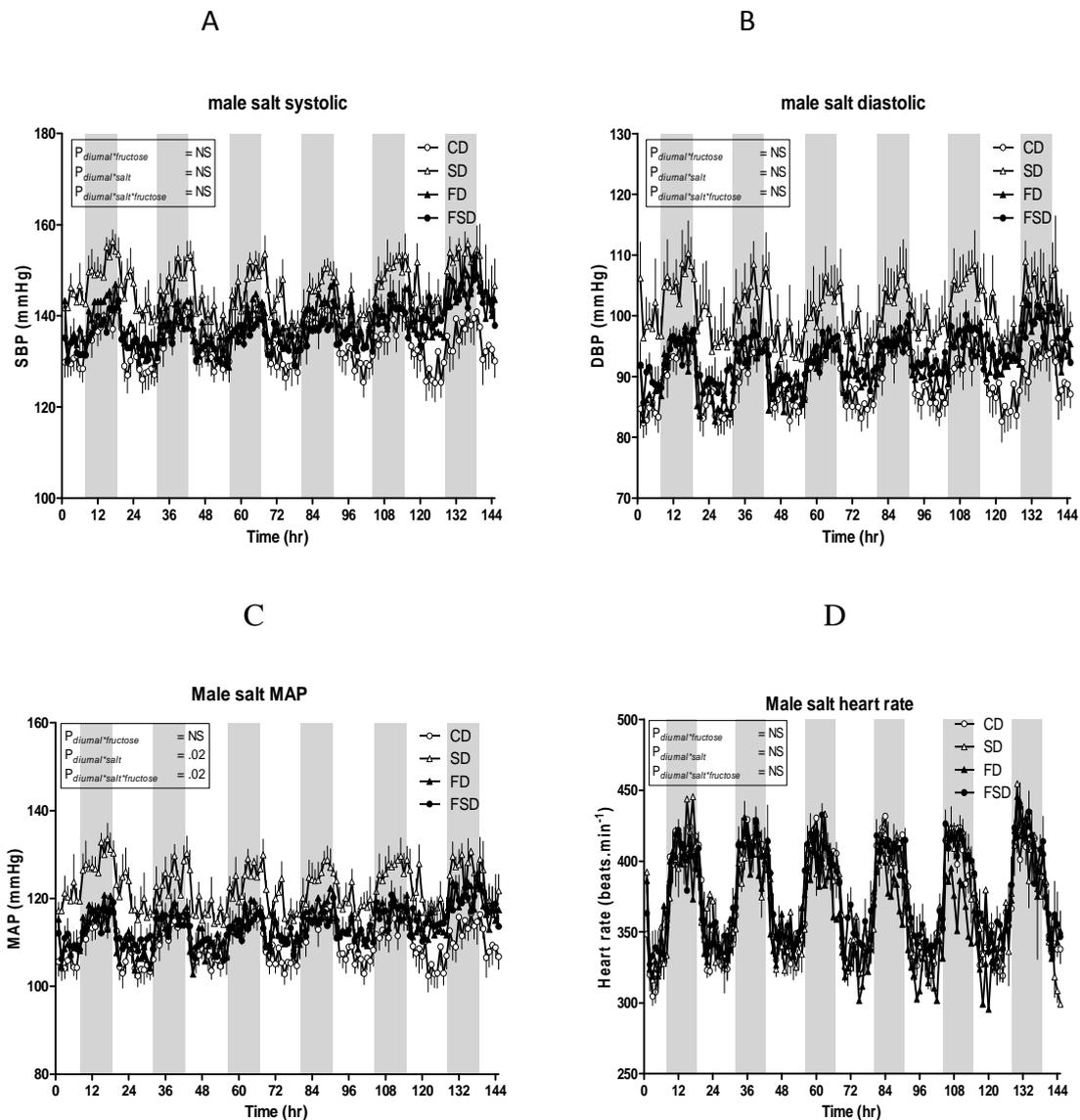


**Figure 30.** Heart rate variability in male and female offspring from dams fed; (○) control diet and water *ad libitum* (n=6), control diet and 10% fructose in water *ad libitum* (n=5), (△) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5, for males and female offspring). Heart rate was derived from the radio telemetric pressure pulse and recorded intermittently (for 30secs every 15mins for the duration [7 days] of each challenge). Heart rate variability (HRV) was calculated as the variance ( $SD^2$ ) in heart rate for each hour of recording. Data were highly positively skewed and were therefore analysed by General Linear Mixed Model with a gamma error distribution and logarithm-link function; back-transformed predicted means are presented (Genstat v13).

### 5.3.11 SALT CHALLENGE

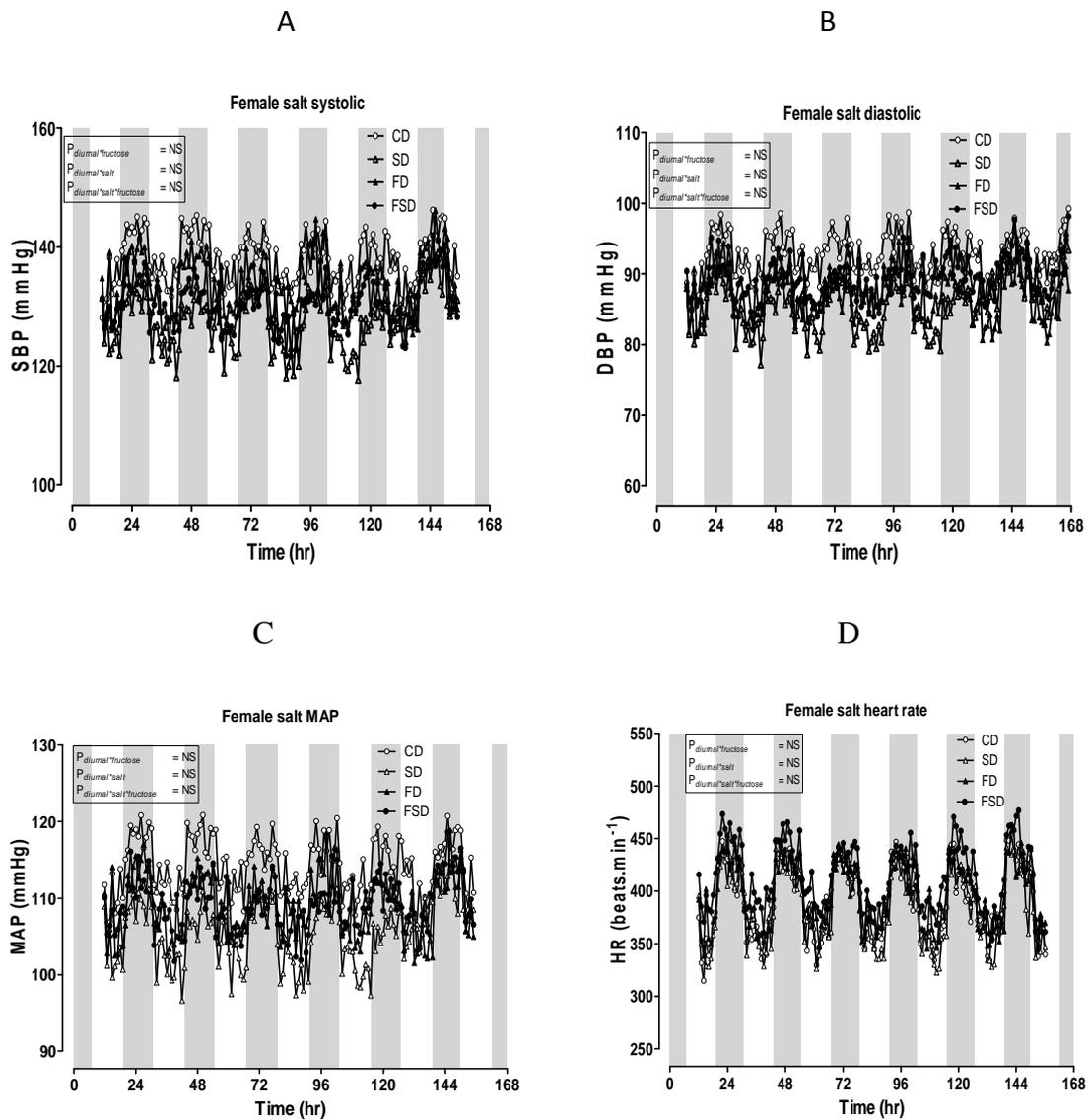
There was no effect of acute salt feeding on offspring basal cardiovascular measures in any group (data not shown). There was no effect of prenatal diet on blunting of circadian rhythmicity in systolic, diastolic or heart rate in males (Figure 31A,B,D) or females (Figure 32A,B,C,D) when fed salt as adults. However, an interaction between fructose and salt was observed on blunting of circadian rhythmicity in male MAP only (Figure 31C ( $P < 0.02$ )). In summary, acute salt feeding for 7 days had little apparent pressor effect in that average systolic and diastolic pressure and heart rate of male offspring remained similar to pre salt-treatment levels, irrespective of the prenatal diet (Figure 31A, B, D). In female offspring, no differences were observed in cardiovascular response to acute salt feeding across all groups (Figure 32A,B,C and D).

**Figure 31. Effect of acute salt feeding and prenatal fructose±salt on the adult male offspring diurnal blood pressures and heart rate**



**Figure31.** Data represents adult male offspring cardiovascular data response to acute salt feeding. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Salt was provided in food (4% inclusion), on average males at  $27.5g.day^{-1}$ . Graph a) represents male systolic pressure (SBP) response to salt, graph b) represents male diastolic (DBP) response to salt, graph c) represents male mean arterial pressure response to salt and graph d) represents male heart rate response to salt. Blood pressure was recorded at 15min intervals for 7 days and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*diurnal\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 32. Effect of acute salt feeding and prenatal fructose±salt on the adult female offspring diurnal blood pressures and heart rate**



**Figure 32.** Data represents adult female offspring cardiovascular data response to acute salt feeding. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Salt was provided in food (4% inclusion), on average females ate 18.8g.day<sup>-1</sup>. Graph a) represents female systolic pressure (SBP) response to salt, graph b) represents female diastolic (DBP) response to salt, graph c) represents female mean arterial pressure response to salt and graph d) represents female heart rate response to salt. Blood pressure was recorded at 15min intervals for 7 days and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*diurnal\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

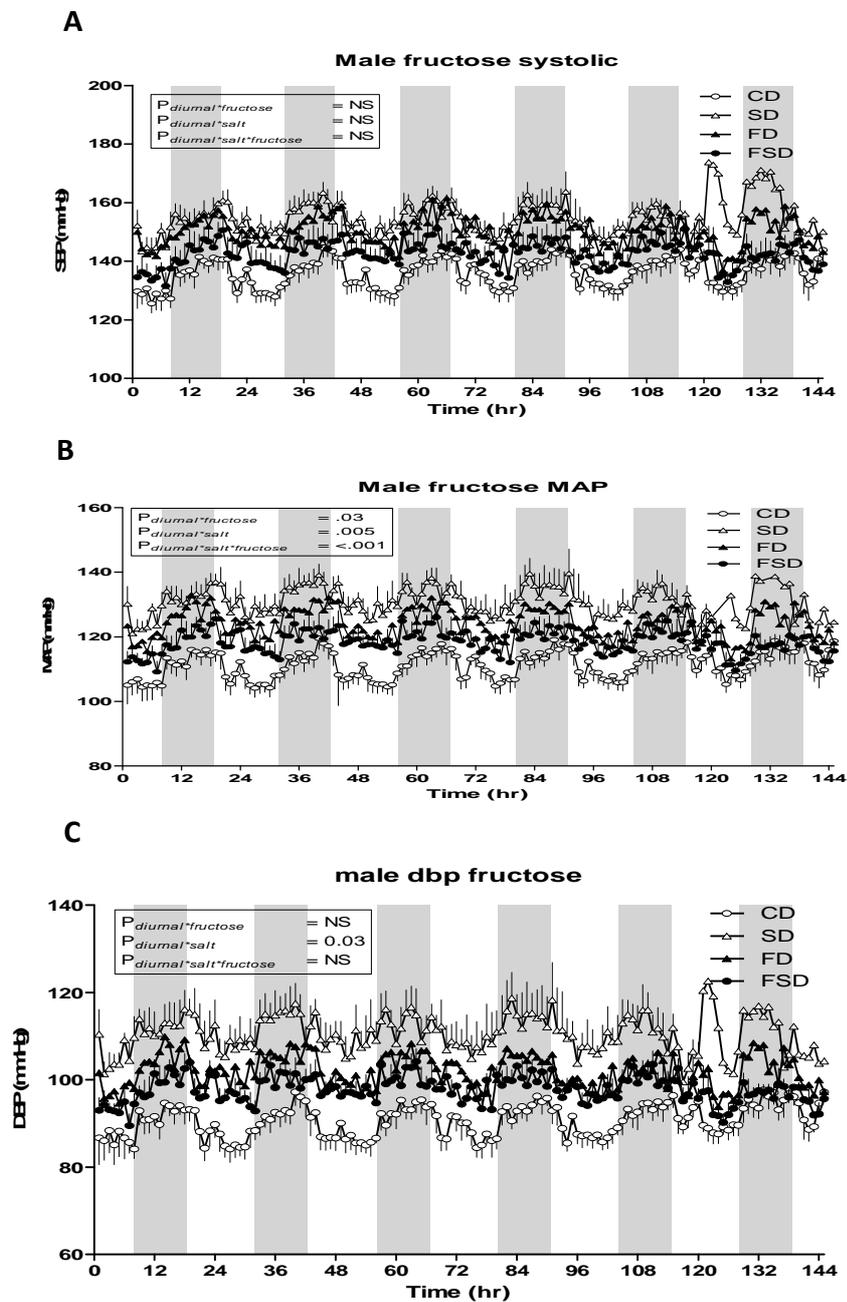
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### 5.3.12 FRUCTOSE SENSITIVITY CHALLENGE

In males, the change in blunting of circadian rhythmicity (non-dipping) diastolic blood pressure between day and night was significantly blunted in prenatal salt-exposed rats when offered fructose in the drinking water ( $P<0.03$ ), being largely due to a relatively increased pressure during the day (or resting) period (Figure 33C). In addition, in males exposed to prenatal fructose, MAP significantly increased when exposed to postnatal fructose ( $P<0.03$ ). Furthermore, a significant interaction of fructose and salt was also observed ( $P<0.001$ ), reflecting a larger increase in male MAP SD offspring in comparison to all other groups (Figure 33B). Circadian blunting of heart rate in males was also influenced by exposure to prenatal salt-diet ( $P=0.06$ ; Figure 34A). There was also an interaction of fructose and salt ( $P=0.023$ ) with regard to the change in pulse pressure when fed postnatal fructose i.e. the diurnal change in pulse pressure became much greater in FD offspring only, without any change in FSD (Figure 34B).

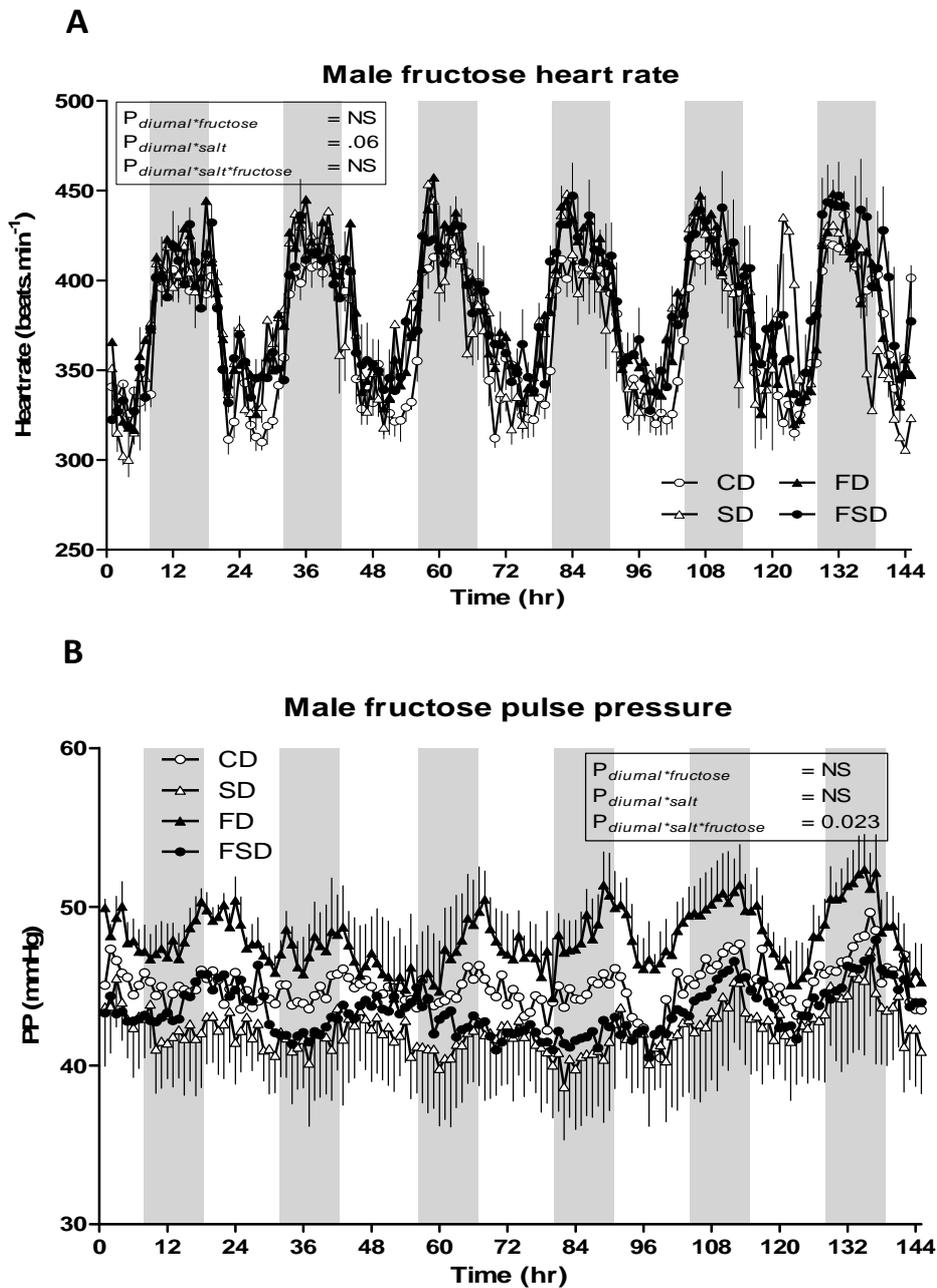
For female adult offspring the change in systolic, diastolic, MAP and heart rate with acute fructose feeding was not affected by prenatal diet (Figure 36A,B,C). However, in similarity to male offspring, a significant interaction of fructose and salt was observed ( $P<0.009$ ) with regard to pulse pressure, i.e. FD offspring displayed a greater circadian variation in pulse pressure with respect to all other groups (Figure 36B).

**Figure 33. Effect of acute fructose feeding and prenatal fructose±salt on the adult male offspring diurnal blood pressures**



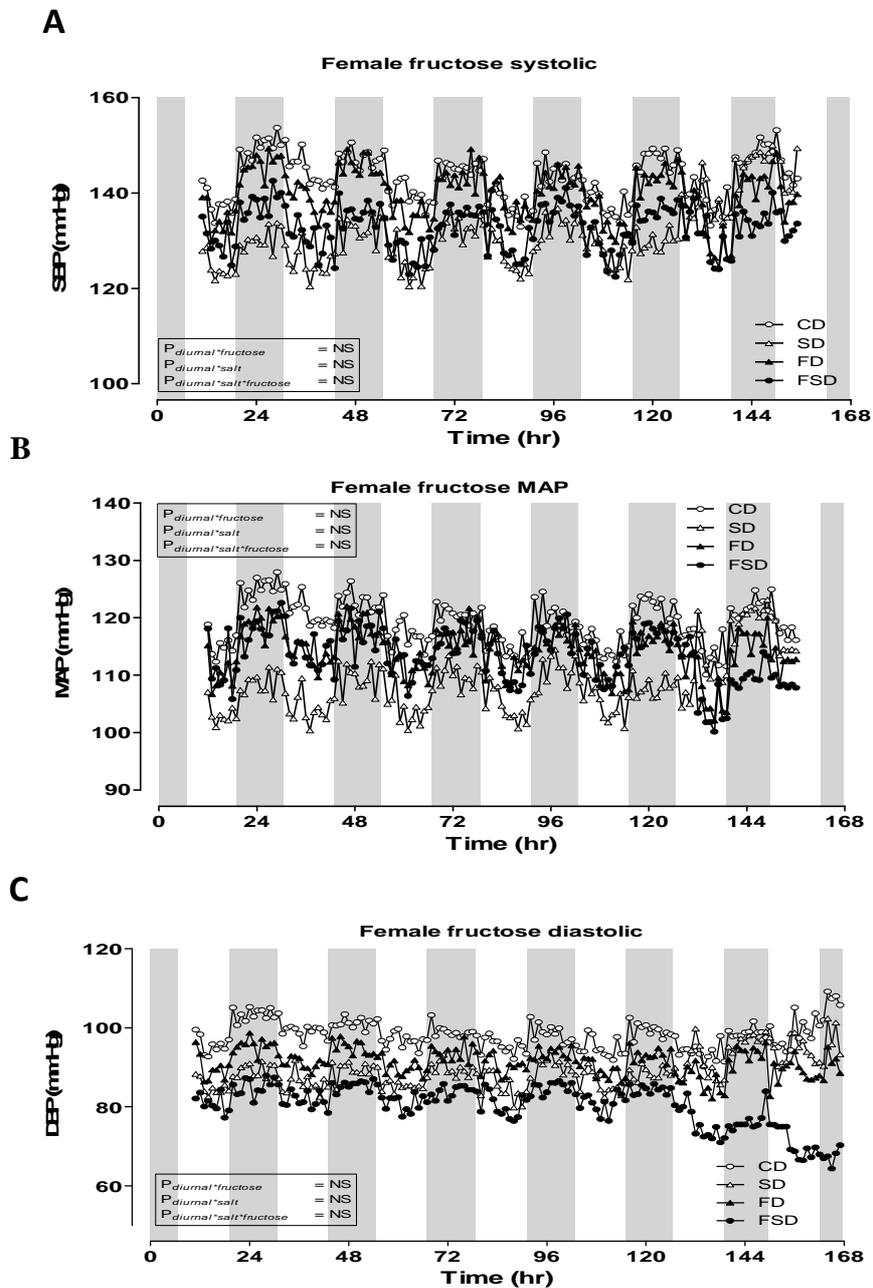
**Figure 33.** Data represents adult male offspring cardiovascular data response to acute fructose feeding at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Fructose was provided in water (10% inclusion). When fructose was added to water, on average males increased intake to 20-40ml.day<sup>-1</sup>. Graph a) represents male systolic pressure (SBP) response to fructose, graph b) represents male diastolic (DBP) response to fructose, graph c) represents male mean arterial pressure response to fructose. Blood pressure was recorded at 15min intervals for 7 days and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*diurnal\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 34. Effect of acute fructose feeding and prenatal fructose±salt on the adult male offspring diurnal heart rate and pulse pressure**



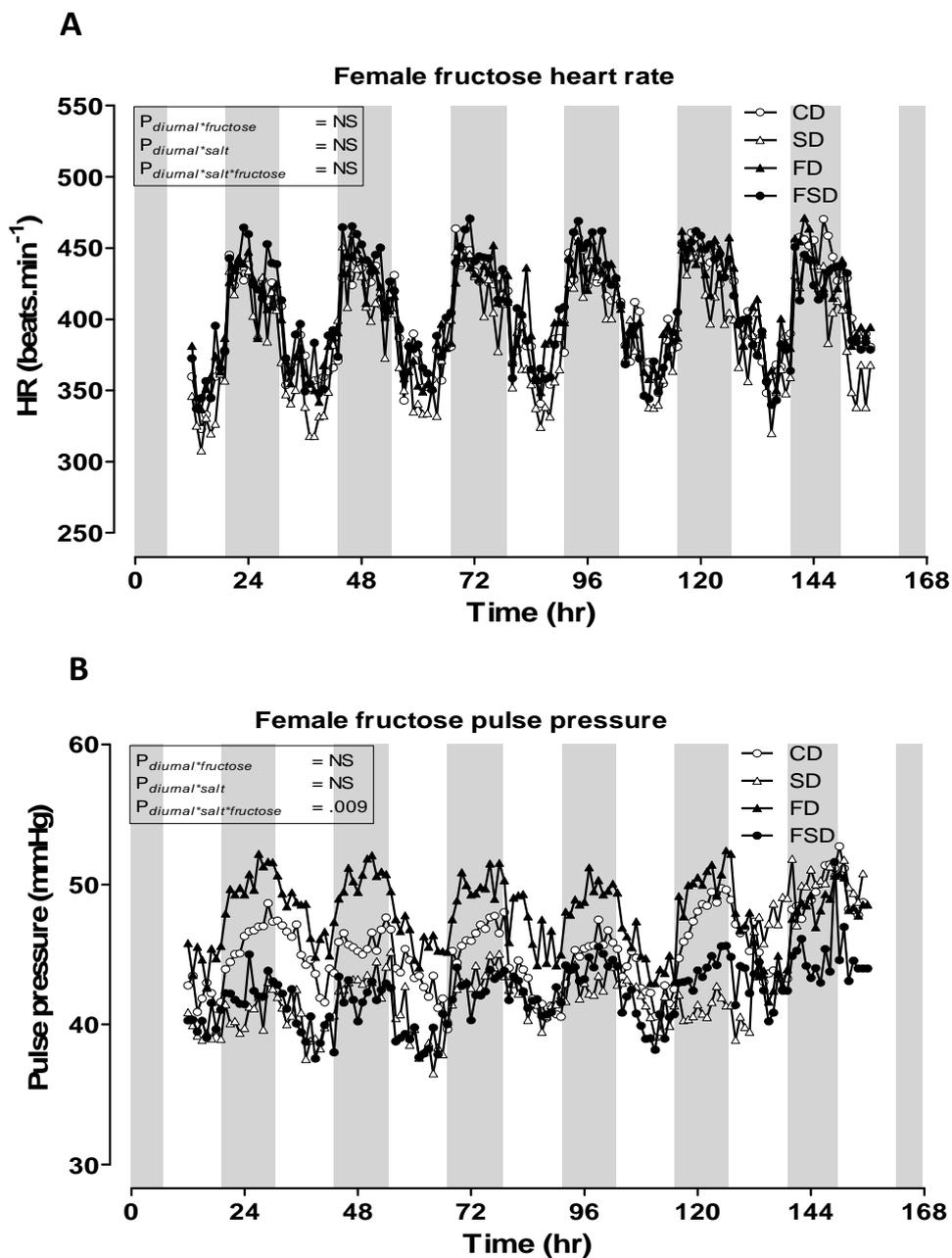
**Figure34.** Data represents adult male offspring cardiovascular data response to acute fructose feeding at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Fructose was provided in water (10% inclusion). When fructose was added to water, on average males increased intake to 20-40ml.day<sup>-1</sup>. Graph a) represents male heart rate (HR) response to fructose; graph b) represents male pulse pressure (PP) response to fructose. Blood pressure was recorded at 15min intervals for 7 days and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*diurnal\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 35. Effect of acute fructose feeding and prenatal fructose±salt on the adult female offspring diurnal blood pressures**



**Figure 35.** Data represents adult female offspring cardiovascular data response to acute fructose feeding at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose  $\pm$  salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Fructose was provided in water (10% inclusion). When fructose was added to water, on average females increased intake to 20-40ml.day<sup>-1</sup>. Graph a) represents female systolic pressure (SBP) response to fructose, graph b) represents female diastolic (DBP) response to fructose, graph c) represents female mean arterial pressure response to fructose. Blood pressure was recorded at 15min intervals for 7 days and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*diurnal\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

**Figure 36. Effect of acute fructose feeding and prenatal fructose±salt on the adult female offspring diurnal heart rate and pulse pressure**

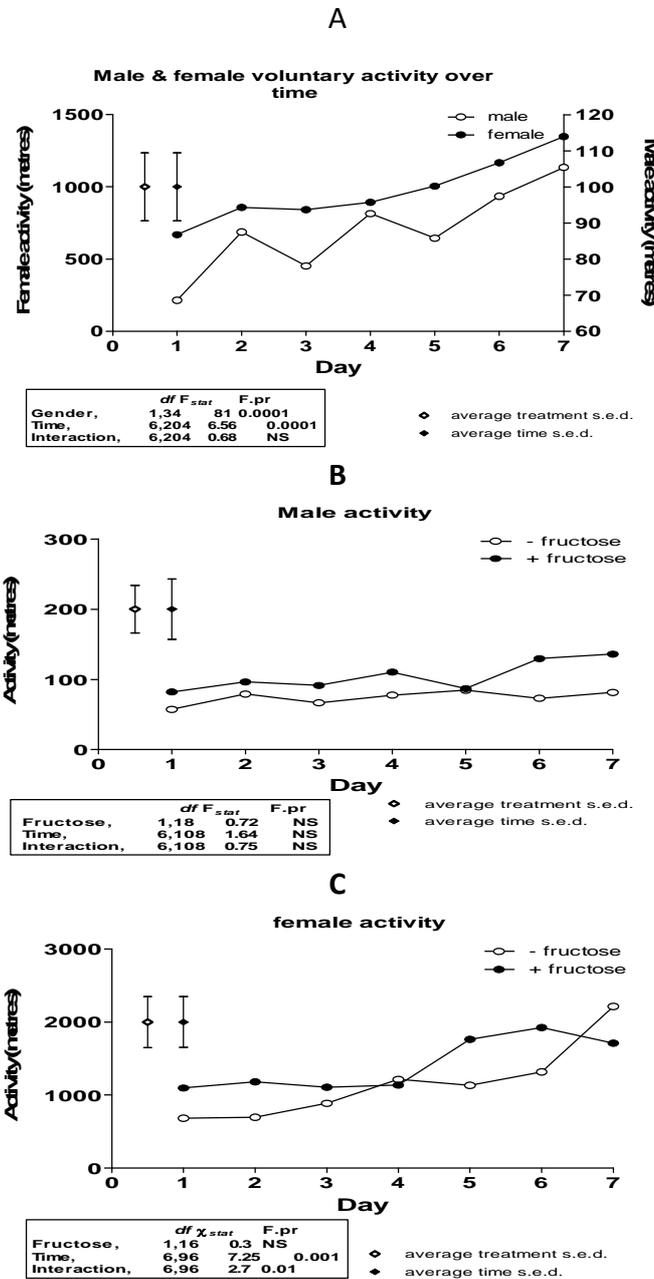


**Figure 36.** Data represents adult female offspring cardiovascular data response to acute fructose feeding at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Fructose was provided in water (10% inclusion). When fructose was added to water, on average females increased intake to 20-40ml.day<sup>-1</sup>. Graph a) represents female heart rate (HR) response to fructose, graph b) represents female pulse pressure (PP) response to fructose. Blood pressure was recorded at 15min intervals for 7 days and data compressed to hourly means. All data analysed as a 2x2 factorial design with fructose\*salt\*diurnal\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK).

### 5.3.13 VOLUNTARY ACTIVITY DATA

As expected, Blood pressures and heart rate increased during activity. Despite no apparent cardiovascular response to prenatal diet during the voluntary exercise period (data not show) differences in total activity between males and females within groups were observed. Total voluntary activity was significantly higher ( $P<0.001$ ) in females when compared to males, being on average  $94\pm 12$ m/day in males in comparison to  $874\pm 109$ m/day in females over the seven day voluntary exercise period. Total activity in males and in females was not different between groups (Figure 37B) but for females, total activity increased significantly with time ( $P<0.001$ ; Figure 37A). A significant interaction of fructose and salt was observed ( $P<0.01$ ) on total activity, reflecting the fact that FD females had higher activity in general as compared to all other groups (Figure 37C).

**Figure 37. Effect of prenatal fructose on the adult male & female offspring voluntary activity level**



**Figure37.** Data represents adult male & female offspring activity data response to voluntary exercise at 14 weeks of age. Offspring weaned from mothers fed a maternal fructose ± salt diet and subsequently maintained on a postnatal control chow diet. CD (n=6); FD (n=5); SD (n=5); FSD (n=5). Adult offspring were individually housed in activity analysis cages for 7 days. Animals had unrestricted access to the running wheels and activity was voluntary. Total activity was measured every 24h for 7 days. Graph a) represents male & female total activity, graph b) represents male activity in response to prenatal fructose and graph c) represents female activity in response to prenatal fructose. All data was analysed as a 2x2 factorial design with fructose\*salt\*gender included (general analysis of variance) using Genstat v11 (VSNi, UK). The data are shown as predicted means with the average standard of error of the difference (s.e.d).

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## 5.4 Discussion

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### *Summary of main findings*

Research into the effects of maternal fructose and salt diet up on the later life cardiovascular health in adult offspring has been previously studied. None however have studied the effect of combining fructose and salt on subsequent adult offspring cardiovascular health. The data in this chapter uniquely considered whether a diet moderately high in fructose and/or salt during pre-gestation, gestation and throughout lactation impacts upon later life cardiovascular health in the offspring. The adverse cardiovascular and renal effects of increased consumption of salt have long been recognized (Bang *et al.*, 1949); whereas, for fructose, the deleterious consequences are the subject of much recent speculation (Johnson *et al.*, 2009a). Mounting evidence suggests, however, that the increased incorporation of fructose in our diets since the development of high fructose corn syrup (HFCS) and sugar-sweetened beverages is impacting upon our cardiorenal and metabolic health (Fung *et al.*, 2009; Axelsen *et al.*, 2010; Jalal *et al.*, 2010). An association between *adult* diet high in salt and/or fructose and disease has long been known (Bang *et al.*, 1949). Furthermore, an association between *fetal* and childhood nutritional environment and later life health and adult disease has been proposed (Barker & Osmond, 1986; Gluckman *et al.*, 2008) with many studies supporting the hypothesis with respect to coronary heart disease (Conen *et al.*, 2010; Crispi *et al.*, 2010; Franks *et al.*, 2010), central obesity (De Lucia Rolfe *et al.*, 2010), Type 2 diabetes (Whincup *et al.*, 2008), and hypertension (Law & Shiell, 1996). Prolonged exposure

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to these risk factors throughout childhood and into early adulthood has been observed to significantly increase the risk of premature mortality (Barker *et al.*, 2005; Franks *et al.*, 2010). It is now clear that fetal, childhood and/or sustained adult unfavorable nutritional environments are associated with risk of adult non-communicable disease. Through the use of animal studies, specific mechanistic aspects of variation in the pattern of nutrients and periods of nutritional exposure that may developmentally induce an adverse phenotype can be elucidated. However, the developmental trajectory toward a specific adult disease has yet to be clearly defined by previous studies.

***Summary of findings in adult offspring physiology, metabolism and renal function***

- Prenatal diet did not have an effect on water, food intake or weight gain in either sex
- All adult male and female offspring metabolic and plasma profile appears to be unchanged at this stage.
- Adult female offspring from fructose-fed mothers had relatively heavier livers.
- Adult male offspring from fructose-fed mothers had increased visceral fat deposition, decreased heart size and a trend for heavier livers.
- Both male and female adult offspring display a significant elevation in plasma osmolality and plasma Na<sup>+</sup> and K<sup>+</sup>.

***Summary of findings in adult offspring cardiovascular health***

- Male offspring from mothers fed a moderately high salt diet were hypertensive at 10-15 weeks of age. In comparison, female maternally salt-fed offspring were not

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hypertensive but showed a trend to be hypotensive and appear to be protected from the effects of a maternal moderately high salt diet at this stage. An interaction of fructose and salt was also observed with FSD male offspring not displaying increased basal blood pressures.

- No effect of prenatal diet was observed on activity levels in adult offspring. However, females were observed to do the greatest activity in comparison to males.

***Summary of findings in adult offspring cardiovascular responses to dietary challenges***

- Adult offspring did not elicit any baseline sensitivity to acute salt feeding. However, a blunting of circadian variation was observed in SD male MAP.
- When adult offspring were fed L-NAME, MAP and heart rate circadian variation in male salt fed offspring was reduced.
- An isolation anxiety challenge elicited no overt blood pressure or heart rate differences between diet. However, a blunted increase in heart rate in response to increased MAP was observed in salt fed offspring when regression slopes were analysed.
- Adult male offspring from fructose-fed dams showed modest elevation in MAP and both male and female offspring of all groups showed a marked difference in circadian pulse pressure levels in response to acute fructose feeding.

One of the main aims of this thesis and work presented in this chapter was to assess any differences in adult offspring metabolism, renal function and subsequent cardiovascular

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health. Given the original hypothesis, we expected an increase in ambulatory blood pressure in adult offspring from mothers fed fructose and salt in addition to an elevation in salt-fed offspring. The hypothesis must be rejected, as in the current study cardiovascular control of the adult offspring was only markedly affected by increased maternal consumption of salt without an effect of salt and fructose combined. Male offspring had an average increase in resting blood pressure of  $\approx 15$ mmHg whereas the female offspring had an average decrease in resting blood pressure of  $\approx 10$ mmHg. This is a quite considerable overall effect size ( $\approx 25$ mmHg) of maternal salt intake considering that the male and female offspring shared the same intrauterine environment and previous research has not observed such a marked difference as presented in this thesis. Of the limited data available, other studies have also shown evidence of programmed hypertension in adult offspring from mothers fed salt diets at varying levels of additional dietary NaCl (Contreras & Oparil, 1992; Contreras *et al.*, 2000; Swenson *et al.*, 2004). Previous studies show conflicting results, with 3-8% of salt feeding resulting in no change and hypertension. In the current study, feeding a 4% salt diet presented itself as the most appropriate for our current experimental model with previous studies having mixed results using a 4% salt diet. Grollman and Grollman showed that 2% NaCl in drinking water was sufficient to induce hypertension in adult offspring (Grollman & Grollman, 1962). Similar studies observed 4% and 1.5% NaCl added to chow did not cause a rise in resting pressure, although King and colleagues did report a hyper-responsiveness to stress in adult female offspring fed prenatal 4% NaCl, whereas a 2.3% NaCl maternal diet (in chow) did not cause hypertension (Myers *et al.*, 1985). Additionally, timing of the prenatal

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NaCl can also affect the progression of hypertension in adult offspring previous work has suggested that a high salt diet (8%) restricted to gestation only (Contreras *et al.*, 2000) or lactation only (Contreras *et al.*, 2000) is sufficient to produce chronically high blood pressure in the offspring. In contrast, feeding rats during the pre-gestational period, through gestation and lactation results in hypertension in male offspring (Alzamendi *et al.*, 2010). Interestingly, the higher blood pressure of these offspring was not related to plasma osmolality (Contreras & Williams, 1989). Whilst predictable effects of a high salt intake (4%) were observed (increased maternal kidney and heart tissue), the main finding of the current study was that a high maternal NaCl diet caused an exaggerated development of hypertension in male offspring and a hypotensive trend in female offspring without an interaction of fructose and salt.

There is only limited literature on the effects of combining fructose and salt. Catena and colleagues showed an association between the amount of sodium in the diet and severity of fructose induced hypertension. Systolic pressure was observed between normal and high-salt fed rats, without an increase in the low-salt group. They postulated the effect was due to a failure to decrease renal insulin receptors in rats fed fructose and high salt (Catena *et al.*, 2003). Further studies feeding 4% salt and 10% fructose in water did not show increased blood pressure more than just feeding fructose alone (Vasdev *et al.*, 2007). Given the limited literature, none have considered the potential effects on the resultant offspring.

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The work carried out in this thesis has therefore lead us to reject the main hypothesis *“Pregnant Spague Dawley rats consuming excess fructose and salt will transmit a hypertensive phenotype to the male and female offspring as adults”*. Only male offspring from salt-fed dams displaying an average increase in resting blood pressure of  $\approx 15$ mmHg and female SD offspring displaying an average decrease in resting blood pressure of  $\approx 10$ mmHg without an increase in FSD male offspring and similar decreases observed in FSD females, shown as a significant interaction of salt and fructose and their effects on MAP, systolic and diastolic cardiovascular measures. This reflects the significant increases observed in SD male blood pressure variables in comparison to all other groups. While we show distinct effects on litter size and fetal growth in this group (FSD), programming of the cardiovascular system was extinguished when fructose is also offered with salt in the maternal diet. In many respects these FSD offspring display an intermediate cardiorenal phenotype e.g. osmolality, placental morphology and renal function are intermediate between groups SD and CD. Considering that maternal salt intake was less in FSD, due to the reduced energy intake from food ( $\sim 15$ - $20\%$ ) then it appears that there is a threshold effect of maternal salt intake with respect to cardiovascular programming of the offspring. Thus, preventing FSD males and female offspring from developing the same phenotype observed in SD offspring.

Whilst the levels of, blood pressures of the adult male and female offspring in the current study were only influenced by maternal salt consumption, an effect of maternal fructose consumption on cardiovascular status cannot be ruled out. Late gestation growth

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retardation was greatest in those offspring exposed to a maternal salt and fructose diet and 'catch-up growth' was similarly increased in offspring from dams fed fructose alone; both these groups exhibited an 'intermediate' phenotype i.e. blood pressure in male and female offspring tended to be higher and lower, respectively, relative to controls but the difference was not statistically significant unlike the SD offspring. Furthermore, subtle delayed effects on circadian oscillation in pressure and heart rate were noted. I.e. in both male and female offspring of fructose-fed dams, the amplitude of variation in heart rate within the light: dark cycle was less and, in females only, a similar effect was observed for pressure. There were also some effects on pulse pressure observed in the fructose challenge indicative of altered fructose sensitivity. This finding is intriguing considering the temporal separation in exposure they received; none had consumed any fructose since they were weaned at 3 weeks of age, yet non-dipping pressure was evident. A number of studies have reported a pressor effect of fructose either acutely (using high doses [66% of total energy intake (Hwang *et al.*, 1987)]) or chronically (using lower doses (Jalal *et al.*, 2010)) and others have reported no effect on blood pressure (D'Angelo *et al.*, 2005; Axelsen *et al.*, 2010). A recent study suggests an altered pattern of prostanoid release may be a contributing factor of fructose-induced hypertension, showing fructose-fed rats (10% w/v) at different time points (4,9,15 & 27 weeks) and effects on prostanoid release and thromboxane release. They reported fructose feeding in the rat decreases production of prostanoid (at 9, 15 & 22 weeks) and an increase in thromboxane at 9 weeks. It was postulated that the thromboxane was responsible for an increase in total peripheral resistance (Puyo *et al.*, 2009). Indeed, the development of

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increased vascular tone in the fructose-fed rat can also be due to a number of vasoconstrictor and anti-natriuretic pathways and the subsequent reduction in activity of vasodilatory agents such as NO, resulting in endothelial dysfunction. In the current study uric acid levels observed in the mother were independent of the fetus and adult offspring at this stage offspring are relatively protected from increased maternal uric acid concentrations and this seems unlikely to play a role. Likewise, fructosamine, an indicator of fructose-induced protein glycosylation (Johnson *et al.* 2003) as well as average circulating glucose levels were not different in any of the adult offspring metabolic analysis, suggesting AGE formation also had little influence, with respect to endothelial function in maternally fed fructose offspring. Further mechanisms of fructose induced hypertension include chronic fructose feeding and development of insulin resistance (Reaven, 1991), increased kidney reabsorption enhanced by hyperinsulinemia (Rocchini *et al.*, 1989) and increased sympathetic activity (Reaven *et al.*, 1996) having effects upon endothelial function and subsequent hypertension. The absence of a stimulation of the sympathetic nervous system after acute fructose feeding observed in the current study, due to the lack of blood pressure changes independent of one another, also shown in humans (Vollenweider *et al.*, 1993; Van Gaal *et al.*, 1999) contrasts with numerous reports of increased sympathetic activity in rodents fed high fructose diet (Young *et al.*, 2004). This is likely to be associated with increased adiposity and body fat being a determinant of sympathetic activity seen in rodent models of high fructose feeding (Scherrer *et al.*, 1994). However, whilst giving a mechanistic insight to acute fructose feeding and hypertension in acutely fed animals none of these studies examines the offspring of fructose

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fed animals and falls short of explaining the apparent altered cardiovascular responses and sensitivity to fructose currently observed in maternal fructose-fed offspring. Further cardiovascular challenges proved to show little enhanced effect of maternal diet in females. In males, subtle delayed effects on circadian oscillation in pressure and heart rate were observed when exposed to L-NAME, salt and anxiety challenge, such that the amplitude of variation in heart rate and circadian rhythmicity during the light to dark cycle was less in SD males. Given that SD males have a higher ambulatory blood pressure it would perhaps be expected that cardiovascular variability in response to subsequent dietary challenges in these males would be cardiovascularly less flexible in comparison to non-hypertensive controls. However, during the L-NAME challenge consideration must be given to the differences in individual offspring L-NAME treated water consumption. As discussed (Section, 2.7.4 & 5.5.1), adult offspring were observed to consume on average 27ml day<sup>-1</sup> without an effect of prenatal diet. The previous assessment of water intake would lead us to believe that water intake was consistent across all groups and in both genders, without a significant effect or negative impact on total L-NAME exposure amongst individual or dietary group and subsequent cardiovascular analysis.

Despite maternal diet having little residual effect in male and female adult offspring plasma metabolic profile and urinary excretion, male offspring of fructose-fed dams appeared to retain larger visceral adipose to body weight ratio. Given that glucose is the principal metabolic substrate used by the growing fetus (Hay *et al.*, 1978), it is not known whether

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fructose could replace glucose in the maternal diet without an effect on fetal growth and development. There is evidence to suggest fructose is not metabolised as efficiently during pregnancy as glucose (Ballard & Oliver, 1964), and because of the unregulated metabolic pathway of fructose, fructose causes alterations in lipid metabolism and may be causing further alterations in offspring fat deposition. Fergusson and Koski showed during pregnancy, fructose was inferior to glucose when fed as the sole carbohydrate source. Dams fed 4% glucose had heavier placentas, higher mating rates, maternal and fetal weight gain when compared to 4% fructose fed dams. Similar to results observed in FSD fed dams in the current study, the fructose dams in the study by Fergusson and Koski also reduced energy intake from food which ultimately affected total glucose intake (Fergusson & Koski, 1990). An altered carbohydrate ratio (fructose to glucose) during pregnancy may contribute to the trend observed in fructose fed offspring and the apparent differences in regional fat distribution. Given an excessive supply of fructose *in utero*, offspring may be utilising more fructose than glucose in this instance, particularly when the liver reaches full maturation, promoting an increased deposition of visceral fat where the effects of fructose have been shown to alter fat deposition in adult rats in the current study. Another aim of this chapter was to analyse differences in metabolic status and the possible vertical transmission of NAFLD as observed in dams fed fructose. Interestingly, McCurdy and colleagues showed a maternal high fat diet led to overt NAFLD, not only in the recipient of the diet, but also in the developing fetus (McCurdy *et al.*, 2009) leading to the suggestion that offspring from mothers consuming a poor-quality diet may be susceptible to NAFLD, regardless of their

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habitual adult diet. Data in this chapter provides some support for this hypothesis in that the male and female offspring of fructose-fed dams retain a larger liver: body weight ratio despite having a similar and relatively 'normal' blood metabolite profile. However, subsequent work on offspring livers has shown no differences in intra-hepatic lipid concentrations between groups but has displayed a trend ( $P=0.08$ ) in a reduced dry liver weight in maternally fed fructose offspring indicating the FD and FSD offspring livers have an increased water content. Further postnatal fructose consumption, as would most likely occur in the 'real world', is envisaged to only exacerbate this metabolic phenotype.

The finding that further observations in both male and female offspring from dams fed salt-diet alone had a  $\approx 50$  Mosmole increase in plasma osmolality is intriguing. Extra-cellular fluid osmolality is usually tightly controlled to  $\pm 3\%$ , but here we are witnessing a 15-20% chronic shift. Renal osmolar clearance is similar between groups suggesting that the kidneys are not retaining more  $\text{Na}^+$ , or other ions *per se*, but that either central sensitivity to ECF osmolality has adjusted, and/or  $\text{Na}^+$  is being retained from sites other than the kidney, such as the gastrointestinal tract and/or other ECF osmolytes are contributing to the effect. For the latter, we have measured many of the important osmolytes (glucose, urea, albumin) and see no specific increase and therefore conclude that either extra renal retention of  $\text{Na}^+$  or an altered set-point for osmolar regulation via:

- Hypo-stimulation of the hypothalamic-posterior pituitary and an overall reduction in circulating ADH and/or down regulation in osmoreceptors.

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- Decreased expression of ADH receptors with a secondary reduction of aquaporins in the collecting duct of the kidney must underpin the effect.

Interestingly, the direction of trend for offspring osmolality parallels that observed in the dam i.e. dams fed salt alone had the greatest increase in plasma osmolality, suggesting an association between maternal ECF electrolyte environment, and male offspring osmolality. Since fetal osmolality tends to reflect maternal osmolality (Ladella *et al.*, 2003b; Boyce *et al.*, 2008), it would be expected that the fetal environment of salt-only fed dams would have an increased osmolality and future work clarifying the effect of this environment on fetal vascular development and osmolar regulation is currently progressing. Previous studies are contradictory with regard to the relationship between maternal and fetal osmolality with studies reporting a relative buffering (Patterson *et al.*, 2010) or paralleled change in maternal/fetal osmolality (Ladella *et al.*, 2003a) and further clarification in the current model is warranted. The intermediate cardiorenal phenotype (*e.g.* osmolality, blood pressure) of the offspring from fructose-salt-fed dams (group FSD), that have relatively reduced energy intake from food *vs.* dams fed salt alone argues for an effect of maternal salt intake *per se* and the concomitant effects it engenders as being the initiating factor important with respect to cardiovascular programming in the current model.

### **Conclusion**

The data suggest that increased maternal intake of sugar sweetened water or of salt has significant effects on the cardiovascular system of the offspring, including altered resting levels of blood pressure but also hypersensitivity to an anxiety-related stimulus (male

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prenatally salt-exposed offspring only) and, in terms of pressor activity, to further fructose intake. The data therefore lend support to the recent cautionary statements regarding increased fructose and/or salt intake in the Western diet.

## 6 General discussion

### *Summary of main findings*

The concept that changes in maternal diet during pregnancy may negatively impact upon the metabolism of her offspring during later life is now widely accepted. Known as the fetal origins hypothesis, it proposes that ‘an adverse fetal environment may lead to permanent physiological and metabolic alterations, establishing a pre-susceptibility to adult disease’. Correlations between maternal malnutrition and poor offspring health have been noted for many years and even today studies find that under or over nutrition during pregnancy predispose offspring to low birth weight and cardiovascular disease. The aim of the work described in this thesis was to further explore the concept of fetal programming and determine whether the previously described phenotype of hypertension in prenatally salt fed animals was exacerbated by, or interacted with, the inclusion of fructose in the diet and to investigate the mechanisms by which hypertension may be programmed *in utero*. The results in this thesis support a number of studies of salt and fructose feeding in rats and humans. The current work also confirms the existence of this previously described phenomenon of fetal programming and demonstrates clearly that an unhealthy diet during pregnancy can cause predisposition to cardiovascular pathologies via maternal nutritional manipulation of metabolic status without any further dietary manipulation in the offspring subsequent to weaning in adult offspring. These data effectively demonstrate that the course of an individual’s development is not only influenced by a mixture of genetic and environmental factors, but also that maternal nutritional status in the form of excessive salt

and or fructose during gestation has physiological and metabolic implications for both mother and offspring. Work in this thesis has shown some novel effects of a moderately high maternal diet in fructose and/or salt. To our knowledge, no other study has explored potential interactions between maternal intake of salt and/or fructose with regard to the basal and stimulated cardiovascular status of the offspring.

***Summary of findings in pre-gestational and pregnant dams***

- Dams consuming fructose prior to and during gestation increased caloric intake from fructose water with a consequential decrease in total energy intake from food. Increases in plasma glucose (without an effect on insulin), triglyceride, non-esterified fatty acids, cholesterol and uric acid were all increased by the consumption of fructose in pre-gestational females.
- Dams receiving salt prior to and during gestation elicited an increase in cardiac and kidney tissue mass. All females consuming 4% salt within their diet also presented a severe increase in plasma osmolality as a consequence of increased plasma Na<sup>+</sup> and K<sup>+</sup>.
- During pregnancy, an effect of fructose was observed on plasma glucose, cholesterol and albumin; all other pre-gestational effects were attenuated with the onset of pregnancy.
- Fructose-fed dams also displayed a significant redistribution of regional fat depots i.e. visceral fat increased whilst gonadal fat decreased. Fructose also increased liver

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weight and intra-hepatic triglyceride levels were also observed to be increased with the consumption of fructose.

#### ***Summary of findings in the fetus at day 20***

- At day 20 of gestation fetal plasma profiles and tissues were relatively unchanged and the fetus appears to be protected at this stage.

#### ***Summary of findings in the fetus and neonate***

- Overall rats increased in weight by 85% from day 20 to term. This increase was less in response to fructose and, to a greater extent, salt. Combining fructose and salt did not reduce growth any more than feeding salt alone.
- At weaning, FSD male and female rats were significantly lighter than all other groups. This reflected a reduction in growth rate in this group during lactation.
- Fructose affected sex ratio. On average, skewing the sex ratio 60:40 in favour of males to females. Dams consuming fructose and salt (FSD) displayed a significant reduction in litter size compared to all other diets.

#### ***Summary of findings in placental phenotype***

- Placentas from dams fed 4% salt showed a reduction in proportion of labyrinthine area and an increase in trophoblast relative to total placental area.
- Placentas from dams fed 10% fructose (FD) showed a reduction in proportion of trophoblast layer and an increase in labyrinthine area relative to total placental area.

***Summary of findings in adult offspring physiology, metabolism and renal function***

- Prenatal diet did not have an effect on water, food intake or weight gain in either sex
- All adult male and female offspring metabolic and plasma profiles appeared to be unchanged at this stage.
- Adult female offspring from fructose-fed mothers had relatively heavier livers.
- Adult male offspring from fructose-fed mothers had increased visceral fat deposition, decreased heart size and a trend for heavier livers.
- Both male and female adult offspring display a significant elevation in plasma osmolality and plasma Na<sup>+</sup> and K<sup>+</sup>.

***Summary of findings in adult offspring cardiovascular health***

- Male offspring from mothers fed a moderately high salt diet were hypertensive at 10-15 weeks of age. In comparison, female maternally salt-fed offspring were not hypertensive but showed a trend to be hypotensive and appear to be protected from the effects of a maternal moderately high salt diet at this stage.
- Combining fructose and salt partially attenuated the effects of salt alone on baseline blood pressures.
- No effect of prenatal diet was observed on activity levels in adult offspring. However, females were observed to do the greatest activity in comparison to males.

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***Summary of findings in adult offspring cardiovascular responses to dietary challenges***

- Adult offspring did not elicit any baseline sensitivity to acute salt feeding. However, a blunting of circadian variation was observed in SD male MAP.
- When adult offspring were fed L-NAME, MAP and heart rate circadian variation in male salt fed offspring was reduced.
- An isolation anxiety challenge elicited no overt blood pressure or heart rate differences between the different dietary groups. However, a blunted increase in heart rate in response to increased MAP was observed in salt fed offspring when regression slopes were analysed.
- In response to acute fructose-feeding adult male offspring from fructose-fed dams showed a modest elevation in MAP and both male and female offspring of all groups showed a marked difference in circadian pulse pressure levels in comparison to baseline.

Since the habitual intake of simple sugars and salt are considerably higher in a Western vs. Paleolithic ancestral diet, this thesis aimed to characterize the immediate and delayed cardiorenal physiological effects that such a pattern of intake may have had on the pregnant dam and her developing and adult offspring. Strengths of the experimental design included the use of a purified diet (developed in association with Harlan), in which the macronutrient content and energy density was identical with only the mineral content being different (i.e. increased inclusion of salt, as NaCl), to avoid confounding by other aspects of the diet that

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might otherwise differ between treatment groups (Warden & Fisler, 2008) and the use of a 2x2 factorial design to increase study power and minimise animal numbers (Kilkenny *et al.*, 2010). The levels of intake of fructose and salt were considered moderate when adjusted for the relatively higher energy intake per unit weight of the rat vs. larger mammals (i.e. allometric scaling). Nevertheless, a 4% salt diet represents a high level of salt inclusion, but for a salt-resistant rat strain like the Sprague-Dawley, is intermediate between previously published reports of low salt intake (0.1%, (Kirksey & Pike, 1962)) and high salt intake (8%, (Porter *et al.*, 2007)). A 10% fructose beverage available *ad libitum* equates energetically to a total percentage energy from fructose of 15-20% for non-pregnant rats and 20-30% for pregnant rats (when the reduction in food intake is accounted for) in this study, much lower than previously fed to rats in their diet to induce hypertension (66% energy, (Hwang *et al.*, 1987)) and not dissimilar to that consumed by the general US population (Johnson *et al.*, 2009b).

The most striking effects observed in this thesis were that maternal intake of excess salt had a considerable sex-specific influence on the resting, ambulatory blood pressure of the offspring; adult male offspring were hypertensive (MAP increase  $\approx$ 15mmHg) with a higher heart rate whereas adult female offspring were relatively hypotensive (MAP decrease  $\approx$ 10mmHg). Salt intake by the dams increased maternal plasma osmolality but, rather surprisingly, was also vertically transmitted to the adult offspring whom, as individuals, remained unexposed to salt diet. Maternal intake of excess fructose recapitulated a

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moderate non-alcohol fatty liver phenotype in the dam and led to larger livers in the unexposed adult offspring, whom also displayed blunted variation in circadian cardiovascular function. In addition to the apparent programming effects on baseline blood pressures, subsequent dietary and stress challenges on adult offspring revealed subtle changes in cardiovascular responses to further exposure in later-life to fructose, salt and stress response. A marked response observed in telemetered rats in response to their cage mate being removed clearly provides evidence that it is stressful to remove cage mate in pair housed or groups. This may be of interest with regards to the future of animals' cardiovascular monitoring of baseline blood pressure variables in similar studies to the current one. Further analysis of baroreflex response revealed differences in salt fed males not females, indicating that heart rate rises more per unit pressure. This reciprocal rise in blood pressure and heart rate could rule out a role of baroreflex sensitivity, as baroreflex innervation would have displayed a negative relationship of heart rate decreasing with an increase in blood pressure, which was not the case in the current study. Nevertheless, the observation that male SD offspring exhibited a greater anxiety response, reflected as a steeper positive relationship between heart rate and blood pressure may suggest elevated sympathetic activity or an altered set-point (independent of pressure set-points) in these animals. In order to test this assertion, however, further specifically designed studies would be required. Furthermore, since the constitutive production of nitric oxide within the vascular endothelium is key for determining baseline arteriolar tone, and increased salt intake has been reported to inhibit endothelial nitric oxide synthase activity (Li *et al.*, 2009),

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we have tested whether maternal salt and/or fructose intake may influence offspring vascular nitric oxide balance by administering nitric-oxide blockade using L-NAME and monitoring cardiovascular responses. Similarly to baseline blood pressures analysis of the data revealed differences in salt fed males not females. L-NAME administration elicited a significant rise in blood pressure in blood pressure and heart rate in both males and females. However, this was not affected by diet and nor did diet affect maximal change in response to L-NAME. Ultradian pattern of heart rate variability was also analysed and was not influenced by anything obvious, except normal daily activity. SD male not female offspring were observed to have a blunted circadian variation during the challenge in MAP and heart rate but like most significant responses observed (e.g.Salt,L-NAME, fructose and anxiety) the changes are likely to reflect the already greatly increased basal blood pressures. Diurnal responses to diet were not measured in basal data, however differences would have been expected and because of the increased blood pressures and apparent non-dipping (as seen in all other analysis) we would conclude that this may be the over-riding factor in the consistent blunted circadian effect across a number of responses in SD male offspring. Therefore, increased blood pressures were not dependent on constitutive NO activity, although regional variations may exist but could not be assessed in the current study. Likewise, maternally salt-fed male offspring displayed a much greater 'non-dipping' pattern in response to L-NAME treatment. The effects upon salt\*diurnal interactions observed in MAP & heart rate are more than likely to be an artefact of the increased basal blood pressure than an overall effect of diet as seen in L-NAME-fed offspring.

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Overall, we feel confident SD offspring are no better or worse at this age during salt loading and therefore, not salt-sensitive (perhaps revealed later in life as they become less able to handle salt). Of interest in terms of programming, as prenatal fructose offspring respond more to acute fructose-feeding, fructose caused a greater cardiovascular pressor effect than salt when fed acutely to adult offspring. For the first time, the study has also provided evidence that increased maternal fructose consumption influences adult offspring cardiorenal function in the absence of direct ingestion of fructose by the offspring; resting blood pressure was unaltered but the circadian oscillation in pressure and heart rate was significantly blunted, reflective of a 'non-dipping' pattern – previously identified as a significant risk factor for later cardiovascular disease (de la Sierra et al., 2009). Previous studies have witnessed an acute pressor effect of high fructose intake per se (66% energy intake (Hwang et al., 1987)) or a chronic pressor effect when offered at lower doses over a longer period of time (Jalal et al., 2010), although others are contradictory, reporting no change (D'Angelo et al., 2005; Axelsen et al., 2010). Thus, it is all the more remarkable that such a temporal (fetal and neonatal only) exposure to fructose reveals significant cardiorenal changes in the unexposed offspring and further experiments to ascertain the effect of longer-term fructose consumption with cardiovascular profiling is clearly warranted.

In addition to changes in cardiovascular responses to adult dietary alteration, when offered access to running wheels, rats voluntarily began using them on the first day, but gradually increased their activity over the course of the 7-day period. Female rats on average engaged in an order of magnitude more activity than age-matched male rats. The amount of

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voluntary activity was also moderately influenced by prenatal diet; the male, but not female, offspring of salt-fed dams did slightly more activity than controls, but the study did not observe any significant effect (relative to the baseline control period) of physical activity on resting day or night-time blood pressure or heart rate.

The adverse cardiovascular and renal effects of increased consumption of salt have clearly been shown in this thesis and long been recognized (Bang *et al.*, 1949); for fructose, the deleterious consequences are the subject of much recent speculation (Johnson *et al.*, 2009a). Mounting evidence suggests, however, that increased consumption of fructose after the introduction of high fructose corn syrup and sugar-sweetened beverages has had a negative impact on cardiorenal health (Fung *et al.*, 2009; Jalal *et al.*, 2010). Considering that early Man is thought to have evolved eating approximately  $\approx 0.25 \text{ g}\cdot\text{day}^{-1}$  salt and derive no more than 2% energy from simple sugars, the current estimated average consumption of 8-12  $\text{g}\cdot\text{day}^{-1}$  salt and 18-25% energy from simple sugars places a considerable physiological burden on our cardiorenal and metabolic systems. Pregnancy and development of the fetus are remarkable anabolic and physiological challenges, requiring specific patterns of nutrient delivery to fuel tissue expansion; however, when coupled with the energy-dense but nutrient-poor modern diet, it is unsurprising that we see vertical transmission and developmental programming of an adverse phenotype (McCurdy *et al.*, 2009). The current work shows that increased maternal consumption of salt has predictable effects on the dam (enlarged kidneys and heart, increased urine production; (Kirksey & Pike, 1962; Kirksey *et al.*,

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1962; Bird & Contreras, 1986)) and renders offspring hypertensive (Contreras *et al.*, 2000) but the thesis has additionally illustrated how this effect is markedly sex-specific. Indeed, from a relatively young, but considered 'adult' age (10+ weeks), male offspring had an average increase in mean arterial pressure of  $\approx 15$ mmHg whereas female age-matched offspring had an average decrease in mean arterial pressure of  $\approx 10$ mmHg; a quite considerable overall effect size ( $\approx 25$ mmHg) in siblings. To our knowledge only one study in mice (Huang *et al.*, 2006) and one in rats (Song *et al.*, 2004) has described the effect of additional dietary fructose and salt intake on cardiorenal function; none have considered their interaction when fed either to pregnant dams or potential effects on the resultant offspring. We recapitulated many of the well-characterized effects of salt and/or fructose *per se*, such as effects on urine flow rate and blood glucose (Song *et al.*, 2004; Huang *et al.*, 2006). While the combined (FSD) diet reduced litter size and fetal growth, the cardiovascular programming observed with dietary salt inclusion was partially extinguished when fructose was additionally available; indeed, FSD offspring only displayed an intermediate cardiorenal phenotype e.g. blood pressure, osmolality and renal function were intermediate between groups CD and SD. Considering that maternal salt intake was less in FSD, as dams offered fructose reduce their food intake  $\approx 15$ -20%, suggests a dose-dependent effect of maternal salt intake *per se* on cardiovascular programming in the current model. Indeed, the present results also tend to rule out programmed changes in endothelial nitric-oxide function as the pressor effect of chronic nitric-oxide inhibition elicited similar responses in all groups, albeit from different baselines.

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Postnatally, the fetus and adult offspring were relatively normal in terms of tissue weight, plasma and urine metabolites. Discounting the marked increase in hyperosmolality in both male and female prenatally salt-fed offspring, no clear physiological indicator of hypertension was observed. However, sex specific effects seen in the current study and the large difference seen in blood pressure between maternally salt fed male and female sibling's warrants further research. Sex-specific effects are often observed within the developmental programming paradigm (Sinclair *et al.*, 2007; Grigore *et al.*, 2008) but, to our knowledge, none as marked as presented here. Further investigation and experimentation is warranted to identify mechanisms that were beyond the scope of the present study, but a testable hypothesis would be to examine a potentially 'protective' role of oestrogen in the pre-menopausal female adult. Some excellent recent studies that have shown programming of a sex-specific cardiovascular phenotype (such as increased blood pressure in male but not female offspring) have identified an absence of oestrogen in males as a causal factor (Ojeda *et al.*, 2007; Patterson *et al.*); in effect, oestrogen acts as a 'pro-survival factor' mitigating (perhaps epigenetically) the adverse consequences of a nutritionally-poor developmental environment until concentrations decline in middle-age and morbidity and mortality rates (e.g. for cardiovascular outcomes) in females begin to rise – the basis for oestrogen replacement therapy (Stampfer *et al.*, 1991). However, this is not to discount a fundamental effect of being conceived as a male or female and alterations in the way with which the blastocyst or fetus is interacting with the immediate (intrauterine) environment differently. For example, Sinclair and colleagues reported that peri-conception exposure to a maternal

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methyl deficient diet for only 6 days (day 0 to day 6) revealed significant sex-specific differential DNA methylation of CpG islands in the fetal livers at day 90 gestation i.e. of the altered loci as a result of the dietary treatment, 53% were specific to male and only 12% specific to female (Sinclair *et al.*, 2007). Clearly, this warrants further investigation and experimentation to identify potential mechanisms but a testable hypothesis is for a protective role of estrogen in the pre-menopausal female adult and a scheme of work has been formulated for the near future.

The offspring, insofar as our measurements extend, were also relatively phenotypically 'normal'; they were of similar size, had few residual effects on organ masses, plasma and urinary biochemistry and their kidney function appeared relatively acceptable (reflected in similar clearance values between groups. Although, further novel findings, insofar as both male and female offspring from dams fed salt-diet alone had a 35-50 mosmole L<sup>-1</sup> increase in extra-cellular fluid (plasma) osmolality, a physiological variable usually very tightly controlled (to  $\pm 3\%$ ). Given that baseline renal osmolar clearance was similar between groups suggests that the kidneys of SD offspring were not retaining more Na<sup>+</sup> or other ions *per se*. Interestingly, high osmolality in SD offspring paralleled high osmolality in SD dams, suggesting a causal pathway (at least in the male offspring). Previous studies are contradictory with regard to the relationship between maternal and fetal osmolality reporting relative buffering (Kirksey *et al.*, 1962) or parallel changes (Ladella *et al.*, 2003) and clarification of this effect in the current model is indicated. However, consideration must be

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given to the analysis used in this thesis and could be termed as a proxy for renal function. The studies employed in this thesis give a more general overview of renal function and whilst being adult offspring, the offspring too could be defined as young adults with a relatively high degree of renal metabolic flexibility in comparison to aged rats. Perhaps a more in depth analysis of the adult offspring renal function or kidneys may reveal more subtle alterations in renal structure rather than overall function at this early stage. Studies have shown there is a strong correlation between the number of nephrons and risk of hypertension in humans (Keller et al 2004) and in the rat model (Langley-Evans *et al.*, 1999), of which nutrition and environment can influence overall glomerular formation and subsequent filtration (Woolf & Welham, 2002). Fewer nephrons may result in the reduction of filtration surface area, impairing  $\text{Na}^+$  reabsorption, leading to an expansion of extracellular fluid volume and a consequential rise in blood pressure (Brenner *et al.*, 1988). In protein deficient-fed offspring Alwasel reported increases in NKCC, NCC,  $\text{NHE}_3$ ,  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha 1$  and  $\beta 1$  in the kidney have been shown. Alwasel and colleagues proposed increased renal  $\text{Na}^+$  retention may be driving a higher basal blood pressure in these offspring. They reported that the basis of naturiesis was tubular because GFR was not different and no effect on circulating aldosterone was observed. Moreover, naturiesis was still elevated in 'programmed' offspring (Alwasel & Ashton, 2009). Further renal structural and molecular work is clearly warranted to elucidate any true effects of prenatal diet upon renal function. Clearly the effects of maternal salt and increases in plasma sodium and osmolality in dams and the apparent vertical transmission of hyperosmolality in the offspring

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warrant further investigation. Further work to define the effects of hyperosmolality upon fetal development and if indeed the fetus itself is hyperosmolar at this early stage or if the hyperosmolality is age related. Ongoing studies are working to define what effect elevated osmolality has on branching organogenesis in the kidney and any subsequent effects on nephron deficit and total kidney vascularity.

In our study maternal fructose consumption led to mild NAFLD in the dam (decreased food intake, hepatomegaly with increased lipid deposition) and marked internal re-distribution of her fat depots with the offspring of fructose-fed dams having increased liver:body weight ratio despite a similar and relatively 'normal' blood metabolite profile. The adverse metabolic consequences of increased consumption of extrinsic sugars, in particular fructose, has been widely reported (Rawana *et al.*, 1993; Tappy & Le, 2010) and the predominant effect is on the obligatory site of fructose metabolism – the liver in adult female rats. Non-alcoholic fatty liver disease (NAFLD) is increasingly prevalent in the Western world and a recent study in non-human primates using a maternal high fat diet led to overt NAFLD in the dam but also in her developing fetus (McCurdy *et al.*, 2009) leading to the suggestion that offspring from mothers consuming a poor-quality diet may be more susceptible to NAFLD, regardless of their habitual adult diet. Data in this thesis provides some support for the hypothesis.

Many mechanisms have been proposed as key factors underpinning the fetal origins hypothesis such as; altered kidney development (Langley Evans , 1996), specifically nephron

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deficit, over exposure to glucocorticoids (Gardner et al 1997), perturbed angiogenesis and altered vascular function and deficit in total peripheral vasculature (Hemmings et al 2005, Pladys et al 2005) have all been suggested to play a role. It remains unclear whether these pathological states can be programmed *in utero* by alterations in maternal diet during fetal development. The placenta may play a key role in the programming of adult disease (Myatt, 2006). With reference to markedly different placental phenotype observed in salt fed placentas shown in this thesis, quantification of placental-fetal transfer capacity and/or of the vasculature by corrosion casting as well as further micrograph analysis is clearly warranted. Reductions in spiral and fetal blood vessels as well as reductions in labyrinthine area have all been proposed to reduce maternal-fetal nutrient transfer (Godfrey, 2002; Myatt, 2006) and can lead to reduced angiogenesis (Pladys *et al.*, 2005) with a hypothesized reduction in overall peripheral vasculature or reduced endothelial functionality or dysfunction. Differences in placental growth and function as shown by Rossant and Cross has been reported in human to have serious detrimental effects upon the health of the fetus and may further play a role in the reduction of litter size and through exposure to excessive physical force i.e. maternal hypertension or osmolality may alter physiological set-points within the developing fetus. Data presented in this thesis, and previously reported data, emphasizes the need to investigate further the mechanisms that regulate placenta growth and integrity. It is important to note that placental weight is a poor marker of function and placental efficiency and architecture are needed to understand the central role of which the placenta may play in fetal programming of cardiovascular disease. The 'programmed' effects

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observed in the current study may involve several pathways of phenotypic change within individual organs exerted by maternal diet moderately high in salt and fructose. In particular maternal salt and subsequent elevated basal blood pressure observed in male offspring from salt-fed dams, of which the effects can be broken down into five possible mechanisms:

- The number of cells within the organ may have decreased with associated relative proportion or distribution of different cell types within the tissue or organ may be unbalanced such as nephron count, endothelial dysfunction and placental integrity.
- Normal blood supply to the organs/periphery may not have developed due to placental dysfunction, microvascular rarefaction and possible increased Na<sup>+</sup> plasma concentration affecting endothelium and subsequent dysfunction.
- Up or down-regulation of natriuretic factors such as water channels and sodium transporters.
- Up or down-regulation of hormone receptors such as aldosterone receptors,
- Increased glucocorticoids and decreased 11bHSD2 and effects on the HPA contributing to a resetting of feedback and other physiological control mechanisms altering overall systemic set points.

However, there are still many directions of work that can be considered from the current study. Firstly, further analysis of data presented here could unravel many changes observed that have not yet been explored. Analysis using global approaches (i.e. gene, microarrays)

may prove useful and provide more information with regard to specific tissue such as heart, kidney, hypothalamus and changes in the different phenotypes observed. The combination of *in vivo*, *in vitro* and genetic research can provide substantial mechanistic evidence to identify causal regulatory pathways in the pathogenesis and role of salt and fructose in fetal development and later life health. Hypertension is clearly multi-factorial in nature and to pinpoint one mechanism or pathway linking maternal nutrition, fetal development and later life cardiovascular health is not possible with many mechanisms explored to date still needing further investigation. The present study has started to elucidate possible routes of further study from which mechanisms that underpin the development of hypertension in salt fed offspring but leaves more questions unresolved than answered. These results with other previous studies help add to a working hypothesis that may explain the developmental programming of hypertension.

We have developed and characterized an animal model that recapitulates many of the clinical symptoms of increased salt and/or fructose intake that marks current dietary trends. Uniquely, we have elucidated two distinct phenotypes in the dam as a result of moderate salt intake (effects on cardiovascular and osmolar regulatory function) or moderate fructose intake (effects on the liver) and show how these are vertically passed to the offspring to increase their expression of risk factors for cardiovascular disease and systemic alterations respectively, despite little or no exposure to the diets themselves. The study emphasizes the importance of *quality* rather than *quantity* when assessing maternal diet, particularly in

terms of its mineral and simple sugar content and the importance of studying outcomes in both sexes as it is clear that what is observed in one (e.g. males) will not necessarily be observed in the other (e.g. females). The implication is that continued exposure to such refined, poor-quality diets has the potential to feed-forward adverse health outcomes through generations that may explain and contribute to the exponential increase in cardiovascular disease, kidney failure and NAFLD over the last 40-50 years and also the increase in childhood cases. The data also emphasize the importance of a nutritionally-balanced diet when pregnant and the model offers a robust nutritional model in which mechanistic aspects of sex-specific developmental programming may be usefully and carefully explored. The current study has used exemplary techniques throughout the study (e.g. telemetry for cardiovascular data, osmometry and analysis of electrolytes, advanced statistical models such as Fourier plots and random effects models) to reveal some very clear and some subtle effects of the maternal diets on adult offspring physiology.

### ***In summary***

This thesis has confirmed previous reports of a fructose induced lipogenic plasma profile, hypoglycaemia and evidence of NAFLD. In addition, it has been demonstrated that offspring from dams fed a diet moderately high in fructose and/or salt demonstrate a vertical transmission of parts of the dam's phenotype. The main finding during this series of experiments has revealed long-term cardiovascular effects of increased maternal salt or fructose intake in the adult offspring, despite little direct exposure themselves. The delayed

cardiovascular effects are distinctly sex-specific and some may be related to differences in levels of voluntary activity. It is concluded that any anticipated cardiovascular deterioration with age, for example with prolonged, rather than short-term, exposure to such refined nutrients, is likely to manifest differently in males vs. females. Of course, non-communicable disease such as cardiovascular diseases, hypertension, non-alcoholic fatty liver disease and insulin resistance are multi-faceted. However, research in the area of developmental programming has, in this thesis and a great number of other studies utilising many models demonstrate that developmental programming of CVD, hypertension and altered metabolic profile are detrimental to later life health of the offspring. Although, the contribution of developmental programming to the overall population of people suffering from non-communicable disease may be minor compared to the overriding effects of environment and lifestyle factors it is still an important risk factor. Dietary intervention or antenatal advice gleaned from programming research within this very large population will inevitably benefit a significant amount of people. Finally, this thesis has highlighted the importance of a balanced, healthy diet during pregnancy and the possible detrimental effects of an unbalanced or maternal excess. This thesis taken together with published data will serve to encourage further research within the programme of work already carried out and further investigation of the development origins of hypertension and other non-communicable diseases.

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**Conclusion & perspectives**

Feeding pregnant rats increased fructose and/or salt recapitulates many of the physiological and anatomical effects of increased fructose and/or salt intake that mark current dietary trends. The main findings of this study provide evidence that a high salt diet during pregnancy leads to hypertensive male offspring and hypotensive female offspring and limiting salt intake during pregnancy (FSD) is sufficient to cause an intermediate phenotype. Uniquely, we have elucidated two distinct phenotypes in the dam as a result of moderate salt intake (effects on cardiorenal function) or moderate fructose intake (effects on the liver) and show how these are vertically passed to the offspring to increase their expression of risk factors for coronary vascular disease and metabolic syndrome respectively, despite little exposure to the diets themselves. The implication is that continued exposure to such refined, poor-quality diets has the potential to feed-forward adverse health outcomes through generations that may, in part, explain the exponential increase in NCD over the last 40-50 years. The data also emphasize the importance of a nutritionally-balanced diet when pregnant, and the model offers a robust nutritional paradigm in which mechanistic aspects of sex-specific developmental programming may be usefully and carefully explored. Based upon the data presented we would echo the recent commentaries and initiatives to reduce both the quantity of salt (He *et al.*, 2010) and fructose (Johnson *et al.*, 2009b) consumed in a Western diet specifically during pregnancy. A more complete understanding of how a prenatal high salt diet affects mechanisms that cause cardiovascular insult may aid in the prevention of adult disease. However, only once the mechanisms and processes involved

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have been characterised can novel therapeutic strategies be invoked to combat the progression towards disease.

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