UNIVERSITY OF SOUTHAMPTON

FETAL PROGRAMMING OF APPETITE FOR MACRONUTRIENTS AND OBESITY

by

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE
HUMAN NUTRITION

Doctor of Philosophy

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Epidemiological studies have demonstrated that poor intrauterine growth is linked to non-communicable diseases in adulthood, such as type II diabetes, hypertension and coronary heart disease (Barker 1993). Obesity is a major risk factor for both these diseases and also appears to be related to early growth. Using a rat model, previous studies have demonstrated that nutritional inadequacy *in utero* induces hypertension in rats maintained on standard laboratory chow (SLC) post-weaning (Langley & Jackson 1994). The mediator(s) of this hypertension is unknown, but dietary and/or endocrine factors could be involved. This thesis was designed to investigate the effects on metabolism, dietary intake and body composition by manipulating the pre and postnatal diets in a rat model.

All offspring were produced by feeding rat dams either 9% or 18% casein diets during pregnancy. In both male and female offspring, glucose tolerance test profiles were similar between 9% casein and 18% (control) groups. The whole glucose load had been cleared by 60 minutes in both groups. Initial peak insulin response was lower in the 9% group however, in this group, a second peak in plasma insulin concentration (PIC) such that after 60 minutes, PIC was significantly higher than the 18% group. This suggests a degree of insulin resistance in these animals. Total carcass energy and %fat were both significantly greater in the 9% group following macronutrient self-selection feeding (MSS). These results show that exposure to a low-protein diet in utero alters fetal programming which manifests itself as an increase in body weight due to an increase in body fat.

Systolic blood pressure (SBP) was significantly elevated in the 9% group upon weaning, but this difference was only sustained when rats were maintained on standard laboratory chow (SLC). A MSS diet lowered SBP in rats from the 9% group without affecting SBP of rats from the 18% group. The relative hypertension of the 9% group was expressed again when SLC was consumed. The effects appeared more pronounced in male offspring although the trend was clearly visible in female offspring. These results indicate that diet during adulthood is critical for the expression of hypertension in this model. Since the major difference between SLC and MSS is the ratio of carbohydrate : fat ingested, it is likely that the metabolic handling of these macronutrients may, in part, underlie the expression of hypertension in this model. As these studies appeared to show similarities with the present rat model and SHR, the macronutrient intake was compared. The SHR consumed significantly less protein than both the 9% and 18% Wistar groups, and weighed significantly lighter by the end of the study. The SHR male offspring consumed less energy than the Wistar group, although this was not the case in the female offspring.

The final study compared pre-natal and post-natal diets. The SLC results confirmed those shown previously. The high-carbohydrate diet also maintains high SBP in the 9% offspring indicating that it is not a specific component in SLC causing the increase in SBP. The high-fat feeding quickly abolished and high-protein feeding gradually reversed the hypertension in the 9% group without affecting the age-related increase in SBP in the 18% group. Post-natal diet had a greater effect on PIC with the high-fat fed females requiring higher insulin levels return the glucose levels towards baseline. In female offspring there was a significant move towards an increase in the proportion of type II fibres as a result of feeding a high-protein or high-fat diet in the 9% groups which was not seen in the 18% group. These results support that both pre- and postnatal diet influence the expression of the hypertension and possibly carbohydrate metabolism in this model. This suggests that a low dietary carbohydrate intake may underlie the hypotensive effect.

To summarise, the changes which have been seen in these studies occurred without moving to the extremes of deficiency during pregnancy and could therefore be of great importance in understanding the possibility of the onset of factors which could lead to obesity and Type II diabetes in the next generation.

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Systolic blood pressure (SBP) was significantly elevated in the 9% group upon weaning, but this difference was only sustained when rats were maintained on standard laboratory chow (SLC). A macronutrient self-selection diet lowered SBP in rats from the 9% group without affecting SBP of rats from the 18% group. The relative hypertension of the 9% group was expressed again when SLC was consumed. The effects appeared more pronounced in male offspring although the trend was clearly visible in female offspring. These results indicate that diet during adulthood is critical for the expression of hypertension in this model. Since the major difference between SLC and MSS is the ratio of carbohydrate : fat ingested, it is likely that the metabolic handling of these macronutrients may, in part, underlie the expression of hypertension in this model. As these studies appeared to show similarities with the present rat model and SHR, the macronutrient intake was compared. The SHR consumed significantly less protein than both the 9% and 18% Wistar groups, and weighed significantly lighter by the end of the study. This was matched in the male offspring with less energy consumed although this was not the case in the female offspring.

The final study compared pre-natal and post-natal diets. The SLC results confirmed those shown previously. The high-carbohydrate diet also maintains high SBP in the 9% offspring indicating that it is not a specific component in SLC causing the increase in SBP. The high-fat feeding quickly abolished and high-protein feeding gradually reversed the hypertension in the 9% group without affecting the age-related increase in SBP in the 18% group. Post-natal diet had a grater effect on plasma insulin concentrations with the high-fat fed animals requiring higher insulin levels return the glucose levels towards baseline. In female offspring there was a significant move towards an increase in the proportion of type II fibres as a result of feeding a high-protein or high-fat diet in the 9% groups which was not seen in the 18% group. These results support that both pre- and postnatal diet influence the expression of the hypertension and possibly carbohydrate metabolism in this model. This suggests that a low dietary carbohydrate intake may underlie the hypotensive effect.

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CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

APPENDICES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

5-HT	5-Hydroxytryptamine
11ß-OHSD	11ß-Hydroxysteroid dehydrogenase
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
AIN	American Institute of Nutrition
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
CCK	Cholecystokinin
CHD	Coronary heart disease
CRF/H	Corticotrophin releasing factor/hormone
DoH	Department of Health
DRV	Dietary reference value
ECG	Electrocardiogram
e.d.l.	extensor digitalis longus
FFA	Free fatty acids
GABA	Gamma aminobutyric acid
GAL	Galanin
IBAS	Interactive biological analysis system
I-BAT / I-WAT	Interscapular brown / white adipose tissue
IGF-I	Insulin-like growth factor I
IVGTT	Intravenous glucose tolerance test
LDL	Low density lipoprotein
MSS	Macronutrient self-selection diet
NA	Noradrenaline
NIDDM	Non-insulin dependent diabetes mellitus
NPY	Neuropeptide Y
NS	Not significant
NSB	Non specific binding
NSP	Non-starch polysaccharide
PDH	Pyruvate dehydrogenase
PUFA	Polyunsaturated fatty acid
RIA	Radioimmunoassay

ABBREVIATIONS

SBP	Systolic blood pressure
SDS	Special diet services
SEM	Standard error of the mean
SHR	Spontaneously hypertensive rat
SNS	Sympathetic nervous system
TAG	Triacylglyercerol
TCA	Tricarboxylic acid
TSH	Thyroid-stimulating hormone
VLDL	Very low density lipoprotein
WAT	White adipose tissue

PUBLICATIONS

Pickard CL & McCarthy HD 'Influence of pre- and postnatal diet upon skeletal muscle fibre composition in the rat' Proc Nutr Soc 1998; submitted

Pickard CL, Hurley M & McCarthy HD 'Body fatness and circulating triglyceride concentration in relation to pre- and postnatal dietary experience in the rat' Proc Nutr Soc 1998; submitted

Pickard CL & McCarthy HD 'The response of blood pressure to feeding composite diets of differing macronutrient composition in rats with hypertension of fetal origin' Proc Nutr Soc 1996; 56(1A) : 11A

Pickard CL, McCarthy HD, Browne RF, Jackson AA 'Altered insulin response to a glucose load in rats following exposure to low-protein diet in utero' Proc Nutr Soc 1996; 55(1A) : 44A

Pickard CL, Devoto MK, McCarthy HD 'Post-weaning dietary manipulation reduces blood pressure in rats with hypertension of fetal origin' Proc Nutr Soc 1996; 55 (1A) : 103A

McCarthy HD, Pickard C, Speed J, Jackson AA 'Sexual dimorphism of macronutrient selection and regional adipose tissue accumulation following in utero exposure to maternal low protein diet' Proc Nutr Soc 1994; 53(3) : 172A

Chapter 1.0
INTRODUCTION

1.1 Obesity

Obesity in humans is classified by those individuals having a body mass index (BMI), height/weight^2, of over 30kg/m^2, and those between 25-30kg/m^2 are classed as being overweight. Moderate overweight is tolerated, especially by women and the elderly, but once the BMI is greater than 30, there is a linear relationship with increased mortality (Larsson 1991). This is primarily because obesity is a major factor linking non-communicable diseases such as non-insulin dependent diabetes (NIDDM), hypertension and coronary heart disease (CHD), and obesity will increase the severity and likelihood of death in all these diseases.

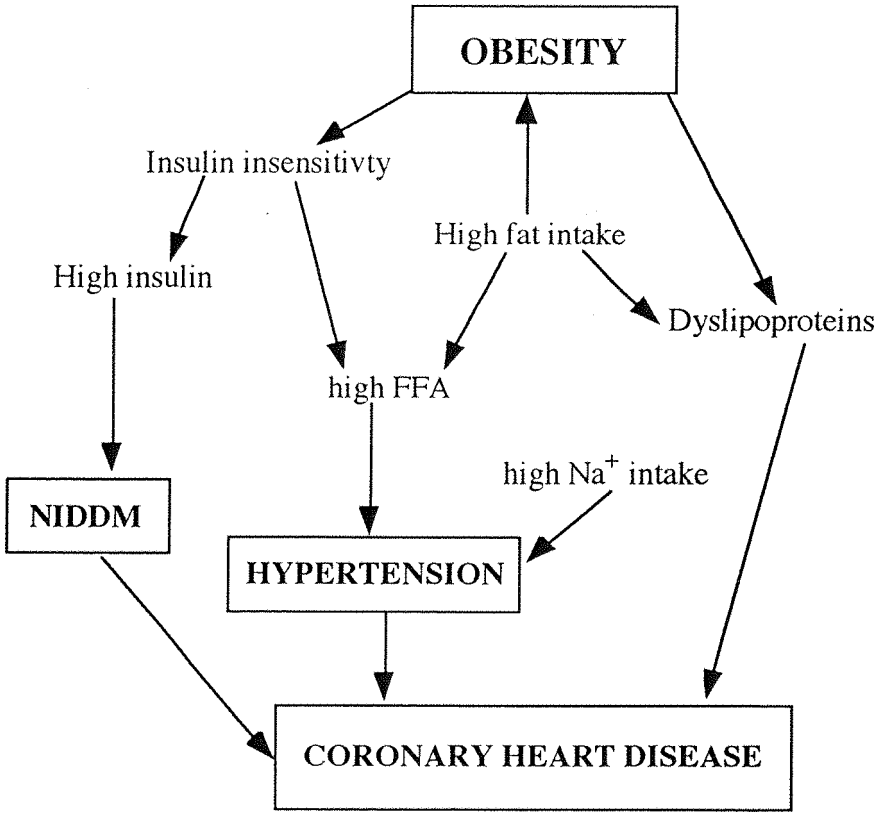

Figure 1.1 The factors that link obesity with the non-communicable diseases discussed. FFA= free fatty acids (Garrow & James 1993)

Obesity is a major public health problem within the UK and throughout the world (Dept. of Health 1992, Gortmaker et al 1987). It is now one of the most prevalent disorders in the USA and in 1990 in the UK, 45% of men were regarded as overweight with 8% in the

obese category and in women 36% were overweight and 12% obese. (DoH 1992). These have since risen to 61% of men overweight, 16% obese and 52% women overweight and 17% obese (DoH 1996). This is despite obesity being targeted by the UK government for specific reduction in these figures.

Obesity has been divided into four types depending on whether it is total fat being considered (type I) or distribution of excess fat (types II, III, IV) (Bouchard & Perusse 1993). There are problems with human studies in the isolation of human genes as it is most likely to involve complex multigenic systems, therefore much interest has been placed on animal models. There have been several models of genetic obesity studied particularly the Zucker rat and the ob/ob mouse. Interest has been focused on the ob/ob mouse recently as the protein from the defective gene in these mice has been cloned (Zhang 1994) and when this has been injected it has been found not only to regulate the weight of the obese ob/ob mouse but also of their lean counterparts (Halaas et al 1995, Pellymounter et al 1995). Although these genetic mutations have their own interest, this programme of work is more concerned with those which have no known genetic defects.

1.1.1 Energy Balance

The primary cause of obesity is a change of energy balance where over a period of time, energy intake exceeds energy expenditure leading to energy storage as excess body fat within the body. Normally, once an individual has reached adulthood, weight is maintained at a relatively stable level. In fact if there were only an imbalance of 2% over 10 years this would lead to a weight change of over 25kg (Saltzman & Roberts 1995).

The mechanisms that adjust energy intake to expenditure, or visa versa, are more critical to those which influence these separately (Flatt 1995). The variation of energy intake or food intake is generally much greater than that of energy expenditure (Flatt 1995), although a reduced energy expenditure appears to lead to weight gain in those susceptible to obesity (Saltzman & Roberts 1995). An increase in physical activity increases energy expenditure only by a very small percentage. This is because out of total energy expenditure, physical activity only accounts for approximately 15-20%. However physical activity may be associated with an increase in resting energy expenditure and also changes in sensitivity to insulin and other hormones which ultimately may affect total energy expenditure (Poehlman et al 1994). There is also a small increase in energy expenditure in the short term after feeding when the meal is being digested, assimilated and oxidised. Energy expenditure changes in this case however have been shown not to differ between lean and obese individuals. In a study by Schutz (1993), after one day of overfeeding the change in respiratory quotient was identical

between the lean and obese subjects and the efficiency of substrate utilisation and storage was also not influenced by obesity.

Energy intake in terms of food is composed of fat, carbohydrate and protein. Alcohol also provides energy but is not classified as a food. Eating behaviour is not only regulated by what the body requires but also by sensory perception and social habits. In particular fats and sugars have a positive sensory appeal which crosses all cultures (Drewnowski 1995).

The ratio of amino acid oxidation to protein intake and therefore nitrogen balance is maintained on a large range of protein intakes irrespective of the amount of carbohydrate and fat in the diet unless energy is limiting (Flatt 1988), and these two macronutrients provide the bulk of energy used in weight maintenance. Originally it was thought that fat and carbohydrate intakes were controlled separately but since then studies have shown that they are integrated, as the combined inhibition of fatty acid and glucose metabolism caused a greater stimulation to eating than each of them separately (Friedman 1995). High-fat foods are easily overeaten due to the fact they have a positive sensory effect, low satiety and are more energy dense (Rolls & Hammer 1995).

Although there have not been any long-term measurements of energy expenditure and physical activity levels on the UK population, proxy measurements of inactivity such as number of hours spent watching television and car ownership, have dramatically increased Prentice & Jebb 1995). This suggests that rates of energy expenditure have been decreasing at a greater rate than the decrease in energy intake. Also there has been a shift in the proportions of energy in the UK diet derived from fat and carbohydrate. Percentage energy for carbohydrate has been decreasing and percentage energy for fat increasing (Prentice & Jebb 1995).

For women, there is a strong correlation within social class with the lower social classes (IV & V) having the highest rates of obesity and also the highest levels of inactivity and television viewing, compared to those in the highest social class (I) which have the lowest rates of all three variables (Prentice & Jebb 1995).

1.1.2 Fat Consumption

The link between dietary fat and obesity has been known since 1955 when Mickelsen demonstrated this by feeding a diet with 85% of energy from fat to rats. This caused them to achieve weights of over 1kg (Oscai et al 1984). Although this connection has been well documented (Hill et al 1990, Hill et al , Lucas et al 1989, Triscari et al 1985), precisely how the intake of fat leads to chronic obesity is not clear. Hyperphagia is an obvious way in which obesity occurs (Lucas et al 1989) but this is not the answer in all cases. In one study by Boozer et al (1995) they found that when they fed four groups of rats isoenergetic

diets with increasing fat content (ranging from 12% to 48% energy) there was a dose-response relationship between the amount of fat in the diet and the amount of body fat accumulated. If this were also the case in humans, it could mean that those people who are eating a high-fat diet but in small amounts so there is no overall weight gain, may still be depositing fat in the visceral depots which are associated with increased risk of obesity. Since World War Two the proportion of fat in the diet has increased at the expense of complex carbohydrate consumption (Prentice & Jebb 1995). This has occurred at the same time as a steady rise in the proportion of the population who are obese. Similar data was found through epidemiological studies in Denmark, where an increase in the proportion of fat intake was closely related to an increase in obesity in those entering National Service (Lissner & Heitmann 1995).

The type of fat appears also to be important in determining the degree of obesity, at least in experimental animals. It has been shown that rats fed a diet with the majority of fat coming from polyunsaturated fat (supplied using corn oil) became heavier and fatter than those fed a saturated diet using lard (Hill et al 1992). However it is also dependent on which unsaturated fat is used. N-3 fats which are present in fish seem to be protective against obesity compared to n-6 fats (Pan et al 1994). This may be due to the different enzymes required for their metabolism.

CHD is strongly associated with the proportion of saturated fat consumed. This is because these fats raise the levels of plasma low density cholesterol (LDL) and high levels of this cholesterol found in CHD cause the thickening of arteries. N-6 polyunsaturated fats (PUFA) decrease plasma LDL cholesterol, however at high amounts they can also decrease the HDL which is detrimental as the HDL removes cholesterol from the blood to the liver. N-3 PUFA which are found in fish oils, do not have an effect on the plasma cholesterol levels but do decrease triglycerides another type of fat found in the blood. The main effect of the n-3 PUFA is in decreasing platelet aggregation.

However fat in the diet is not the only determinant for dietary obesity. Obesity can also be sugar induced. (Bock et al 1995, Kanarek et al 1979, 1982, 1987, Oscai et al 1984). As with fat the type of sugar is important, whether it is glucose, sucrose or fructose (Kanarek et al 1982) as well as the concentration of minerals. It was found in rats that increasing the zinc, chromium or selenium caused a decrease in weight gain and insulin concentration and improved glucose tolerance (Bock et al 1995).

1.1.3 Fat Distribution

There are many metabolic factors which are associated with, or changed in obesity (Mayer 1963), but whether many of these are the cause or result of the obesity is unknown. Blood levels of glucose, lipid and total cholesterol can be increased with an increase in lipogenesis and cholesterogenesis. There are changes in enzymatic activities leading to abnormalities in adipose tissue metabolism and liver glycogen turnover.

Other factors which are also changed include intestinal absorption, acetate pool and turnover, steroid hormones and ketone levels which are all increased. The body composition is changed with enlarged liver, heart and pancreas together with high body fat. However the degree of metabolic changes is dependent on the location of fat distribution in the body. It appears that particularly in women, a measure of a waist-hip ratio will give an indication of the relative distribution of adipose tissue. This ratio indicates whether the fat is located predominately within the abdominal cavity (waist) or otherwise subcutaneously covering the limbs (hip). There is a relationship between the relative risk of CHD, NIDDM and this ratio, in that the greater the waist-hip ratio, the more fat being laid down in the abdominal cavity and hence an increased risk of CHD and NIDDM (Lapidus et al 1984). As early as the 19th century insurance companies defined an abdominal girth which was greater than the expanded chest as a greater risk, and those with increased abdominal obesity had increased mortality compared to those who were obese but not abdominally (Kahn & Williamson 1994).

It has been found that in upper body or central obesity, plasma lipoprotein levels are altered with an increase in low density lipoprotein and a decrease in high density lipoprotein compared with both the lean individuals and those who were obese but their distribution of fat was more subcutaneous (Despres 1991, Zamboni et al 1994). Insulin action was impaired in both men (Zamboni et al 1994) and adolescent girls (Caprio et al 1995) who were abdominally obese. In the obese girls this correlation was only observed with visceral fat and not with waist/hip ratio or subcutaneous fat.

1.1.4 Obesity Resistance

The susceptibility of an individual to become obese on a high-fat diet varies between individuals. This is the case in both humans and animals. Studies on humans have limited use due to the differences in genetic background and the influence of many other environmental factors which can more easily controlled in a laboratory situation. However, it does appear that in some individuals, there is an altered response to the fat eaten in the diet and the metabolic handling of this substrate is altered, in that there is little or no increase in fat oxidation with increasing fat intake. Why this happens in certain individuals is unknown

(Astrup 1993), but a consequence of this is a partitioning of dietary energy from fat into triglyceride stores in adipose tissue.

Animal studies with rats from the same genetic pool have been used to characterise obesity-prone and obesity-resistant individuals. In one study the time course for differences to be seen between the two groups took only one week after feeding a high-fat diet, with the obesity-prone rats preferentially depositing fat, whereas the obesity-resistant rats had an increase in circulating B-hydroxybutyrate concentrations, possibly contributing to a decrease in total food intake (Pagliassotti et al 1994). In other studies investigating the metabolic features of obesity-prone and obesity-resistant rats, energy absorbed was identical in both groups (Chang et al 1990, Lauterio et al 1994, Pagliassotti 1994). Mean plasma glucose concentration was increased in the obesity-prone rats once obesity had been established and the obesity-resistant rats showed a greater fat oxidation and lower plasma free fatty acids (Chang et al 1990), whereas urinary nitrogen and creatinine excretion and water balance were all unchanged (Lauterio et al 1994). The reason why these differences exist is as yet unknown, however an alteration in the sympathetic nervous system has been hypothesised (Jeanrenaud 1985), with particular reference to changes in insulin and brown adipose tissue function (Kortner et al 1994, Levin et al 1987, Jeanrenaud 1985, Shetty et al 1981)

1.2 Coronary Heart Disease

Deaths from CHD account for 27% of all mortalities, equivalent to 180 000 deaths each year in England and Wales. These figures are still probably an underestimate of cardiac deaths and together with over 10% of working days lost due to CHD related illness, it can be seen that the problem is widespread (Garrow & James 1993). Currently a high proportion of funding is used for the treatment of CHD and related diseases. This is very short term and efforts need to be concentrated on its prevention.

Although death from CHD is declining slowly, the UK rates are still one of the highest in the world being over six times as high as those in Japan (DoH 1992). This decline is partially due to better treatment such as bypass surgery as opposed to a large decrease in incidence. There is a large regional difference in the rates of CHD nationally, with lower death rates in the South of England and it is more common in increased smoking incidence and lower social class areas (DoH 1992. The greatest rates are in the Northwest and Northeast England, South Wales, Scotland and Northern Ireland.

Coronary heart disease has many risk factors associated with it. Some of these cannot be influenced by the individual such as age, sex, and family history. However of those which are able to be manipulated, such as obesity, hypertension and hyperlipidaemia, diet can play a

key role. Smoking and low levels of physical activity are also key risk factors. Smoking is by far the biggest individual risk factor, however if attention is paid to diet, several risk factors such as the ones mentioned above may be able to be reduced. For example, a high fat consumption and obesity are two of the risk factors for type II diabetes, CHD and related diseases (Bray 1976).

Diet can influence CHD in many ways. Primarily it can cause hyperlipidaemia, one of the major risk factors for CHD, by changing the levels of specific cholesterol factors and triglycerides in the body. It can also change the likelihood of thrombosis as prostaglandins and tromboxanes involved in this process are influenced by the fatty acid content of the diet. Diet can also influence the amount and types of antioxidants that protect cells and vessels from damage by free radicals. Vitamins A, C, and E are antioxidants, acting on various points in the sequence of free radical formation.

Additionally diet can contribute to an increase risk of hypertension and obesity. A raised blood pressure, which is known to increase the risk of CHD, is strongly related to an increase in body weight, particularly when this is related to an increase in intra-abdominal fat deposition.

1.3 Hypertension

Hypertension is the sustained elevation of the systemic arterial pressure. The aim in clinical management of hypertension is to reduce systolic blood pressure to below 160mmHg and diastolic to below 90mmHg. Over 25% of the adult population in the UK are treated for hypertension. The main problem is that of this 25% only 10% can be assigned to a clearly identifiable disease such as renal disease. The other 90% have essential hypertension where the underlying cause is not clear (Bowman & Rand 1990). There are several dietary factors which can increase the risk of hypertension developing including excessive sodium, alcohol and coffee intakes.

Hypertension (which in most cases is the result of peripheral resistance) puts a strain on the pumping action of the heart, which then causes the cardiac muscle to hypertrophy. The risks of myocardial infarction, arteriosclerosis and heart failure are all increased.

Hypertensive individuals are also likely to have an increase risk of cerebral haemorrhage and renal failure even if this is not the primary cause. Obesity is also associated with hypertension (Stamler et al 1978), and weight reduction can decrease blood pressure (Dornfeld et al 1985). This decrease in blood pressure, both systolic and diastolic was correlated with the degree in change of weight. A weight loss of 9.5-18kg correlated with a

decrease in systolic blood pressure (SBP) of –7.8±5.5mmHg, whereas a weight loss of more than 27kg gave rise to a decrease in SBP of –24.5±8.2mmHg (Dornfeld et al 1985).

As for obesity, a genetically hypertensive animal model has arisen, the spontaneously hypertensive rat (SHR), and the changes which occur within this strain have been investigated in order to understand further the pathogenesis of hypertension. Many similarities occur between the SHR and essential hypertension in humans, although there are also some important differences.

Various hormones and neurotransmitters have been implicated in the maintenance of blood pressure in the rat. Takezawa et al (1994) showed that in female rats, blood pressure changed depending on the time of day and also on the oestrus cycle. They hypothesised that oestrogen was responsible for these changes. Chen & Meng (1991) also found differences between the blood pressure of males and females in the SHR, which were explained by the background steroid hormone status. In this study when the animals were gonadectomised, blood pressure was only affected in the males and the development of hypertension was retarded. When testosterone was administered to both sexes, a male pattern of blood pressure was seen. Although the sex steroids exert an influence the regulation of blood pressure, the peptide hormone insulin appears to exert a more influential effect.

1.3.1 Insulin

Insulin is a polypeptide secreted from the ß-cells of the pancreas. It stimulates glucose uptake into cells of muscle and adipose tissue and synthesis of glycogen and protein whilst inhibiting its degradation. Therefore the net effect of its actions lead to the storage of carbohydrate, protein and fat.

In addition to its widespread role in the intermediary metabolism of glucose, fatty acids and amino acids, insulin has a number of other experimental effects which may also function physiologically. For example, in both animal and human studies, insulin has been shown to influence the sympathetic nervous system (SNS), at both central and peripheral sites. (Anderson 1993, Daly & Landsberg 1991, Krieger & Landsberg 1988, Landsberg 1986, 1990, 1992a, 1992b, 1993, 1994, Landsberg et al 1991, Landsberg & Krieger 1989, Modan et al 1985). These effects could further influence blood pressure directly or indirectly via hyperinsulinaemia and insulin resistance which are observed in obese type II diabetic subjects (Welborn 1966).

The stimulation of the SNS causes an increase in salt and water retention, via action on the kidney, leading to raised blood pressure. In type II diabetes, where hyperinsulinaemia is present, this could cause excessive SNS stimulation and hence hypertension. However, many

of the human studies in this area have been carried out on subjects who were both obese and hypertensive. In these subjects both the basal and post-glucose load insulin levels were higher (Daly & Landsberg 1991), and plasma noradrenaline concentration was increased, indicating increased SNS activity (Landsberg 1994). However insulin resistance has also been found in non-obese subjects (Lachaal & Jung 1992, Ferrannini et al 1987). Ferrannini performed a euglycemic insulin-clamp on normal weight subjects aged 38 years and concluded that hypertension was an insulin-resistant state and that it was glucose metabolism involved not lipid or protein.

The relationship between insulin resistance and hypertension has been further studied in animal models including the SHR. It has been found that several insulin-related abnormalities exist in the SHR including, hyperglycaemia, hyperinsulinaemia, elevated plasma free fatty acids, compared with normotensive controls (Swislocki & Tsuzuki 1993), abnormalities in insulin secretion, action and catabolism (Mondon & Reaven 1988) and attenuated insulin-stimulated glucose utilisation (Hulman et al 1993), where skeletal muscle was identified as the major site of insulin resistance in these animals. However these differences are shown in a specific genetically altered strain of rat and although this appears to be a good animal model for essential hypertension, it needs to be assessed as to whether these defects are specific to the SHR, or is a feature of other models. Differences exist also between strains of rats and the relative insulin-sensitivity of their adipocytes, with the Sprague-Dawley rats having increased sensitivity compared to the SHR and Wistar rats (Lachaal & Jung 1992).

Conflicting evidence exists in this area (Anderson 1993, Katyama et al 1994, O'Hare et al 1989, Vettor et al 1994). For example, in a single gene hypertensive rat strain, it was found that glucose utilisation was unaltered in skeletal muscle and both white and brown adipose tissues (Vettor et al 1994), compared with normotensive controls. Furthermore, fasting levels of glucose, insulin and free fatty acids were also normal. In addition in the SHR, Katyama (1994) showed that giving an antidiabetic drug (CS-045) improved insulin resistance and glycaemia, but there was no alteration in blood pressure. However this was just the case in the SHR and not in the obese Zucker rat questioning whether the SHR can be fully accepted as a model for essential hypertension in humans.

It has also been suggested that the insulin-resistance seen in many obese subjects is a mechanism recruited to limit further weight gain and stabilise body mass (Daly & Landsberg 1991, Landsberg 1986, 1990, 1992a), as by acting on the SNS, insulin enhances thermogenesis and this then limits weight gain (figure 1.2). Insulin-resistance also limits glucose and free fatty acid uptake by adipocytes.

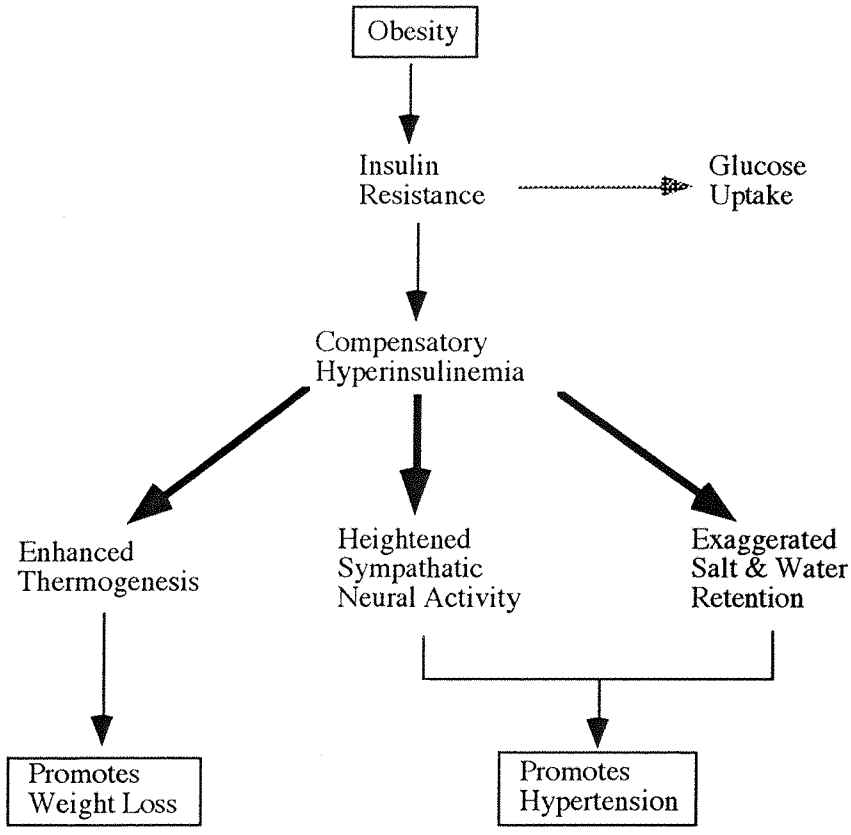

Figure 1.2 Schematic representation of the hypothesis that insulin plays a role in the development of hypertension (Anderson 1993)

1.3.2 Dietary Fat and Hypertension

The studies examining the relationship between fat and hypertension vary in the methodology and types of fat examined and also, especially with humans, it has been difficult to control for the exact amount and type of fat in the diet. In humans the role of polyunsaturated fatty acids (PUFA) in hypertension has been reviewed by Iacono & Doughty (1993). They found that n-6 fatty acids such as linoleic acid caused a decrease in blood pressure in both normotensive and mildly hypertensive subjects who were free-living. However other studies showed no change in blood pressure when feeding linoleic acid (Brussaard et al 1981, Goldberg et al 1992, Margetts et al 1985). Generally it could be concluded that when hypertensive subjects were studied, their blood pressure decreased when being fed a diet rich in n-6 fatty acids (linoleic) although the data on normotensives was less conclusive. N-3 fatty acids such as marine or fish oils (e.g. eicosapentanoic) also decreased both systolic and diastolic blood pressure in several studies (Iacono & Doughty 1993).

Monounsaturated fatty acid (e.g. oleic acid) intake was also inversely correlated to blood pressure (Brussaard et al 1981).

Animal studies in determining the effect of dietary fat on blood pressure have been performed as the manipulation of diet can be controlled to a much greater extent. These studies have shown that feeding a high fat diet can lead to an elevation of blood pressure. For example rabbits fed diets containing 40% energy as fat demonstrated increased blood pressures by 15-20% above baseline (Bursztyn 1987). This is even more marked if the fat fed is saturated as shown by Langley-Evans & Jackson (1995), where rats fed a 10% coconut oil (predominantly saturated fat) diet had systolic blood pressures on average 30mmHg above those fed on a polyunsaturated fat diet (corn oil). However, when rats were fed a diet high in n-6 or n-3 fatty acids, blood pressure was decreased (Hoffman et al 1986, Iacono & Doughty 1993, Langley-Evans et al 1996).

Of most interest and importance to this study feeding a high-fat diet to SHR decreased blood pressure independent of whether it was mainly saturated (Wexler 1981), or unsaturated fat (Hoffmann 1986). This suggested that the expression of hypertension in this model was critically dependent upon a high-carbohydrate diet, or some related aspect, such as insulin secretion, concentration or action. Secondly, the Wistar rats became obese whereas the SHR showed a decrease in weight gain compared to the Wistar rats and also when compared with the SHR fed standard laboratory chow. The SHR fed the high-fat diet had hypopituitarism together with an increase in adrenal and thymus sizes. It was thought that these changes lead to changes in endocrine and metabolic factors causing the decrease in blood pressure. However, no measurements of dietary intake were recorded in these studies. It is known that some strains of rats do not find a high-fat diet palatable or acceptable and in this case, it is possible that the prevention of hypertension and reduced weight gain was simply a result of a low energy intake. Indeed Young et al (1978) have also demonstrated that energy-restricted SHR do not express the hypertension to the degree of those fed ad lib. Secondly, as previously mentioned, insulin action and related glucose metabolism is altered in the SHR. Both a high-fat diet and energy-restricted diet would, presumably reduce circulating insulin concentration which could also account for the fall in systolic blood pressure.

1.3.3. Dietary Carbohydrate and Hypertension

The suggested involvement of insulin in hypertension could imply that dietary carbohydrate could promote an elevation in blood pressure and this has been examined in a number of human and animal studies. In the SHR, feeding an increased carbohydrate intake such as a sucrose load, increased blood pressure (Young & Landsberg 1981), whereas a

restriction in carbohydrate intake decreased blood pressure (Wright et al 1981, Young et al 1978). The addition of a sucrose load had no effect on normotensive rats. This data agrees with the theory that increases in SNS activity, which can be diet-induced, leads to an increase in blood pressure.

Carbohydrate overfeeding also prevented the normal fall in blood pressure in the last weeks of pregnancy in the SHR (Ahokas et al 1986), while the urinary noradrenaline but not adrenaline content was increased in these animals indicating an increase in SNS activity. These results were mirrored in a study by Fagerberg (1984) where two groups of obese, normotensive subjects were given weight-reducing diets. The first group were given a diet with 24% of metabolisable energy as carbohydrate and their blood pressure, heart rate and noradrenaline all significantly decreased, whereas the second group which were given a diet with 59% of metabolisable energy as carbohydrate showed no significant decreases in these parameters. These studies support the proposal that dietary carbohydrate and hypertension are linked, possibly via insulin and SNS actions.

1.4 Non-Insulin-Dependent Diabetes Mellitus

Non-insulin-dependent diabetes mellitus (NIDDM) or Type II diabetes is widespread and its prevalence is as high as 50% in Pima Indians. Asian Indians and the Finns are also high-risk groups (Fuller 1994). NIDDM is a major public health problem and is one of the diseases which has been targeted specifically in the Health of the Nation white paper (DoH 1992). Up to 3% of the population suffer from NIDDM, with the incidence of NIDDM nearly six times higher in those of Asian origin. This is an underestimate as many diabetics go undetected as highlighted by Barker et al (1993).

NIDDM is characterised by insulin resistance which generally leads to impaired glucose uptake by cells, although insulin resistance alone theoretically should not lead to NIDDM, it is influenced by other risk factors such as obesity, increased intra-abdominal fat distribution and low levels of physical activity or exercise (Bergman & Ader 1994). Increased lipid oxidation is generally observed in diabetes and it has been suggested that this is responsible, at least in part, for the insulin resistance (Felber et al 1987). This resistance prevents the inhibitory effect of insulin on the mobilisation of free fatty acids from adipose tissue, but what causes this is still unknown (Bjorntorp 1994).

In NIDDM, glucagon secretion is not suppressed therefore the liver still releases glucose which then stimulates insulin secretion. The increase in plasma insulin leads to a down regulation of insulin receptors on muscle and adipose tissue. This causes the insulin to have less effect therefore glucose levels after a meal are higher than normal which further

stimulates insulin secretion (Garrow & James 1993). Increasing age can spontaneously cause insulin resistance as insulin sensitivity decreases (Fuller 1994), or insulin-stimulated glucose utilisation decreases (Narimiya et al 1984).

The resistance to insulin in the major target tissues such as skeletal muscle, adipose tissue and liver, results in hyperinsulinemia and glucose intolerance. This in turn can also result in abnormal regulation of free fatty acid metabolism. Certain individuals with hypertension are also insulin resistant, but what triggers the events which cause NIDDM is still unclear. There seems to be a series of related variables which occur in individuals suffering from NIDDM. These have been termed 'Syndrome X' and include an increase in very-low-density lipoprotein triglycerides, a decrease in high-density lipoprotein cholesterol, glucose intolerance, hyperinsulinaemia, hypertension and central obesity (Reaven 1988). However it has been hypothesised that the hyperinsulinemia may be a compensatory factor to restore body weight during weight gain by stimulating SNS activity (Tremblay et al 1995).

The symptoms of Syndrome X have all been shown to increase the risk of CHD and it is known that individuals with diabetes are particularly likely to develop CHD (Kleinmann et al 1988).

Hypertension and obesity are also common in NIDDM and these add to the risk factors for CHD. If a patient is obese they are much more likely to die from NIDDM than an average weight person. This increase can be as high as eight fold for women and five fold for men (Lew & Garfinkel 1979). The distribution of body fat is again also important. It is particularly upper-body obesity or visceral obesity which is associated with NIDDM (Bjorntorp 1992, Frayn & Coppack 1992, Kissebah 1991). There are several reasons for this, including an increased sensitivity to lipolytic agents and resistance to insulin to decrease lipolysis compared to other fat depots and also the liver may be involved with VLDL-TAG synthesis and decreased insulin extraction (Frayn & Coppack 1992). Also free fatty acid release from the intra-abdominal site is carried directly to the liver via the portal system.

There is also the possibility of the hypothalamic-pituitary-adrenal axis being involved as androgens are linked with abdominal obesity and cortisol is increased in abdominally obese women. This relates to NIDDM as cortisol is known to cause insulin-resistance together with testosterone (Bjorntorp 1992).

1.4.1 Diet and NIDDM

Diet has been the major factor in managing NIDDM, by controlling plasma glucose and lipid levels and body weight (Sheard 1995). Although fat intake also stimulates insulin release, the composition of the fat is important, as it has been shown that in rats, severe insulin resistance is caused by diets high in saturated, monounsaturated or n-6

polyunsaturated fatty acids (Storlien et al 1991). Insulin-resistance is reduced by feeding n-3 PUFA (Storlien et al 1987, 1991). The reasons for these differences are not clear, but it is suggested that it could be due to changes in membrane fluidity and hence insulin-receptor binding, or their relationship in inhibiting VLDL synthesis in the liver. Recently studies in NIDDM patients have shown that after ingestion of a diet rich in monounsaturated fatty acids compared to the traditional high-carbohydrate/ low fat diet, a more favourable lipid/glucose, insulin profile was obtained (Bonanome et al 1991, Campbell et al 1994, Parillo et al 1992).

1.5 Programming in Humans

The concept of programming has been defined by Lucas (1991) as 'the process whereby a particular stimulus or insult in the fetus establishes a permanent or long-term change in the biological response of the organism' and is based on the theory that there are critical periods of development throughout pregnancy. If there is a deficiency (or possibly an excess) of a particular nutrient at this time, then the impairment in development is irreversible, leading to changes in metabolism and possibly a greater susceptibility to certain non-communicable diseases in adult life. Such impairment could be structural e.g. islet size, functional or even at the level of metabolic pathways and enzyme action. This theory was first proposed after investigating mortality rates across the UK. Barker & Osmond (1986) found that those areas which had the highest incidence of death from ischaemic heart disease also had the highest infant mortality rates and highest numbers of low birth weight babies. From this initial information, a follow-up study was carried out in Hertfordshire where detailed birth records were available from 1910. Men from these records were traced and an association between death from coronary heart disease and birth weight was again found, with an inverse relationship between the two (Barker et al 1989). This relationship held for weight at one year also. Stroke mortality showed similar trends. These correlations remained even after correcting for socio-economic conditions such as social class, smoking and physical activity levels. From these original findings a number of studies have ensued, investigating the relationships between low birth weight and a number of non-communicable diseases later in life.

1.5.1 Programming of CHD risk factors

Once the link between birth dimensions had been established the question arose as to what caused this link. Barker's next study was in Preston, again using birth records. These dated from the 1930's, the males were recruited at age 50 years and blood pressure measurements and their medical history were recorded.

It was found that there was an increasing incidence of hypertension with decreasing birth weight with the heaviest babies associated with the lowest blood pressure. An inverse relationship was also found with placental weight, in that the larger the placenta, the higher risk of hypertension. These two factors were also independent from each other in that those who were born small with a large placenta were most at risk (Barker et al 1990). Further studies indicated that the relationship was more specific, in that a baby which as thin compared to its length, i.e. had a low ponderal index (weight (kg) / length^3 (m)), was most at risk (Barker et al 1992, 1993c). All these relationships were found with full term not premature babies. The premature babies are of small birth weight but if this is the correct weight for their gestational age then they are at no higher risk than other babies the average weight for their age that are born at term.

Additional studies have shown a continuing association between low birth weight and hypertension as highlighted by the Preston study (Barker et al 1990, Fall et al 1995, Gennser et al 1988, Law et al 1993, Leon et al 1996). The low birth weight discussed in the above studies is not at the extreme end of the population but within the normal distribution of birth weight and the increasing risk is a gradual change not one which switches on at a particular weight. Blood pressure also increased as placental weight or the ratio of placental to birth weight increased. This increase in blood pressure is not found in the neonatal stage but is amplified with age (Barker et al 1995, Law et al 1993).

One study by Leon et al (1996) suggested that it was the failure to realise growth potential *in utero* which gave the worst outcome of blood pressure in later life. This was measured by the individual being light at birth but tall as an adult. Further, more recent studies have explored this area of catch-up growth and consequently the highest rate of coronary heart disease has been found in men who were thin at birth but displayed catch-up growth so that they were an average, or above average body mass at age 7 years (Eriksson et al 1999). The possible reason for this is that those who are small at birth have less muscle mass and therefore if heavier in childhood, this must relate to an increase in body fat. The reasons why a low birth weight may lead to increased blood pressure are unknown although the diet and nutritional status of the mother have been implicated as potential risk factors. It may be that those who are born small, but then are relatively tall as adults failed to reach their growth

potential *in utero* (Leon 1998) and the stress of catch-up may have implications on their metabolic systems.

Godfrey et al (1994) showed that blood pressure increased in children whose mothers had decreased triceps skinfold thickness at 15 weeks gestation and decreased circulating haemoglobin concentration. It had previously been shown that decreased birth weight was associated with increased haemoglobin levels (Godfrey et al 1991). More recently the ratio of carbohydrate to protein in the diet of the pregnant woman has shown to influence the blood pressure of her offspring. When a maternal intake of less than 50g animal protein was consumed daily, an increase in carbohydrate intake lead to an increased blood pressure, whereas if the animal protein intake was above 50g, it was a lower carbohydrate intake which was associated with an increased blood pressure. Also these effects depended on the stage of pregnancy, with an increase in carbohydrate early in pregnancy associated with a decrease in placental growth (Godfrey et al 1996). Why these patterns of macronutrient intake were accompanied by changes in the offspring's blood pressure is not known.

Other risk factors for coronary heart disease possibly influenced by a 'programming effect' include plasma fibrinogen levels, which increase with decreasing weight at one year (Barker 1992), increased triglyceride concentrations and decreased high density lipoprotein (Fall et al 1995), increased apolipoprotein B (Fall et al 1992) and increased cholesterol (Barker et al 1993b). The factors appeared to be related to the size and dimensions of the baby.

1.5.2 Programming of NIDDM

In addition to CHD and hypertension, there has also been found a relationship between low birth weight and insulin resistance associated with NIDDM. Hales et al (1991) investigated the programming of glucose metabolism and found that men aged 50-60 years, with impaired glucose tolerance or diabetes were on average 0.5lb lighter at birth and 1lb lighter at age one year. Interestingly the same men were heavier at the time of being tested with a higher body mass index. Both fasting levels of glucose and insulin were increased significantly as was their systolic blood pressure. These values fell progressively with increasing birth weight and weight at age one year.

The study was repeated by Phipps et al (1993), but this time in both men and women from Preston. Comparable results were found, where the prevalence of impaired glucose tolerance fell from 27% in those of low birth weight (< 5.5lb) to 6% in those over 7.5lb. Increased fasting concentrations of glucose and insulin have been correlated with decreasing

birth weight (Fall et al 1995). The prevalence of non-insulin dependent diabetes mellitus (NIDDM) also falls in a similar manner (Barker et al 1993a).

One suggestion for this birth weight-glucose tolerance relationship is that poor nutrition during a critical period in pancreatic development *in utero*, leads to impaired ß-cell function and hence impaired insulin secretion (Hales et al 1991). However a more recent study investigating the relationship between NIDDM and birth weight suggested it was in fact mediated through insulin resistance rather than impaired ß-cell function as the insulin response to an intravenous glucose load had no correlation to birth weight (Lithell et al 1996).

One possible reason for the association of insulin resistance and birth weight is that babies who are thin at birth lack skeletal muscle and this is the main peripheral site of insulin action and glucose disposal. It may be impairment of this development *in utero* which leads to insulin resistance and NIDDM later in life (Barker et al 1995). The associations with low birth weight and NIDDM, CHD and hypertension are all amplified if the individual is obese.

1.5.3 Programming of Obesity

The question of fetal programming of adult obesity has been addressed to some extent. One early observation in this area comes from studies of the Dutch winter famine, which was a 'natural experiment' of energy and nutrient deprivation in pregnancy (Lumey et al 1992, Ravelli et al 1976, Susser & Stein 1994). Towards the end of the Second World War the German occupation of Holland formed an embargo on food supplies entering the Western part of the country which resulted in a winter of famine. Once the embargo was lifted towards the conclusion of the war, food quickly became readily available. The timing of the famine was very precise and did not last the duration of the human gestation period.

Ravelli et al (1976) carried out a cohort study on men whose mothers were pregnant during this time. Records were obtained from men who were recruited at age 19 into the army, their date and place of birth was recorded to pinpoint the duration, timing and degree of famine exposure. The majority of subjects came from the large cities which were the worst hit. Controls were obtained from areas which were unexposed to the famine but had a similar population density.

It was found that there was an increased incidence of obesity in the group of subjects whose mothers had been exposed to the famine in the first and second trimesters of their pregnancy, while those who were exposed to the famine in the third trimester and neonatal periods of life showed a decreased incidence of obesity compared to the unexposed controls and compared with the expected incidence of obesity in that population. Obesity in this case was defined as a value of weight for height greater than 120% of the standard as described by Jelliffe (1966). Although this was a landmark study in the case of programming of obesity, it

lacked important information such as birth weight, and an indication of body fat distribution, such as waist circumference or waist : hip ratio. Since, as will be seen later, further epidemiological evidence suggested that obesity linked to poor early growth was mainly of the intra-abdominal type.

Although this study was only done in men a similar study was carried out investigating the outcome in female offspring who had been exposed to famine *in utero* and similar results to the males were found (Lumey et al 1992). Ravelli et al, having studied these men at age 19, conducted a follow-up study, investigating obesity at age 50 years in both men at women (Ravelli et al 1999). It was concluded that, at age 50 years, maternal malnutrition was associated with increased BMI and waist circumference in women only, however when asked to recall their weight at 20 years of age, this agreed with the previous findings of male conscripts (Ravelli et al 1976). Linear growth was unaffected in the subjects studied but it appeared that adaptations to exposure to poor nutrition *in utero* resulted in greater accumulation of body fat later in life.

Further studies have also been undertaken in transitional countries such as Jamaica (Godfrey et al 1994) and Gambia (Margetts et al 1991) where the possibility exists that the energy and protein demands of pregnancy may not be satisfied (Landman & Hall 1989). In these cultures there is still a high incidence of obesity, type II diabetes and hypertension.

In all these studies, overall obesity has been considered. However a study by Law et al (1992), explored fat distribution in adulthood and its relationship with birth weight. Again birth records from Hertfordshire and Preston which had been used previously in relationship to coronary heart disease were examined. Over 1000 men were followed-up and waist circumference and hip girth measured. Findings showed that those men who had an increased waist to hip ratio, indicative of abdominal fat distribution, tended to be lighter at birth. This was also the case for those lighter at one year of age. These relationships were independent of environmental factors such as smoking, alcohol consumption, age and social class.

Abdominal fat distribution is associated with an increased risk of CHD, NIDDM and hypertension (Lapidus et al 1984) which may, in part underlie the pathology of these diseases with poor early growth and the phenomenon of 'programming'. The underlying processes linking decreased fetal growth with later abdominal fatness are unknown, however Dietz (1994) has suggested there are critical periods for obesity development and *in utero* experience may reflect on both appetite regulation and adipocyte numbers. To clarify all the changes and disease outcomes of the programming hypothesis a summary is given in figure 1.3.

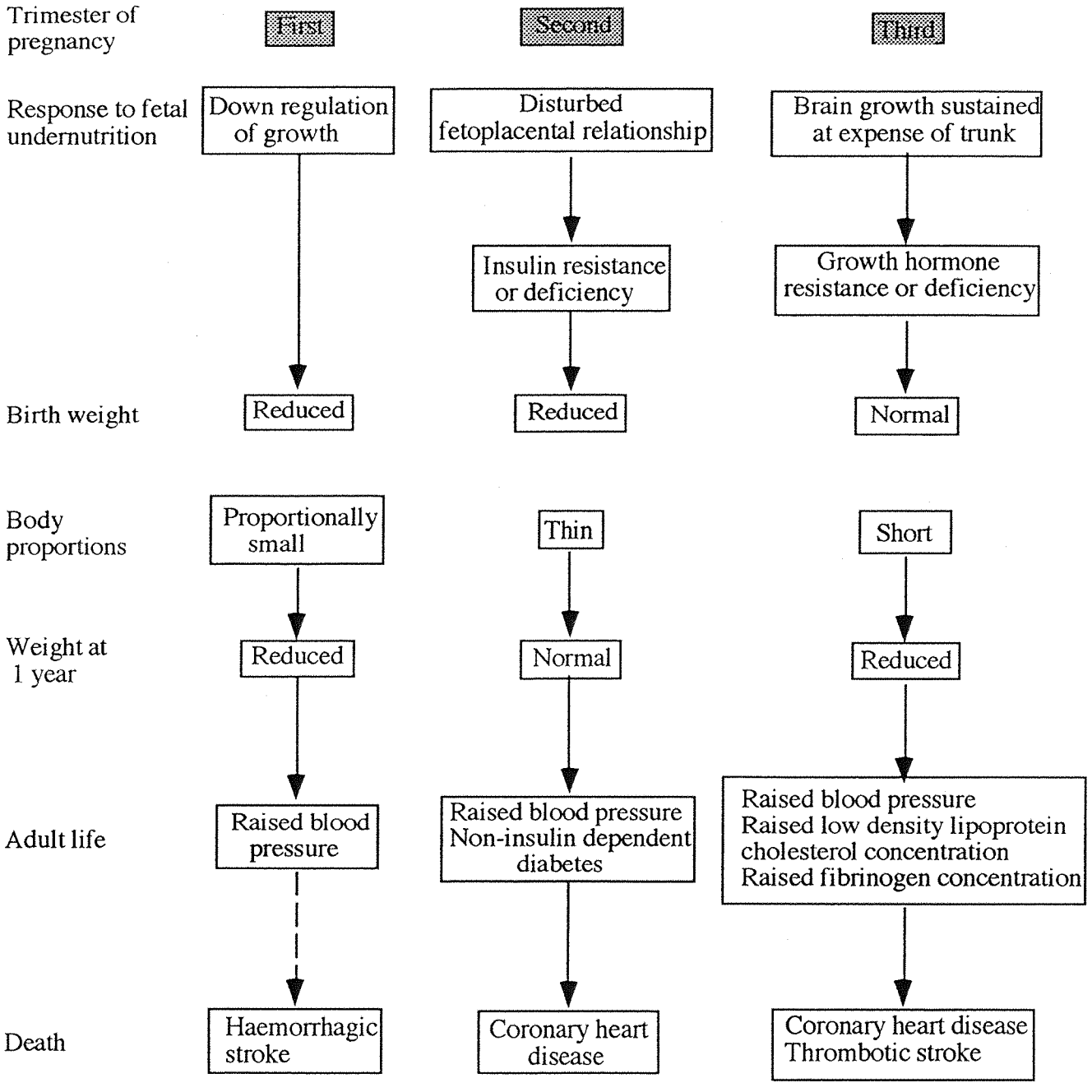

Figure 1.3 Summary of ideas in the fetal origins hypothesis linking fetal undernutrition with later abnormalities (Barker 1995)

1.5.4 Evidence against Programming

Although there is now a large body of evidence for the fetal programming hypothesis, it has not been totally accepted by the scientific community (Paneth & Susser 1995). This largely comes from twin studies as it is known that twins have a lower average birth weight compared with singletons. Three recent studies on twins (Allison et al 1995, Christensen et al 1995, Vagero & Leon 1994) have shown that although of lower birth weight, mortality was not different to that of the general population. However these babies are proportionally small as during the 3rd trimester growth is compromised in twins, and it has been shown that it is the thin or disproportioned baby rather than the generally small baby which is most at risk (Barker 1995, Barker et al 1992, 1993). In fact one of these studies did find that the shorter of the two babies was more likely to die of heart disease than the taller one (Vagero & Leon 1994). A further study by Levine et al (1994) also found correlations between birth weight and blood pressure of twins.

1.6 Programming in Animal Models

Due to problems with interpreting human data, (and some ethical causes) well-controlled studies in animal models may be more appropriate, to precisely define the influence of maternal intake on fetal outcome. An animal model has been developed to examine the influence of maternal dietary intake during pregnancy on the phenomenon of programming in the offspring (Levy & Jackson 1993). Specifically, the protein content of the maternal diet has been varied from 18% casein (control) to 6% casein. A diet this low in protein has still been shown to support pregnancy to term (Hastings-Roberts & Zeman 1977). The diets were fed for two weeks prior to conception and throughout pregnancy. At birth the mothers were transferred to standard laboratory chow thus the dietary intervention focussed solely upon gestational and not lactational influences. When the protein level was decreased from normal, disproportionate patterns of fetal and placental growth ensued (Langley-Evans et al 1996a, Levy & Jackson 1993). The offspring from the mothers who had been exposed to a low protein diet spared the growth of the brain at the expense of the trunk. Rees et al (1999) also showed differences in growth with those offspring exposed to a 9% protein diet *in utero*, in that the 9% exposed fetuses were significantly heavier at day 19 of gestation (7.5%), but by day 21 they were significantly lighter (14%). From the study by Levy & Jackson (1993), mothers fed a 6% casein diet were unable to gain weight to the same extent as those fed an 18% casein control diet and the offspring were significantly lighter at birth. However the offspring from dams maintained on a 9% casein diet were able to maintain postnatal growth similar to those fed the control 18% diet. The 9% diet was therefore the diet used in the rat model for following experiments.

1.6.1 Programming of Hypertension

This dietary intervention has been shown to increase blood pressure in offspring exposed to a 9% casein diet *in utero* compared with a control diet of 18% (Langley & Jackson 1994, Langley-Evans 1994, Langley-Evans & Jackson 1995, Langley-Evans et al 1994). This is present from 4 weeks of age and continues throughout postnatal life (Langley-Evans et al 1994), and is independent of maternal blood pressure.

Once the 'programming' of hypertension was demonstrated, the next step was to elucidate the *in utero* mechanisms by which this rise in blood pressure was brought about and how this increase is sustained in post-natal life. Blood pressure is regulated via a number of neural and endocrine pathways. It was hypothesised that maternal glucocorticoids may be mediating this effect as it was also demonstrated that in rats, excess exposure to the synthetic glucocorticoid dexamethasone *in utero* leads to low birth weight, increased placental weight and hypertension (Edwards et al 1993). Dexamethasone is able to freely cross the placenta as it is not deactivated by 11ß-hydroxysteroid dehydrogenase. Normally the fetus is protected from maternal circulating corticosterone by the placental enzyme 11ß-hydroxysteroid dehydrogenase (11ß-OHSD). This converts the active form, corticosterone, to the inactive form, cortisone. In addition it has been found that those offspring which had the lowest birth weight and highest placental weight also had the lowest levels of placental 11ß-OHSD activity (Benediktsson et al 1993). 11ß-OHSD activity is also affected by maternal protein intake, with decreasing maternal protein intake there is a corresponding decrease in 11ß-OHSD activity in the placenta (Phillips et al 1994). Glucorticoids have been shown to affect both placental size and also the rate of maturation of organs such as the kidney and heart (Seckl et al 1999). Furthermore offspring exposed to a low protein diet *in utero* and whose mothers had either been adrenalectomised or administered metyrapone, which suppresses corticosterone synthesis, showed no increase in blood pressure compared to the controls (Langley-Evans et al 1996b). Although an adrenalectomy is physiologically severe and a crude method of removing glucocorticoids, this procedure may have many more implications as other hormones would also would be affected, for example aldosterone and adrenaline.

Glucocorticoids affect blood pressure via direct vasoconstriction effects and also via mediators such as nitric oxide and angiotensinogen (Seckl et al 1999).

The renin-angiotensin system is a second endocrine system which regulates blood pressure. This may also be involved as offspring exposed to a low protein diet *in utero* showed increased angiotensin-converting enzyme (ACE) activity (Langley & Jackson 1994) and when captopril, an ACE inhibitor, was administered to those animals, the blood pressure was normalised (Langley-Evans & Jackson 1995). It has been found that rats exposed to a

low-protein diet in utero, were more sensitive to low angiotensin II doses, and it may be that glucocorticoids increase angiotensin II receptor expression (Langley-Evans et al 1999). Angiotensin II is responsible for increasing blood pressure.

As the growth pattern of these offspring in this model is altered, it is possible that the growth and hence function of the kidney is also altered. Brenner et al have investigated kidney histology in this model and have suggested that the number of glomeruli and hence nephrons at birth is inversely related to the risk of developing essential hypertension (Brenner et al 1988, Brenner & Chertow 1994, Mackenzie & Brenner 1995).

The spontaneously hypertensive rat may also be a useful model for considering the mechanisms involved in the programming of hypertension as it has been found that the SHR display similar characteristics with respect to fetal growth retardation and an increase in placental weight (Johnston 1995). There does however appear to be a lactational influence in the SHR as cross-fostering of pups to normotensive mothers resulted in a significant reduction in mean arterial pressure compared to SHR controls reared by their natural mothers (McCarty & Fields Okotcha 1994).

1.6.2 Programming of Obesity

The phenomenon of *in utero* programming of obesity in the rat has tentatively been studied by Jones & Friedman (1982). In their studies they restricted pregnant dams to 50% of their preconception food intake for the first two weeks of pregnancy, equivalent to the first and second trimester of human pregnancy. This dietary intervention not only resulted in a reduced maternal protein intake, but also in energy intake (and possibly micronutrient intake).

At birth and weaning both sexes of offspring were of normal weight but once the males reached 5 weeks old they became hyperphagic and had an increase weight gain compared to controls. At age 20 weeks a high fat diet was fed and then adipocyte measurements were made. Both sexes had an increase in fat cell size in those who experienced dietary restriction *in utero*.

Although that study was able to reproduce the Ravelli results, there were some criticisms made (Enns & Wilson 1983), in particular that adipocyte measurements were only made after high fat feeding. Jones et al (1984) therefore repeated the study a year later examining the fat cells both at weaning and in adulthood both before any high fat feeding. Another criticism was that the restricted mothers may have become hyperphagic in an attempt to overcome the restriction so the pregnant dams were pair-fed with controls to prevent this. On this occasion both the male and female offspring became obese and gained more weight

than the control animals. In adulthood there was again adipocyte hypertrophy and also increased carcass lipid content.

In a more recent study with a similar protocol (Anguita et al 1993), obesity was again found in the offspring. In this latter study the female offspring became obese but the males were in fact significantly lighter that the controls at age 53 days. Only the females showed increase carcass lipid content and fat pad weights.

These results are clearly different to those of Jones but it may be explained by differences in the protocol in that in this study although the pregnant dams were restricted for the same period of time, it was 50% of their pregnancy food intake rather than their preconception intake. A lower fat content of the maternal diet was also present in this study, together with a different strain of rats.

More recently McCarthy et al (1994) demonstrated that when rats obtained from the Southampton low-protein diet model were allowed to self-select for macronutrients, the female offspring exposed to a 9% casein diet became significantly heavier than the 18% animals. In particular it was the omental and intestinal adipose sites which had the greatest percentage increase in both males and females, which is an indicator of abdominal obesity.

Anguita et al (1993) hypothesised that decreased brown adipose tissue (BAT) activity and trioodothyroxine (T_3) activity might underlie the obesity and in the latter part of gestation changes in fetal environment can significantly enhance BAT development (Symonds & Stephenson 1999). Jones et al (1982) suggested that hypothalamic function may be altered in these offspring. In support of this it has been proposed that there are critical periods of hypothalamic development during specific times in pregnancy (Widdowson 1971, Widdowson & McCance 1963) and if there is a shortage of protein at these times development is impaired. The hypothalamus regulates appetite (Powley 1977) and energy balance (York 1987).

1.6.3 Programming of Other Parameters

There have been several studies indicating that rats who have experienced protein restriction *in utero* have altered glucose tolerance or insulin responses to a glucose load (Dahri et al 1995, Langley et al 1994, Phillips et al 1994, Smith et al 1975, Tse et al 1995). A low protein diet has been shown to influence the development of the pancreas with a decreased islet cell proliferation, insulin content and islet vascularisation (Dahri et al 1991, Snoeck et al 1990). Further, there are changes in the liver such as insulin growth factor I (IGF-I) gene expression (Munaku et al 1995). Pups born from dams which had been fed a low protein diet during pregnancy had decreased plasma and liver IGF-I and liver IGF-I

mRNA concentrations. The development of hepatic zonation, which are areas within the liver where enzymes are present regulating either energy utilisation or energy production, is also affected (Desai et al 1995), together with growth hormone receptor expression in both the liver and muscle (Barker et al 1995).

Finally this model of programming also alters the acute phase response in adult rats which had been exposed to a low protein diet *in utero* (Langley et al 1994). The response was blunted in the low protein exposed rats with particular reference to the anorectic response, hepatic zinc uptake and pulmonary glutathione uptake.

1.7 Low protein diet

If rats are fed a low protein diet from weaning, body weight is reduced and adipocyte number is decreased (Tulp & Horton 1981). Even when the rats are returned after four weeks to the protein intake of that of the controls, growth does not reach that attained by the controls. If the rats are protein-malnourished for several weeks an attempt to compensate for the deficiency is made by overeating and energy intake relative to body weight is increased. This increase in energy intake is not accounted for in carcass energy and therefore must be dissipated in an increase in energy expenditure, possibly through an increase in thermogenesis (Tulp & Horton 1981).

It has been proposed that the stimulus for the increase in food intake is neuropeptide Y (NPY) (White et al 1994). They showed that rats which had been fed a low protein diet had elevated NPY gene expression and levels in the hypothalamus. NPY stimulates food intake particularly carbohydrate (Morley et al 1987). Cellular protein synthesis decreased apparently due to a change in mRNA translation (Young & Marchini 1990). The overall capacity of the muscle cell is decreased with prolonged exposure to a decrease in protein intake.

Protein degradation in the liver is initially increased on a low protein diet but then declines as the insult continues (Garlick et al 1975). Amino acid oxidation is decreased and it appears that this is specific to the amino acid limiting in the diet (Young & Marchini 1990). When rats were given a low protein diet, they increased the amount eaten to try to combat the deficiency compared to the controls and hence were obtaining a higher energy intake (Levy & Jackson 1993, Manaker & Navia 1973). This was particularly evident during pregnancy.

1.7.1 Pregnancy and a Low Protein Diet

Numerous studies in both humans and animals have investigated the effect of energy or protein restriction on the maternal weight gain and fetal weight. It has been calculated that the fetal weight gain in the last trimester of pregnancy is strongly related to the maternal weight gain, indicating that the nutrients available are shared between the mother and the fetus equally (Rosso 1981).

In severely protein and energy restricted rats the conceptus was not favoured over the mother. When the diet is returned to a control diet, the mother was greatly favoured over the fetus in terms of nutrient partitioning, which indicated that the fetus was not actively parasitic on the mother.

Particular interest has focused on protein-energy malnutrition during pregnancy. Dietary protein is especially important because although it contributes only 8% of weight gained by the mother in pregnancy, over 60% of this goes into fetal and placental tissues compared to only 10% for fat (Garrow & James 1993). Protein is particularly important in the second trimester where there is rapid growth of the fetal organs. Most of these studies have been carried out on laboratory rats.

The normal level of intake of protein for rats is 18-20% protein by weight. Female rats which have been fed a diet low in protein (0-9% by weight) throughout pregnancy give birth to smaller pups (Hastings-Roberts & Zeman 1977, Jones 1976, Levy & Jackson 1993, Mayel-Afshar & Grimble 1983, Shrader & Zeman 1969, Van Marthens & Shimomaye 1978, Zeman & Stanbrough 1969). Protein metabolism in pregnancy has been proposed to be biphasic, with an anabolic phase and a catabolic phase (Naismith & Morgan 1976). The anabolic phase occurs at the beginning of pregnancy when protein has a net deposition within the maternal tissues, mainly within muscle. The catabolic phase is when this protein is broken down to amino acids and used for protein synthesis for the fetus and placenta.

In low-protein-fed rat dams weight gain in the first week of pregnancy was similar to that of the controls, however the protein deficient mothers maintained consistently smaller fetuses (Zeman & Stanbrough 1969). However many of these studies have looked at the effect of dietary protein at the extremes of intakes, either very low (0-6%) or very high (24-30%). At these extremes both cell size as well as cell number were affected across the whole fetus. The timing of protein-deficiency seems also to be important. Early maternal protein deprivation for a 5-6 day period does not affect, in rats, the chance of pregnancy carrying to term, but if this is continued, failure of a litter increases significantly (Zeman & Stanbrough 1969). The organs of the fetus develop at different stages during pregnancy, and at specific timepoints during pregnancy there will be a time of rapid cell division for each organ. If the

protein-deprivation to the dam is within a discrete length of time, then this may rise to the possibility of specific organs being affected (Jackson 1992).

Changes in body composition occur if the mother is protein-restricted during pregnancy, caused by changes in both muscle and adipose sites (Glore & Layman 1985, Young et al 1985). This may be due to the fact the mother preferentially uses fatty acids for energy, sparing glucose (Herrera et al 1967). Kidney, liver and intestine weights were decreased although protein or DNA concentrations did not differ (Pond et al 1969).

1.7.2 Offspring from Mothers fed a Low Protein Diet

Several studies have examined the growth of offspring from rat dams that have been fed a protein deficient diet during gestation. Manipulation of the maternal diet revealed that it was specifically protein which stunted the offspring, as restoration of other components such as vitamins or energy intake did not prevent stunting (Hsueh et al 1967). Although birth weight and subsequent growth is decreased (Chow & Lee 1964, Hsueh et al 1967, Levy & Jackson 1993, Pond et al 1969, Schoknecht 1993, Zeman 1969), the brain appears to be spared (Langley-Evans et al 1996a, Pond et al 1969), as the DNA content or concentration is unaffected, although there is a decrease in protein synthetic activity in those offspring exposed to the low protein diet *in utero*.

The pituitaries of these stunted offspring are reduced in size and have a lower concentration of growth hormone (Stephan 1971) and if growth hormone is replaced at weaning the stunting is reversed (Chow & Lee 1964). The stunted offspring also presented with anaemia and decreased resistance to hypothermia which was corrected with the administration of growth hormone (Chow & Lee 1964). The body composition of offspring from protein restricted mothers is not significantly different from control offspring at birth (Allen & Zeman 1971). However protein metabolism is altered in these offspring (Lee & Chow 1965, 1968, McLeod et al 1972), as although there is a normal ability to absorb protein from the diet (McLeod et al 1972), there is an increased excretion of nitrogen in the urine (Lee & Chow 1965, McLeod et al 1972). This is in spite of having normal plasma levels of nitrogenous compounds. The excess urea loss suggest altered protein metabolism and as these offspring have decreased growth hormone, this could be responsible in part as this hormone stimulates protein synthesis (Stephan et al 1971).

Gambarella et al (1990) also showed that in male rats which had been exposed to a low protein diet *in utero*, there were changes in plasma corticosterone, aldosterone and 5-hydroxytryptamine (5-HT) levels. These hormones are all involved in regulating a number of intermediary pathways of carbohydrate, fat and protein metabolism.

Previous studies have explored the feeding behaviour of rats previously exposed to a low-protein diet *in utero*. One previous study showed that offspring of mothers fed a low protein diet throughout pregnancy reflect their mothers low protein intake (Leprohon & Anderson 1980). In a more recent study, rats were allowed to self-select their dietary macronutrients, their mothers having been fed differing degrees of protein content in their diets (Leprohon & Anderson 1992). In this study it was found that weanling rats were able to regulate protein intake separately from energy intake and that their protein intake correlated with the percentage protein of the maternal diet fed throughout gestation and lactation. The changes in protein intake even occurred over relatively normal ranges of protein content in the maternal diet.

The appetite for fat and carbohydrate of rats exposed to protein deficiency *in utero* have not been studied. Changes in the intake of these macronutrients may reflect changes in the handling of these nutrients. This is of particular interest, because of the strong relationship between fat intake and obesity.

1.8 Regulation of Appetite

The exact neurochemical mechanisms which regulate nutrient intake are still not fully understood. The mechanisms are complex and the pathways interact at several levels. Appetite is regulated both by peripheral and central mechanisms with the hypothalamus important in integrating all of the signals. Meals increase the activity of the sympathetic nervous system (SNS) which can be measured using noradrenaline concentrations (Welle 1995). Tryptophan and 5-hydroxytryptamine (5-HT) both decrease food intake (Bray 1987) and these transmitters are found to be increased in brains of malnourished rat pups (Sobotka et al 1974). Neuropeptide Y (NPY) stimulates feeding on a daily basis and also in long-term regulation and food deprivation stimulates its release (Kaiyala et al 1995). NPY is increased in both the obese Zucker (fa/fa) rat (Beck et al 1990) and the ob/ob mouse (Wilding et al 1993).

The peripheral mechanisms include the circulating nutrients, metabolites and hormones such as corticosterone, insulin and cholecystokinin (CCK). Insulin has been shown to be an important hormone in food intake regulation by being sensitive to glucose utilisation and it increases food intake in diabetic rats regardless of the type of diet eaten (Willing et al 1994). Recent work suggests that adult hypertension can develop from rat pups exposed to excess glucocorticoids *in utero* (Benediktsson et al 1993), although the mechanism for this is not clear. Excess progesterone has been shown to stimulate weight gain, food intake and fat deposition, whereas chronic elevation of testosterone has been shown to decrease fat mass and food intake of male rats (Wade & Gray 1979).

The regulation of appetite can be summarised as follows. Protein appetite is thought to be regulated by the endogenous opioids, as the experimental administration of these substances stimulate protein intake (Leibowitz 1992).

Carbohydrate appetite is stimulated by NA, NPY and GABA in the brain in association with corticosterone in the blood. These seem to be specific for carbohydrate since when rats have been given separate macronutrient diets, and then injected with these transmitters, no increase in protein or fat was noticed (Leibowitz 1992). Carbohydrate intake is inhibited by 5-HT and CCK. Finally the neuropeptide galanin (GAL), opioid peptides and aldosterone or glucocorticoid type I receptor all stimulate fat intake by acting on the hypothalamus (Leibowitz 1992). Dopamine, as well as inhibiting protein has the same effect on fat intake.

1.9 Fetal Growth

The term growth refers to changes at various levels from hyperplasia and hypertrophy to differentiation of cell, to a change in form and function. Nutrition is involved at all of these stages through the supply of substrate and cofactors. The fetus receives all its requirements for growth from the mother or her diet via the placenta.

The maternal body undergoes many changes in composition and metabolism during pregnancy and a diet which best meets these changing requirements is needed, for the greatest well-being of the offspring. There are changes in the mother, in hormones during pregnancy which lead to net deposition of energy and nutrients. In early pregnancy, progesterone has been shown to inhibit urea production from the liver and this can been shown by a 40% decrease in arginosuccinase, an enzyme of the urea cycle and a 20% reduction in blood urea concentration. At the same time insulin stimulates blood amino acids to be taken up into maternal muscle. Later in pregnancy, oestrogen stimulates the anterior pituitary which activates the release of amino acids from maternal muscle into the blood to make them available to the growing fetus. Meanwhile the action of insulin on maternal muscle is inhibited.

Although there are species differences, there are general statements that can be made for the transport of glucose, oxygen, carbon dioxide, and certain amino acids across the placenta (Morriss et al 1994). The requirements for each specific nutrient vary as, for some, the mother can maintain fetal development at the expense of her own well being, but for other nutrients the fetus competes equally with the maternal needs.

The formation of the fetus requires a tightly co-ordinated supply of substrate and cofactors, orchestrated by a series of endocrine actions upon the maternal system and the feto-

placental unit. Mistiming of any of the events in fetal development can result on the one hand in congenital malformations (for example neural tube defects), or more subtle shifts in the form or function of the offspring. Indeed Lucas (1991) suggested that nutritional stress be it a deficit or excess at a critical or sensitive period in early (fetal or postnatal) life may influence metabolic, developmental and pathological processes well into adulthood. In this context, Jackson (1990) explained how the once held view that the fetus is afforded protection from the widely varying dietary intake of its mother now needs to be strongly questioned in the light of the recent epidemiological work of Barker et al, and the experimental findings of Lucas et al. This has led to the reappraisal of maternal nutritional requirements.

1.10 Aims

Detailed studies on birth records indicate that decreased birth weight or disproportionate growth and presumably poor maternal nutrition lead to an increased risk of coronary heart disease, hypertension and non-insulin dependent diabetes mellitus (NIDDM). Additionally high dietary fat consumption and adult obesity further increase this risk. The aim of this investigation was to explore, using a rat model, the effect of a poor maternal plane of nutrition on body fat accumulation and distribution and appetite. Secondly, this was further examined in relation to systolic blood pressure and glucose homeostasis and how post-natal diet interacted with pre-natal diet upon these parameters.

Protein is probably the most important macronutrient influencing fetal growth and development. An animal model has been developed (Langley & Jackson 1993) which supports the epidemiological observations of Barker and colleagues (see section 1.5), whereby female rats are fed a 9% protein diet by weight compared to an adequate 18% diet fed to control rats, prior to and during gestation. Using this model it has been reported that offspring exposed *in utero* to the low-protein diet have increased systolic blood pressure and impaired glucose tolerance when fed on standard laboratory chow. In a preliminary investigation we used this model to examine whether appetite and adiposity in post-natal life could be influenced by prenatal exposure to a low-protein diet (McCarthy et al 1994). It was found that patterns of macronutrient appetite were altered in the group exposed to a low-protein diet *in utero* in both males and females. Both sexes reduced their carbohydrate intake which was balanced in the males with an increase in fat intake and in the females with an increase in protein intake. Total metabolisable energy intake was similar between the 9% and 18% offspring for each sex. The offspring from dams in the 9% group also had increased adiposity particularly at the omental and intestinal sites. The female offspring were most affected as all the nine adipose sites measured, were heavier.

Therefore the hypothesis underlying this programme of work proposes that a maternal diet marginal in protein content during a critical period of fetal development alters fetal programming with long term metabolic and morphological consequences for the fetus. This is manifested as changes in appetite for specific macronutrients and alterations in their metabolic handling, and these changes are influenced by the background hormonal environment. Such programming will ultimately increase the risk for the development of non-communicable diseases such as NIDDM and coronary heart disease.

Chapter 2.0
GENERAL METHODS

2.1 Maternal Breeding Protocol

All studies complied with the Scientific Procedures Act 1986. Female virgin Wistar rats from the University of Southampton breeding colony were used with initial body weight ranging between 205g-255g. Rats were housed individually in hanging wire bottomed cages with sawdust trays beneath. The room temperature was maintained at 21°C on a 14:10hr light cycle with lights on at 6.00am. Half of the dams were fed 9% casein diet and the other half, which was the control group, an 18% casein diet. The food was placed within the cage (see table 2.1 for composition). All had free access to tap water.

After an acclimatisation of two weeks on the diets, the rats were mated, which took between one and ten days. Mating was confirmed by the appearance of a vaginal plug and this was taken as day 0 of pregnancy. The dams were maintained on these diets and the mothers' growth measured every two days and food intake every week . On day 15 of pregnancy the animals were transferred to maternity cages, comprising of deep plastic boxes with a thick layer of wood shavings. When term approached, shredded paper towel was also added to the cages.

Within five hours of birth the dams were transferred onto standard laboratory chow (SDS CRM(X) see Appendix A for composition), and hay was placed in the cages. Each litter was culled to eight pups, four females and four males where possible.

At ages twelve and twenty-four days the pups were weighed and at age four weeks the pups were weaned and the mothers killed by carbon dioxide inhalation.

2.2 Maternal Diet Composition

The 18% and 9% casein diets comprised of the following components expressed as % weight.

The constituents were chosen to provide pure sources of protein, carbohydrate, fat and non-starch polysaccharides (NSP). Snowflake is maize starch and Solka-floc is powdered cellulose. The mineral and vitamin mixes were commercial preparations - AIN-76 (American Institute of Nutrition -76 see Appendix B for the composition).

Substance		18% Diet	9% Diet
Casein	(Besnier, France)	18.0	9.0
Snowflake	(Cerestar)	42.5	52.0
Sucrose	(Tate & Lyle UK)	21.0	21.0
Solka-floc	(James River USA)	5.0	5.0
DL-Methionine	(Sigma)	1.0	0.5
Mineral Mix	(AIN-76)	1.8	1.8
Vitamin Mix	(AIN-76)	0.5	0.5
Corn Oil	(CPC UK)	10.0	10.0
Choline	(Sigma)	0.2	0.2
Energy (kJ/g)		15.7	15.7

Table 2.1 : Composition of maternal diets

Choline was added as it was not included in the vitamin mix. Methionine was added to improve the biological value of casein and to prevent sulphur deficiency. There is a high requirement as fur is mainly keratin which has cysteine content.

The diets were made up in batches in an industrial mixer, with each component added separately and time allowed for adequate mixing. The corn oil was added after the dry ingredients and then approximately 2 litres of water, to allow the mixture to hold together. The diets were coloured with food dye, to allow easier recognition. Diets were formed into balls weighing approximately 30-60g, and dried in an oven at 60°C for 2-3 days. They were then cold stored in batches at 0°C until they were required.

Each new batch of diet was analysed for its nitrogen, extractable fat and gross energy content. Details of methodologies can be found in Appendix C.

2.3 Offspring Protocol

At age four weeks the offspring were weaned into individual hanging wire-bottomed cages. A total of eight offspring of each sex from each maternal diet were used per group for the subsequent dietary intake studies. At the end of the measurement period the animals were killed by lethal injection followed by cervical disclocation. Regional fat masses, organs and muscles were weighed. The carcasses frozen for later analysis of total body composition.

2.4 Offspring Diet Composition

2.4.1 Self-Selection Macronutrient Diet Composition

The offspring were fed a choice of three pure macronutrient diets balanced for vitamins, minerals and cellulose. Compositions are shown in table 2.2 below.

Substance		Carbohydrate (% wt)	Protein (% wt)	Fat (% wt)
Lard	(Unecol)	-	-	85.0
Corn Oil	(CPC UK)	-	-	5.0
Casein	(Besnier, France)	-	90.0	-
Snowflake	(Cerestar)	62.0	-	-
Sucrose	(Tate & Lyle UK)	28.0	-	-
Solka-floc	(James River USA)	5.0	5.0	5.0
DL-Methionine	(Sigma)	-	0.1	-
Mineral Mix	(AIN-76)	2.7	2.7	2.7
Vitamin Mix	(AIN-76)	2.0	2.0	2.0
Choline	(Sigma)	0.2	0.2	0.2
Magnesium Hydroxide	(Sigma)	0.1	0.1	0.1
Energy (kJ/g)		15.1	15.1	33.9

Table 2.2 Self-selection macronutrient diet composition

For the carbohydrate and protein diets, all the ingredients were mixed in an industrial mixer and after 15 minutes water was added until the mixture started to hold together. Food colouring was added to the carbohydrate diet to enable spillage to be identified. The diets were then pressed into 2.5cm deep baking trays and dried in an oven for 24 hours at 60°C. When dry, the diets were stored in airtight plastic containers in a cold room (0°C) until required.

For the preparation of the fat diet, the lard was melted in a saucepan until soft. Corn oil and the dry ingredients were then incorporated into the lard. Corn oil was added for its linoleic acid content (50%). The diet was then stored in plastic containers at 0°C.

2.4.2 Composite Diets Composition

The high-protein, high-carbohydrate and high-fat diets were fed as complete diets with the following composition expressed as percentage weight. Magnesium hydroxide was included in these diets to avoid deficiency. The high-fat diet was prepared and stored in the same manner as before (section 2.4.1). The high-protein and high-carbohydrate diets were made up in batches in an industrial mixer. The corn oil was added last and then water to allow the mixture to hold together. The diets were then dried in an oven at 60°C overnight and stored at 0°C until required.

Substance		High-protein (% wt)	High-Carbohydrate (% wt)	High-fat (% wt)
Lard	(Unecol)	-	-	29.5
Corn Oil	(CPC UK)	10.0	10.0	10.0
Casein	(Besnier, France)	49.0	20.0	20.0
Snowflake	(Cerestar)	22.0	51.5	22.0
Sucrose	(Tate & Lyle UK)	10.0	10.0	10.0
Solka-floc	(James River USA)	5.0	5.0	5.0
DL-Methionine	(Sigma)	1.5	1.0	1.0
Mineral Mix	(AIN-76)	1.7	1.7	1.7
Vitamin Mix	(AIN-76)	0.5	0.5	0.5
Choline	(Sigma)	0.2	0.2	0.2
Magnesium Hydroxide	(Sigma)	0.1	0.1	0.1
Energy (kJ/g)		17.2	17.6	23.4

Table 2.3 : Composite diets composition

2.5 Self-Selection Regimen / Weighed Intake

The animals were housed individually in wire-mesh cages with blotting paper on top of the sawdust trays beneath. The three macronutrient diets were weighed into separate bowls and placed randomly in each cage. After 24 hours the remaining food was reweighed, together with the spillage and intakes were then calculated. Fresh food was put back in random positions in the cage and the procedure repeated as indicated in the relevant chapter. Tap water was available ad lib.

2.6 Measurement of systolic blood pressure

The rats were transferred in their home cages to the experimental room maintained at 28°C for 2-3 hours prior to systolic blood pressure (SBP) measurements to allow the tail vein to dilate sufficiently to obtain a blood pressure signal. Once acclimatised, each rat was placed in a clear plastic restrainer with an adjustable nose cone to limit movement without causing discomfort, during the recording period.

The blood pressure equipment comprised of an inflatable cuff mounted in plastic attached to the end of the restrainer, through which the tail was placed. The cuff was connected to the blood pressure monitor (model 229 amplifier, IITC Life Sciences, California, USA). This inflated the cuff and detected the return of the pulse using a light sensor. The data was transferred onto a computer (IBM 386) and was displayed as a graph. The maximum cuff inflation of 300mmHg, intensity of recording and filter parameters, to remove noise of the animal moving, were entered into the computer data acquistion programme. The programme was run and a trace showing the pulse against time was displayed. Once a steady pulse was maintained, the cuff was inflated and then released at a rate of 3mmHg/sec. At 300mmHg the tail artery was occluded and a flat line appeared on the screen. As the cuff was deflated the pulse returned and displayed as a return of an oscillation on the computer screen. Once fully deflated the computer calculated the systolic blood pressure and heart rate from the pressure reading at which the pulse returned.

The procedure was repeated until at least three steady traces were obtained but with no more than five inflations in five minutes and the rat was not kept in the tube for longer than ten minutes. The mean SBP for each rat and for the group was calculated.

2.7 Intravenous Glucose Tolerance Test

The rat was anaesthetised with sodium pentobarbitone (Sagatal, Rhone Merieux) at a dose of 1ml/kg, i.p. Once anaesthesia was established, the rat was placed on its dorsal surface and a thermometer inserted 2cm into the rectum and secured with cotton. A lamp was positioned over the rat to maintain core temperature at 37°C. The skin overlying the throat was excised and the trachea exposed using blunt dissection. A small incision was made between two cartilage rings and a cannula inserted 2cm into the trachea and secured with cotton to maintain a clear passage of air. A 1cm length of each jugular vein was exposed and separated using blunt dissection. An arterial clip was attached to the cordal end and the surrounding area massaged to fill the vein with blood in order aid insertion of the cannula. If the vein failed to fill, two drops of lignocaine solution were dropped onto the vein to enable dilation. The cranial end was then tied off with cotton and a small incision made in the jugular and a cannula was inserted approximately 2cm, using

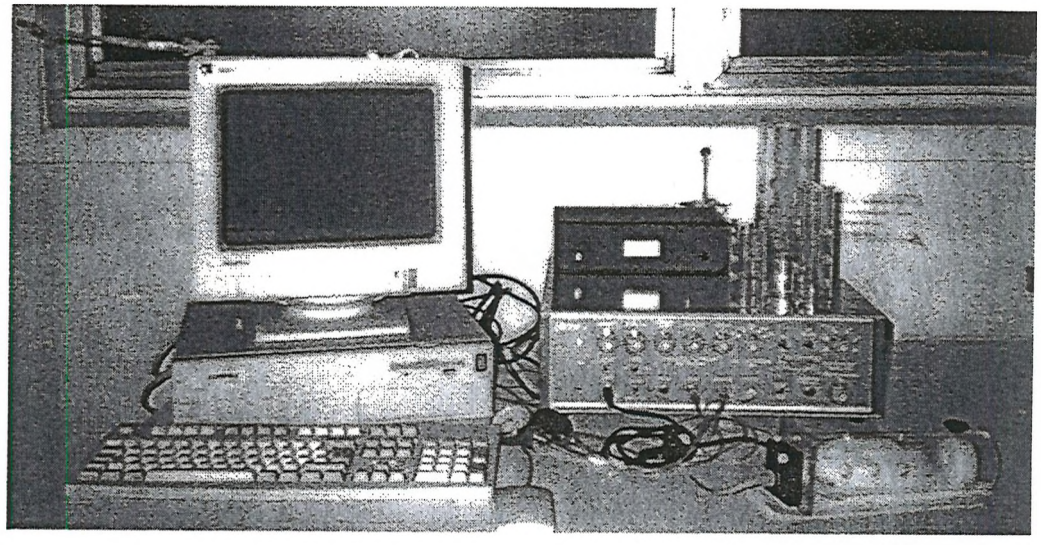

Figure 2.0 : Photograph of set-up for measurement of systolic blood pressure

PP50 tubing. This was attached to approximately 4cm of silicon tubing and connected to a 23 gauge needle attached to a 1ml syringe containing 0.9% saline and 100U/ml heparin. 0.15ml of heparinised saline was injected. This jugular was used to sample blood, with a sterile syringe attached to collect the sample. The original syringe was replaced and a further 0.1ml of heparinised saline injected to clear the tubing. Through the second jugular vein, a glucose solution (1.2mg/ml) was administered at a dose of 2g/kg body weight at time zero. The solution was injected over a period of 20 seconds to minimise cardiac stress. The first 0.1ml of blood removed was discarded at each sampling point and a further 0.2mls of blood was removed at timepoints -10, -5, 0, 5, 10, 15, 20, 30, 40 and 60 minutes. 0.1ml of whole blood was collected in an Eppendorf tube containing 100μl of heparin (100U/ml) and 100μl of tricarboxylic acid (TCA, 10% solution in distilled water).The samples were used for the determination of plasma glucose concentration using a commerically available kit (Sigma. See section 2.8). The remaining blood (0.1ml) was added to a second Eppendorf tube containing 100μl heparin only and used for the measurement of immunoreactive insulin using a commercially available radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories, Texas. See section 2.9). Both tubes were immediately vortexed and kept on ice. The tubes were centrifuged for 5 minutes. Plasma was decanted and frozen at -20°C for later analysis.

A terminal cardiac puncture was performed and 2mls of blood was removed into a heparinised syringe. The animals were then killed by cervical dislocation. The blood was separated and the plasma frozen.

2.8 Plasma Glucose Assay

The glucose (Trinder) reagent was reconstituted by adding 500ml of distilled water. TCA (0.1ml) was added to 1ml reagent as the blank. The spectrophotometer was zeroed at 505nm. 120 1.6ml cuvettes were labelled in duplicate for the standard which was glucose/urea nitrogen 300/30 mg/dl, and then the samples. 1ml of reagent was pippetted into each of the cuvettes. At 15 second intervals 5μl aliquots were added to the appropriate cuvette. The solutions were mixed by gentle inversion before placing in a heated water bath at 37°C for exactly 10 minutes. After this time the optical density was read at 505nm.

Samples were analysed in batches of 120 cuvetttes, with each batch having a new blank and standard recorded.

2.8.1 Calulation of Plasma Glucose Concentration

The glucose concentration was determined from the following equation:

$$Plasma\ Glucose\ Concentration\ (mmol/l) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} * 300 * 0.0555$$

300 = concentration of the standard (mmol/l)
0.0555 converts mg/dl to mmol/l.

2.9 Plasma Insulin Assay

Plasma immunoreactive insulin was removed by radioimmunoassy (RIA), using ^{125}I-labelled insulin as the tracer and unlabelled insulin as standards. The sensitivity of the assay was 0.13ng/ml and the within assay coefficient of variation (CV) was 3.6%.

2.9.1 Reconstitution of Reagents

The reagents, in a protein-based buffer with sodium azide as a preservative, were reconstituted with deionized water prior to the assay. Six insulin standards with concentrations of 0, 0.2, 0.6, 2, 6 and 12 ng/ml of insulin formed the standard curve. The vial containing 0 ng/ml was reconstituted with 5ml and the remaining with 1ml of deionized water, according to instructions in the kit

The radioactive insulin [I-125] was reconstituted with 11ml and the insulin antiserum with 10ml of water. The insulin controls which contained a low and high concentration of insulin, 0.6 and 2 ng/ml respectively, were each reconstituted with 1ml of deionized water. They were all stored at 4°C but then allowed to reach room temperature before use.

2.9.2 Assay

Polycarbonate tubes (LP4) were labelled in duplicate for total counts, non-specific binding (NSB), standards, controls and samples. 100μl of the standards and controls and 25μl of the samples with 75Ul of buffer were added to the appropriate tubes. 200μl of 0ng/ml insulin was added to the NSB tubes.

100μl of insulin antiserum was added to all but the NSB and total count tubes and 100μl of insulin [I-125] was then added to all tubes. These were then vortexed and incubated for 16 hours at 4°C. After this time the precipitating reagent was mixed thoroughly and 1ml added to all the tubes except the total count tubes. After vortexing

they were incubated at room temperature for 15 minutes before centrifuging at 1500x g for 20 minutes at 6°C.

All the tubes apart from the total counts were decanted and any excess liquid blotted away from the inside of the tubes, to leave a pellet containing the bound insulin. Tubes were counted in a gamma counter (LkB) for two minutes.

2.9.3 Calculation of Insulin Concentration

The duplicates were averaged and from the total counts the percentage of binding at each conentration of unlabelled insulin was calculated. A standard curve was plotted using percentage bound versus unbound insulin concentration. From this the concentration was determined in the samples in ng/ml.

2.10 Determination of Skeletal Muscle Fibre Type

The principle of the method used to determine muscle fibre type is that sectioned muscle would be stained for ATPase, which is an indicator of slow-twitch Type I fibres as these stain dark, where as fast-twitch Type II fibres have less ATPase and hence stain lighter.

2.10.1 Sectioning

Immediately after the end of the glucose tolerance test, the rat was killed by cardiac puncture and cervical dislocation. Four muscles of the hind left leg namely anterior tibularis, extensor digitorum longus, soleus and gastrocnemius were removed, weighed and immediately frozen in liquid isopentane precooled to -80°C. Once frozen they were stored at -80°C until required for sectioning and staining.

The muscles were sectioned using a cryostat at -20°C (Reichert-Jung). The muscles were taken from -80°C and allowed to warm to -20°C in the cryostat before a transverse block was cut from the centre of the length of muscle and mounted onto a chuck using OCT compound (Miles). Care was taken that the muscles from separate rats were sectioned from as near the same position as possible. Once mounted transverse sections were cut at 20μm thickness until a clear and straight edge was formed. At least three 10μm sections were cut for each muscle and transferred directly onto a single slide, precoated with poly-L-lysine (Sigma) as an adhesive. This was achieved by soaking the slides in a 0.1% solution of poly-L-lysine for 5 mins, drying for 1hr at 60°C and then storing at 4°C. Upto 50 muscles were sectioned at a time and the slides were placed in a rack ready for staining. The slides were allowed dry at room temperature for 3hrs before staining to demonstrate actomyosin ATPase activity (Guth & Samaha 1970).

2.10.2 Staining

The slides were preincubated for 10 minutes in 50mM solution of potassium acetate before rising twice (1 minute each) in Tris buffer solution (100mM) and incubating in an alkaline buffer solution containing 2.7mM ATP for 15 minutes at 37°C, pH = 9.4. After three 30 second rinses in 1% calcium chloride, the muscle sections were incubated in 2% cobalt chloride solution for 3 minutes. The slides were then washed in distilled water and two 30 second changes of an alkaline buffer solution before finally staining in 1% ammonium sulfide for 3 minutes. The sections were washed in running water for at least 5 minutes before dehydrating in 70% and then 100% ethanol (four changes, 2 minutes each) and clearing in xylene (two changes, 2 minutes each). The sections were then mounted using DPX. Details of the solutions can be found in Appendix D.

According to this method the slow-twitch Type I fibres stained dark whereas the fast-twitch Type IIa fibres were stained pale. The Type IIb fibres were of intermediate staining.

2.10.3 Fibre Type Analysis

The fibre types were anaylsed by counting by eye a set area of each section determined by a graticule. The Type IIa and Type IIb fibres were counted together. The mean of three sections for each muscle was taken and the percentage of Type I to Type II fibres was determined for each muscle.

2.11 Regional Adipose Tissue and Organ Weights

Eight regional adipose sites, four of which were subcutaneous sites (intrascapular white (WAT) and brown adipose tissue (BAT), inguinal and popliteal) and four intra-abdominal sites (epididimal/parmetrial, omental, intestinal, retroperitoneal) and five organs (liver, kidney, adrenal, heart, brain, pituitary) were dissected from each animal, blotted dry and quickly weighed.

Once the muscles were removed the skin of the rat was cut up the midline of the abdomen and then freed from the underlying tissue. The inguinal fat was removed up to the boundary of the femoral vein and down to the back wall. Popliteal fat was removed by cutting the skin away from the right leg and cutting through the biceps femoris. The skin was then dissected from between the shoulder blades to expose the interscapular WAT and BAT sites. The WAT was removed first using blunt dissection and then the BAT, with care being taken to ensure all tissue was removed from behind the rhomboid.

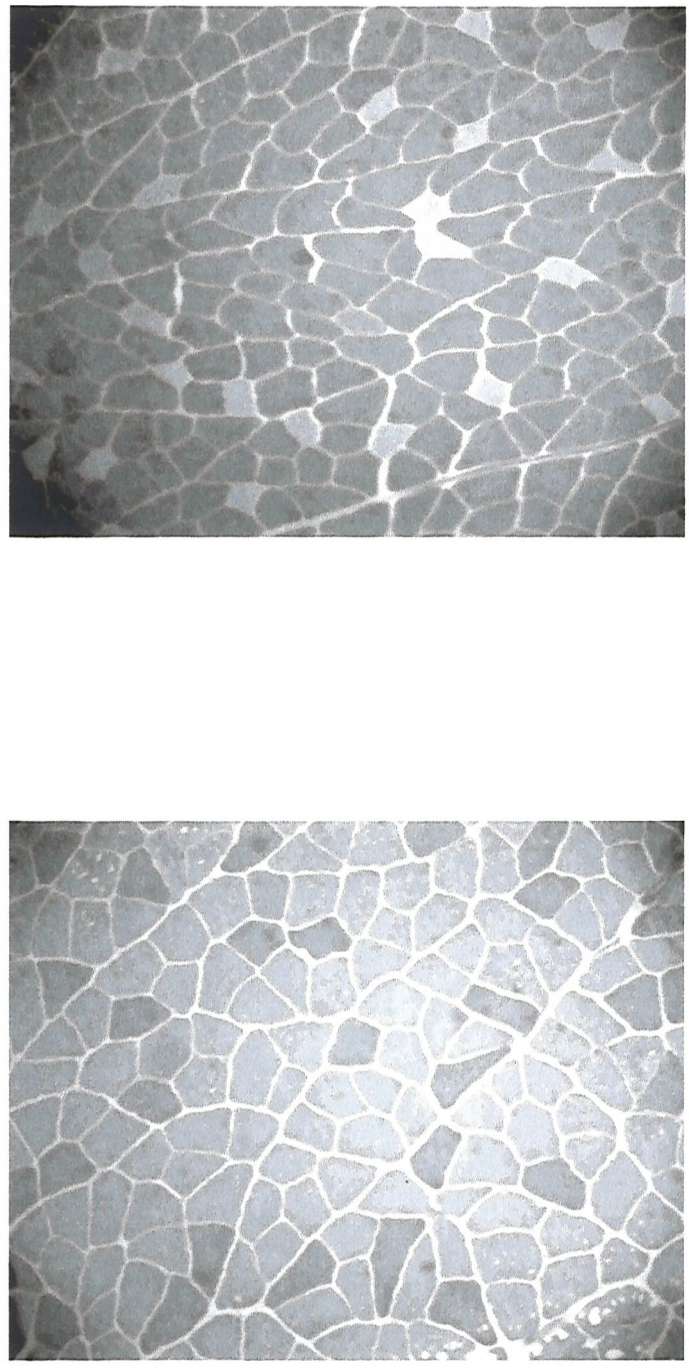

Figure 2.1 : Examples of fibre type staining for ATPase using muscles from animals referred to in Chapter 7.
Type I stained dark, Type II stained lighter

The abdominal wall was then incised upto the xiphisternum. In the male rats the epididimal adipose mass was dissected by gently pulling the fat and removing the whole testicle before carefully dissecting the fat. For the female rats the parametrial fat was dissected by cutting the uterus as close to the cervix as possible and then removing as a whole including the ovaries. It was then carefully stripped of fat. The stomach was then lifted to expose the omental fat and this was removed by teasing it away from the spleen and pancreas.

The intestinal fat was dissected by removing the intestines as a whole by cutting the oesophagus close to the stomach and cutting the colon close to the anal sphincter and then stripping them of fat. The liver was then dissected and weighed before clearing the abdominal cavity of any remaining fat (retroperitoneal) ensuring all was removed from around the kidneys and adrenals and upto the diaphragm. The kidneys and adrenals were then dissected, blotted dry and weighed. The thorax was then opened and the heart removed, clearing any blood clots before being weighed.

The brain was dissected from the top of the spinal cord and gently levering it out from the cranium, ensuring that the olfactory bulbs were also removed. Finally the pituitary was removed from the base of the skull by first breaking its overlying membrane.

All tissues were replaced back inside the abdominal cavity which was then sown up and frozen at -20°C to be later analysed for total body composition.

2.12 Total Body Composition

The frozen rats were weighed and placed in a foil tray before thawing in a fume cupboard. The skulls were then pierced and the abdomen opened. The tray was then wrapped in foil, labelled and weighed and placed in a vacuum oven at 80°C for 4-5 days until a constant weight was obtained. Percentage water was calculated by difference. The carcass was ground in a domestic blender to produce a homogenous sample. This was then divided into plastic bags and frozen for later analysis of body composition.

The percentage nitrogen, extractable fat and energy content of the carcasses were analysed using the methods described in Appendix C. Nitrogen was calculated using an automated Kjeldahl analyser (Kjeltec) and the fat was extracted by Soxhlet extraction in chloroform/methanol (2:1). The body composition was determined using the following equations.

Equation 1: $\frac{test - blank * 0.1 * 14.008}{weight} * 6.25 = \frac{mg\,Protein / gRat}{100} = \%protein(dry)$

Equation 2: $\%protein(wet) = \%prot(dry) * \frac{\%drywt}{100}$

Equation 3: $\frac{beforewt - afterwt}{samplewt} * 100 = \%fat(dry)$

Equation 4: $\%fat(wet) = \%fat(dry) * \frac{\%drywt}{100}$

Equation 5: $Energy\ kJ/g\ (wet) = Deflection * energy\ in\ 1\ deflection * \frac{\%dry\ wt}{100}$

Equation 6: $Total\ Energy = Energy(kJ / g) * Body\ wt\ (dry)$

2.13 Statistical Analysis

All analyses were performed using the Microsoft Excel statistical package (version 5.0). Unless otherwise stated all results are expressed as mean ± SEM and differences between groups were calculated using Student's t-test (for unpaired data) or 2-way ANOVA where appropriate, as stated in the relevant chapter. A P value of ≤ 0.05 was taken as being statistically significant.

Chapter 3.0
Validation of the Macronutrient Self-Selection Dietary Regimen

3.1 Introduction

Qualitative studies on appetite in laboratory animals require specific diets to be constructed in order that individual macronutrient intakes can be determined. However different patterns in macronutrient intake occur under different physiological states such as diabetes, pregnancy, lactation and refeeding after food deprivation (Abadie et al 1993, Castonguay et al 1982, Kanarek 1985, Kon 1931, Leprohon & Anderson 1980, Leprohon & Anderson 1992, Morris & Anderson 1986, Mullen & Martin 1990). Furthermore, macronutrient intake is influenced by sex steroid background (Wade & Gray 1979). The regulation of macronutrient intake in the rat has been studied extensively by various groups, most notably by Leibowitz and her colleagues, in experiments where separate sources of fat, carbohydrate and protein are offered. Generally these studies have shown that rats are able to select an intake of macronutrients in adequate proportions to sustain normal growth and that the proportions of macronutrients consumed changes with development and maturation (Leibowitz et al 1991). For example protein intake has been shown to increase to a peak at puberty.

This has not always been the case however, since when the self-selection dietary regimen was critically reviewed it was found that almost half of the studies showed that the rats were not able to self-select the correct amounts or proportion of macronutrients to sustain normal growth, primarily due to a failure to eat adequate protein (Galef 1991). The fact there are certain studies which show rats unable to select the correct amount and balance of macronutrients suggests that there may be problems (possibly methodological) associated with the feeding of specific macronutrient diets. This may be the case as different intakes have been recorded depending to the type of diet used.

If the diets are examined separately there are differences in intake depending on the individual constituents used. The source of protein used can affect the proportion of animals which do not maintain normal growth and development. For example 70% of rats failed to consume sufficient protein when egg albumin was used whereas this fell to only 30% if casein, lactalbumin or fibrin were the protein sources (Kon 1931). Only 10% failed to grow normally if all three types of protein were on offer. Amongst these three proteins it is casein which has been shown to be the best at promoting optimum weight gain in the rat (Kanarek 1985).

The type of fat is also important in determining the amount selected and also the percentage fat in the separate diet changes the amount selected. These differences were shown in a study by Mullen & Marten (1990). They examined the effect of feeding a saturated fat which was beef tallow compared to a fat diet high in polyunsaturated fatty acids i.e. corn oil. They fed these at different levels in the diet - 5%, 20% and 34% by weight. It was found that both the type of fat and its proportion not only affected fat consumption, but also the amount of protein and carbohydrate. The high saturated fat diet lead to significantly more protein and less carbohydrate being eaten when compared to all the other diets.

The carbohydrate diet is generally constituted from a sugar base, glucose or sucrose and cornstarch or dextrin, both nonsweet carbohydrates (Abadie et al 1993, Larue-Achagiotis et al 1992, Leibowitz et al 1991, Kim et al 1991, McCarthy et al 1994, Mullen & Martin 1990, Shor-Prosnor et al 1994). The proportion of these carbohydrate sources varied greatly between studies ranging from 100% dextrin (Kim et al 1991) to 50% cornstarch - 50% dextrose (Abadie et al 1993) to 100% dextrose (Mullen & Martin 1990). This makes comparing results across studies difficult.

The separate macronutrient diets within an individual regimen also vary in that both texture and taste differ between the macronutrients. The protein diet is usually dry when given but if it has been previously soaked in water, it has been shown that the rats eat up to twice as much (Kanarek 1985). Ramirez (1987) showed marked increases in total energy intake when the diets were given moist, which lead to increases in carcass fat content. If however the texture of the moist diet was changed from firm to fluid by the addition of varying degrees of gum this did not affect the intake. However it has been shown that rats do eat more if given a granular diet as opposed to powdered or in gel form (Kanarek 1985).

Both age and sex affect the macronutrient selection which are other factors to be aware of when comparing results across studies. In both male and female rats upto puberty carbohydrate and protein intakes increase whereas fat consumption is consistently low (Leibowitz et al 1991). As the animals age, total fat intake (and as a proportion of total energy intake) increases with a corresponding decrease in carbohydrate and protein intake (Abadie et al 1993, Kanarek 1985, Leibowitz et al 1991). Male rats have been shown to consume significantly more than females in both protein and fat, as a percentage of energy intake, although females are more likely to sustain normal growth on the macronutrient self-selection regimen than males (Kanarek 1985). Females also have the added confounding effect of the oestrous cycle during which hormonal changes occur. This can also influence macronutrient intake since sex steroids have been shown to specifically influence macronutrient consumption (Wade & Gray 1979). There is some conflict in this area as it is agreed that energy intake varies across the cycle (Wade & Gray 1979), but whether the preference for

any specific macronutrient also varies depends on differing studies (Abadie et al 1993, Leibowitz et al 1991)

Most importantly there are also differences in macronutrient intake across different inbred strains and in lean and obese littermates in obese strains. The obese Zucker rat has metabolic abnormalities such as the preferential partitioning of ingested food energy into adipose tissue and an alteration in protein metabolism which limits growth of the lean body mass. These changes in metabolism affect macronutrient intake. Castonguay et al (1982) offered Zucker rats a self-selection macronutrient dietary regimen for nine days. The obese Zucker rat selected a diet which provided 12% of their total energy intake from protein and 64% as fat.

Many other factors will also affect the macronutrient selection including physical environment where socially reared rats have a greater growth rate that those individually housed (Kanarek 1985), time of measurement and experimental history (Leprohon & Anderson 1980, 1992, Morris & Anderson 1986). For example the level of protein selected by young rats has been shown to be positively correlated with the protein concentration of their first solid food, if offered a choice between a low-protein diet, 10%, and a high-protein diet, 60% (Morris & Anderson 1985). There is a clear diurnal cycle (Abadie et al 1993, Larue-Achagiotis et al 1992, Leibowitz et al 1991) with respect to macronutrient ingestion and furthermore, subsequent meals are influenced by the composition of the previous one (Larue-Achagiotis et al 1992, Li & Anderson 1982). There is however no information on the effect of hypertension on intake.

Therefore the inclusion of macronutrient self-selection diets in metabolic studies must consider all these factors and eliminate, or at least reduce, the variability where possible. As the macronutrient self-selection regimen forms an integral part of this programme of work, the purpose of this study was to analyse the individual day to day variation in macronutrient intake and also, to examine whether the variation in macronutrient intake within a particular group would mask any real differences when comparing separate groups of rats. It was used to determine the length of time required to record an individual rat's intake to obtain the most valid average over the shortest time possible, which would then be used as the standard time for a weighed intake in the rest of the study. This study would also give an indication of how stable and reproducible the intake data of further studies was likely to be.

3.2 Method

Separate diets of protein, fat and carbohydrate were prepared as in section 2.4.1 and fed as blocks of food. The macronutrient self-selection diet was fed to eight adult female rats (mean weight 220g ± 12g) over a period of 16 days. Weighed amounts of the three

macronutrient diets were placed in each cage. Paper was placed on top of the sawdust in the trays beneath to collect any spillage. After 24 hours the food remaining in the cage was weighed together with the spillage. From this total energy and macronutrient intake was calculated. Fresh food was weighed daily and put into each cage in random positions and the procedure repeated. The body weight of the rats were also recorded daily. This protocol was then repeated with weanling rats at age four weeks. For the weaning study the macronutrient diet was fed to sixteen rats in total, eight female and eight male over a period of 20 days from weaning.

The intake of the carbohydrate diet was validated in a separate study which consisted of feeding the macronutrient self-selection regimen as before but this time the carbohydrate diet was offered in different physical forms although the overall composition was the same throughout. Eight weanling rats were used, four of each sex and four different physical forms of carbohydrate diet were used. These were classified as moist (no drying in the oven but 3% water added), and dry uncooked (basic ingredients mixed together no water added), dry cooked (water added and then dried in oven, given as blocks), and dry ground (as dry cooked but ground up). Each batch of diet was given for a period of five days and body weight was monitored throughout the whole study period.

The intake of the protein diet was also separately validated by feeding the macronutrient self-selection diet as before to the 16 rats in the original weaning validation study. This time the protein diet was either offered as a single block or in a ground form. Each form of the diet was fed for five days and then reversed for the following five days.

3.3 Results

3.3.1 Adult Rats

The mean individual amounts of each of the macronutrient diets and total metabolisable energy consumed over the sixteen days are shown in table 3.1. The table expresses the data as the mean with the ranges in the brackets. The coefficient of variation, given as a percentage is an expression of the repeatability of the day to day measurements. It can be seen from the table that was a wide variation for all of the macronutrients with the protein diet having the least variation and the carbohydrate the most. This is particularly evident from the ranges of amount of carbohydrate diet eaten, from 0.1g to 25.7 grams/day. The %CV recorded were higher than expected between 12% and 65%.

The total metabolisable energy intake demonstrated less day to day variation for a single rat than the separate macronutrient intakes. On a weight basis, carbohydrate was eaten in the greatest amount but the macronutrient that was eaten in the least amount was equally divided between the fat and the protein diets. The actual amounts eaten of each diet together

with body weight and total metabolisable energy for each individual rat on a single day basis are recorded in appendix E in both tabular and graphical format

Rat	Amount of Macronutrient Diet Eaten (g)						Energy	
	CHO	% CV	Fat	% CV	Protein	% CV	kcal	% CV
1	6.5±1.0 (1.1-12.3)	59	9.2±0.5 (5.0-12.9)	20	5.3±3.0 (1.8-13.3)	56	116.8±8.6 (63.9-164)	29
2	5.2±0.7 (0.1-10.6)	54	9.3±0.4 (5.6-12.7)	19	5.3±0.6 (2.4-11.5)	42	109.3±5.7 (85.9-139)	20
3	13.3±1.3 (7.1-25.7)	37	4.0±1.5 (1.8-6.5)	37	12.1±0.6 (8.9-16.7)	18	112.6±5.1 (85.1-161)	16
4	7.6±0.9 (1.1-14.2)	47	6.2±0.4 (2.7-8.4)	25	5.6±0.3 (3.2-7.5)	18	96.6±5.1 (66.2-118)	20
5	10.6±0.8 (6.0-16.5)	29	5.8±0.4 (2.6-7.9)	25	7.7±0.2 (7.1-9.1)	12	116.3±7.2 (83.6-138)	24
6	11.1±1.3 (2.8-19.0)	44	5.9±0.3 (4.5-8.2)	19	7.9±0.5 (4.9-10.2)	23	114.6±5.8 (70.2-145)	20
7	6.1±1.0 (0.1-13.3)	65	9.1±0.6 (5.3-14.3)	26	5.8±0.6 (2.1-9.8)	39	113.5±5.3 (65.5-153)	18
8	9.7±0.9 (3.9-15.4)	35	5.5±0.4 (2.5-9.7)	32	5.6±0.3 (3.6-8.5)	34	98.3±4.1 (73.8-128)	16

Table 3.1 Individual Mean±SEM of each macronutrient diet eaten. Range given in brackets

There was a large variation in body weight within the group therefore the intake was expressed as g/kg body weight. The mean intake of carbohydrate is shown by figure 3.1.

It can be seen that there was a wide variation in the amount of carbohydrate diet eaten over the 16 day period of the study. A fluctuation around a plateau is evident upto day 7 whereupon there was a large decrease in the amount of carbohydrate eaten. This reached a lower plateau for the remainder of the study. A rolling average was calculated to reduce the day to day variability, which can be useful when two groups are being compared. The rolling average takes the first four readings and averages them, the second to fifth readings and averages them and then the third to sixth and so on. This method reduces the day to day variation so that comparisons between groups can be made more easily. It provides more consistent and reproducible data. Figure 3.2 shows the rolling average for the same

carbohydrate intake data as shown in figure 3.1. An interval of 4 days was used as there appeared to be a cycle of this length of time from the original graph, a cycle longer than this gave no significant changes in the average and so the shortest time span was chosen.

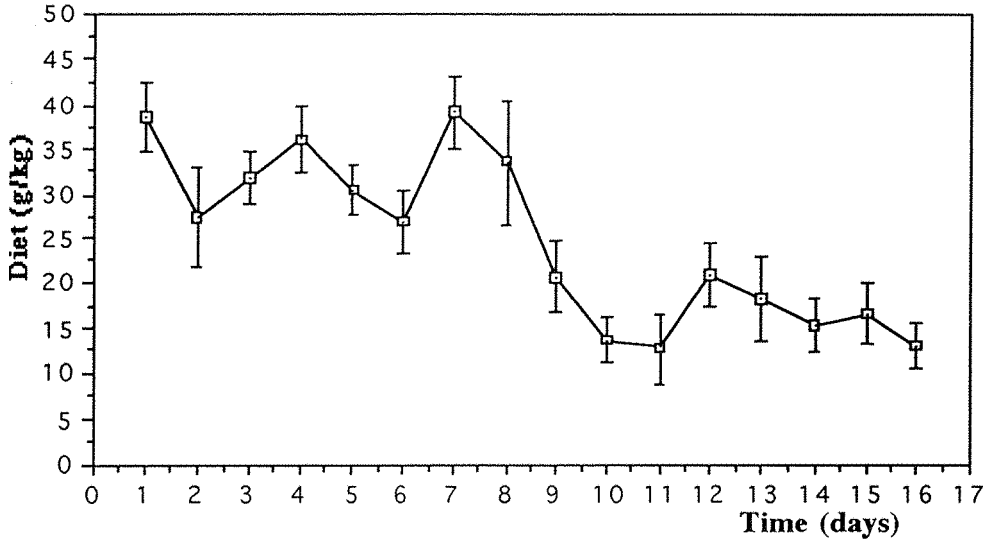

Figure 3.1 Mean daily amount of carbohydrate eaten by the group expressed as g/kg body weight (±SEM)

The graph of the rolling averages shows the two plateaux more clearly with the coefficient of variation (%CV) over the first five measurements being reduced to 3.6% from 18.5%. Similarly the %CV over the last five measurements is 8.1% compared to 19.9% in the original figure.

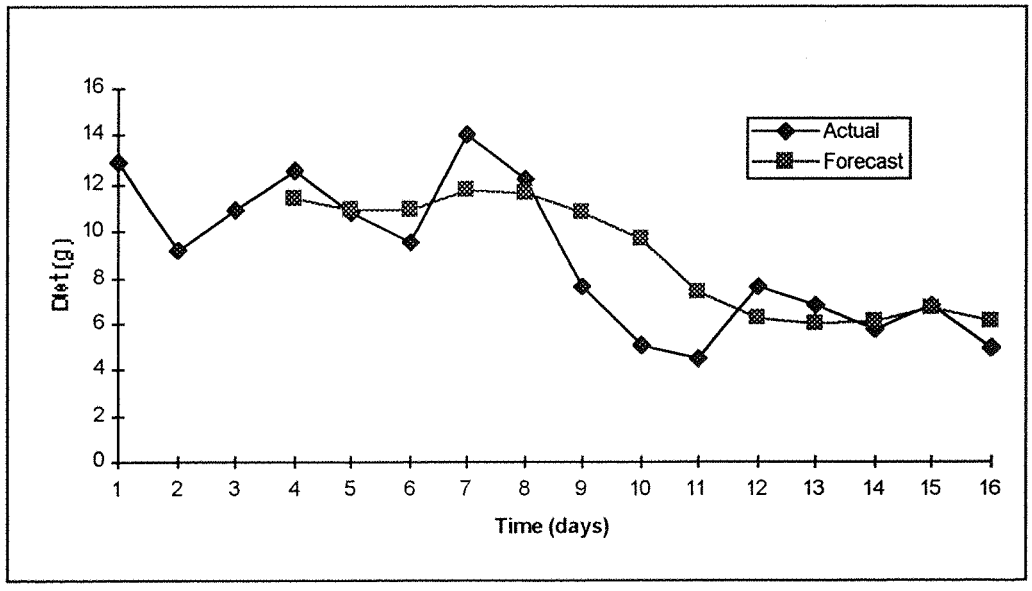

Figure 3.2 : Rolling average (cycle 4 days) for mean carbohydrate diet eaten

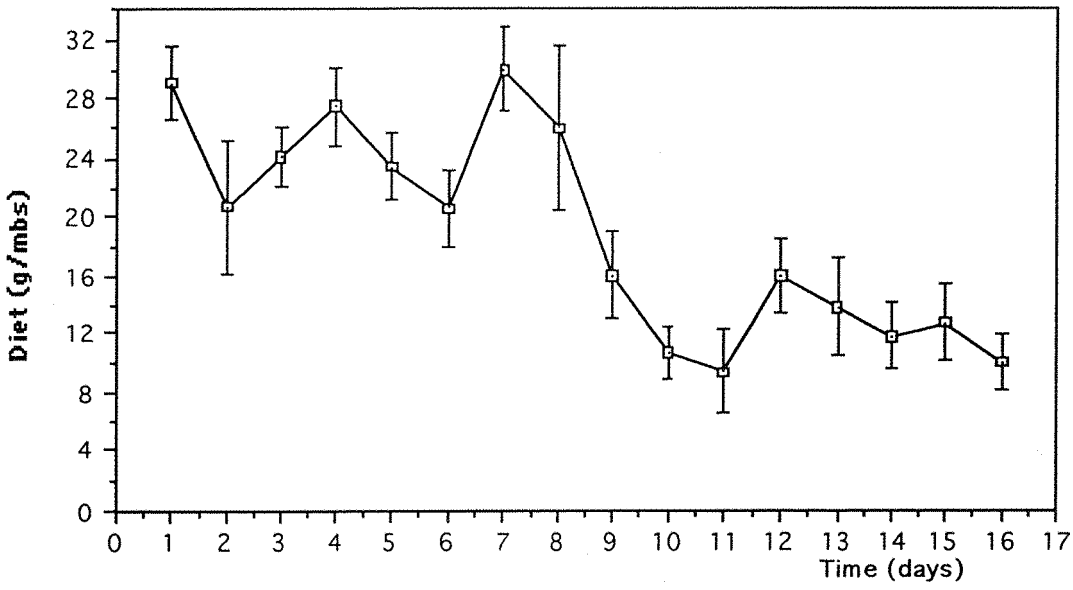

Figure 3.2a : Mean daily amount of carbohydrate eaten by the group expressed as g/mbs (metabolic body size) (±SEM)

The results were also expressed in relation to metabolic body size (figure 3.2a). Metabolic body size (mbs) is an expression to reduce differences in body size by calculating body weight$^{0.75}$. Although difficult to determine from comparing figure 3.1 and 3.2a, there is a reduction in the variability although the same basic pattern of consumption is reproduced.

When fat consumption is examined, it can be seen from figure 3.3 that this was a regular intake of around 20g per day, apart from a sharp nadir at day 7 which corresponded with the peak seen at that time in the carbohydrate diet consumption.

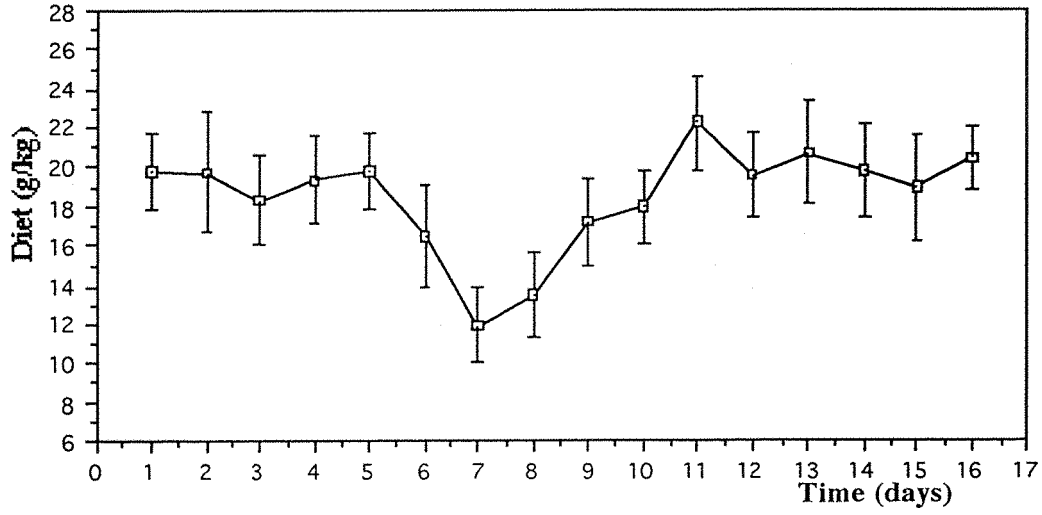

Figure 3.3 Mean daily amount of fat diet eaten by the group expressed as g/kg body weight

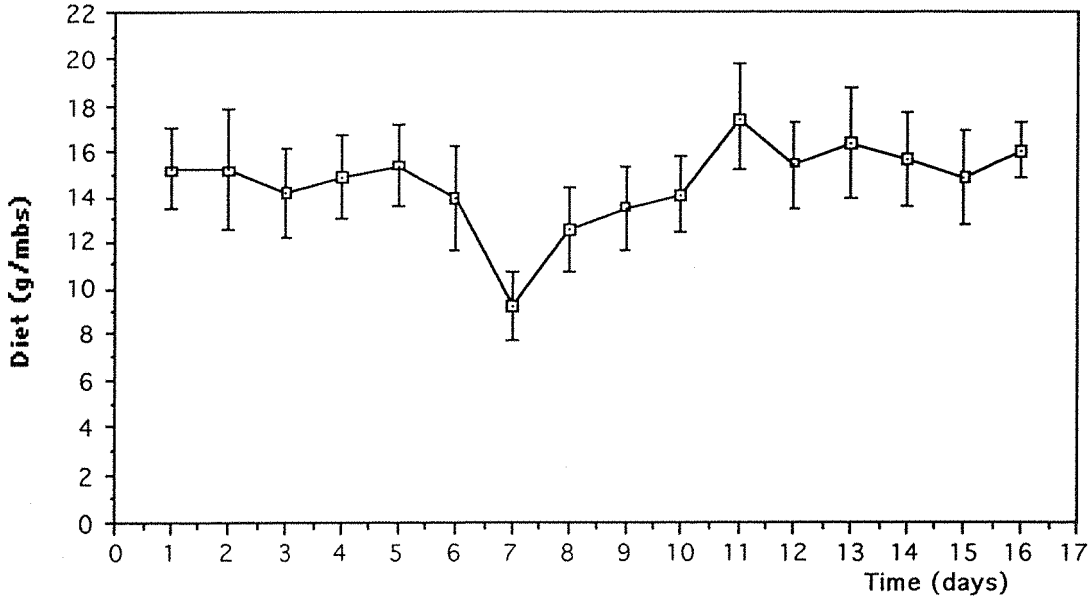

Figure 3.3a Mean daily amount of fat diet eaten by the group expressed as g/metabolic body size

Figure 3.3a shows the fat consumption as expressed per metabolic body size, as with the carbohydrate intake, there is little difference with the pattern of consumption when comparing it to the g/kg results. Again if the rolling average is taken as shown by figure 3.4 the variability is reduced and a much more constant value was obtained for the duration of the study. If the %CV is calculated over the same timeperiod as for the carbohydrate, the variability is reduced from 13.6% to 7.3% over the first plateau and from 7.5% to 5.8% over the final plateau.

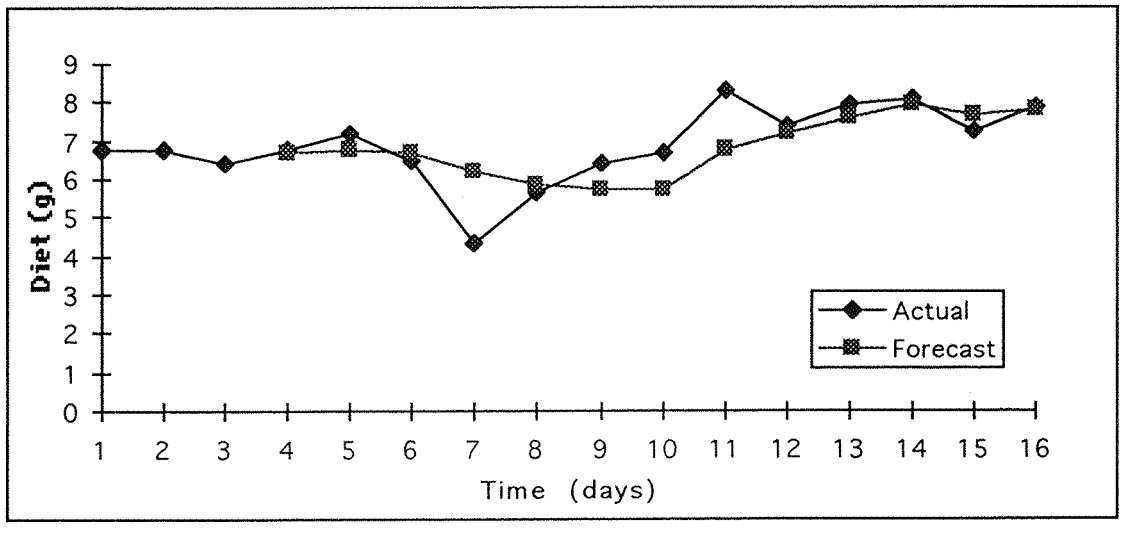

Figure 3.4 : Rolling average (cycle 4 days) for mean fat diet eaten

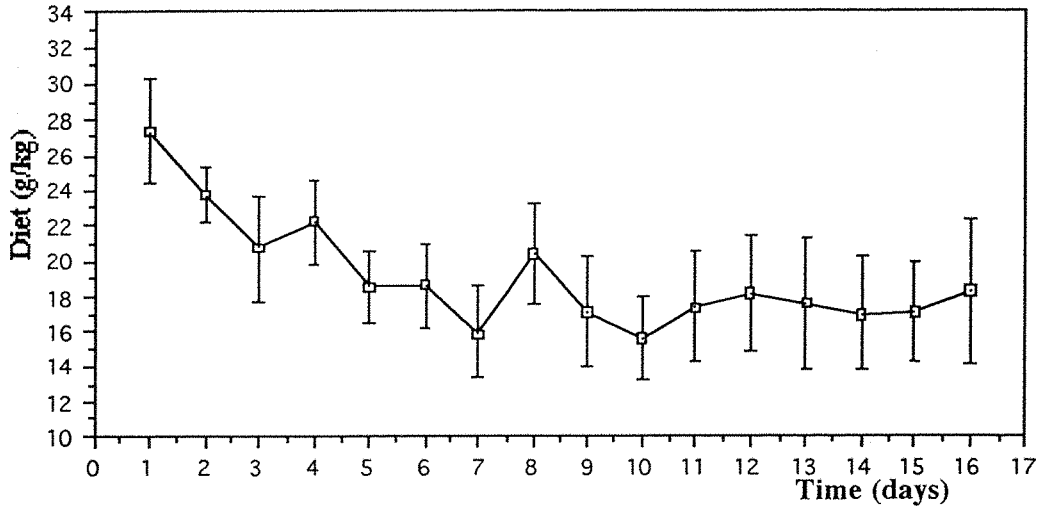

Figure 3.5 : Mean daily amount of protein diet eaten by the group expressed as g/kg body weight

Figure 3.5 shows the amount of protein eaten and it can be seen that after an initial fall over the first five days, the protein consumption plateaux at around 17±3g/kg body weight. From day five the 4 day cycle can be seen as in the results for carbohydrate, although this graph shows less variation than for fat and carbohydrate. There is still a standard error at each timepoint of ±3g. This was due to the fact that although each individual rat consumed a fairly constant intake the range, *between* individuals was still fairly large as seen from table 3.1. The rolling average graph is shown in figure 3.6 and as the plateau is more clear, the %CV does not vary as much as for the other macronutrient diets. The intake decreases slightly over time due to the fact that these animals were mature females who reached their growth plateau.

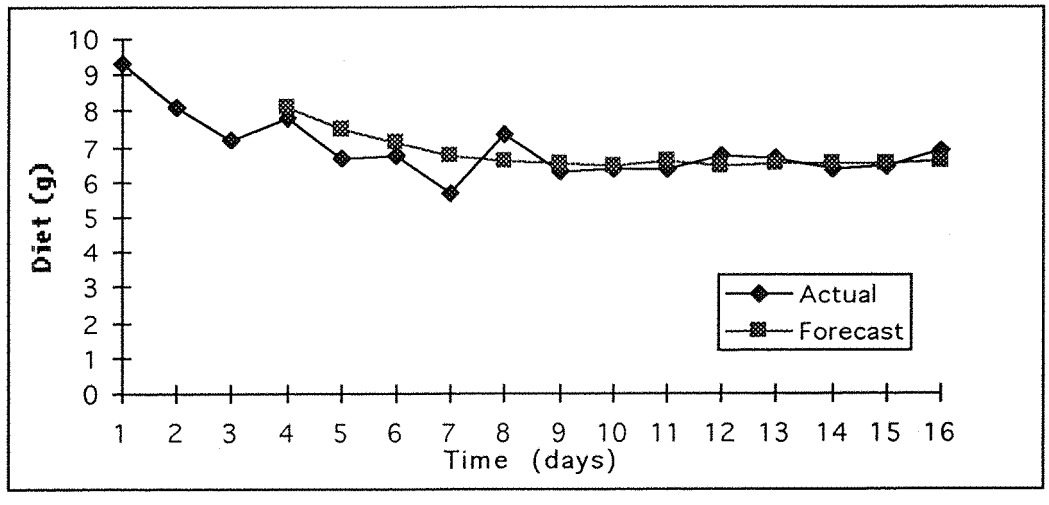

Figure 3.6 : Rolling average (cycle 4 days) for mean protein diet eaten

The reduction of variability is clearly seen from figure 3.6 as the rolling average graph is very similar to that of the actual measured values. Similarly the expression of the results as per metabolic body size varies little from that of the results expressed per g/kg. This shows that there was a steady protein intake but variable fat and carbohydrate intakes. This may suggest that protein intake is much more tightly regulated than the other two macronutrients.

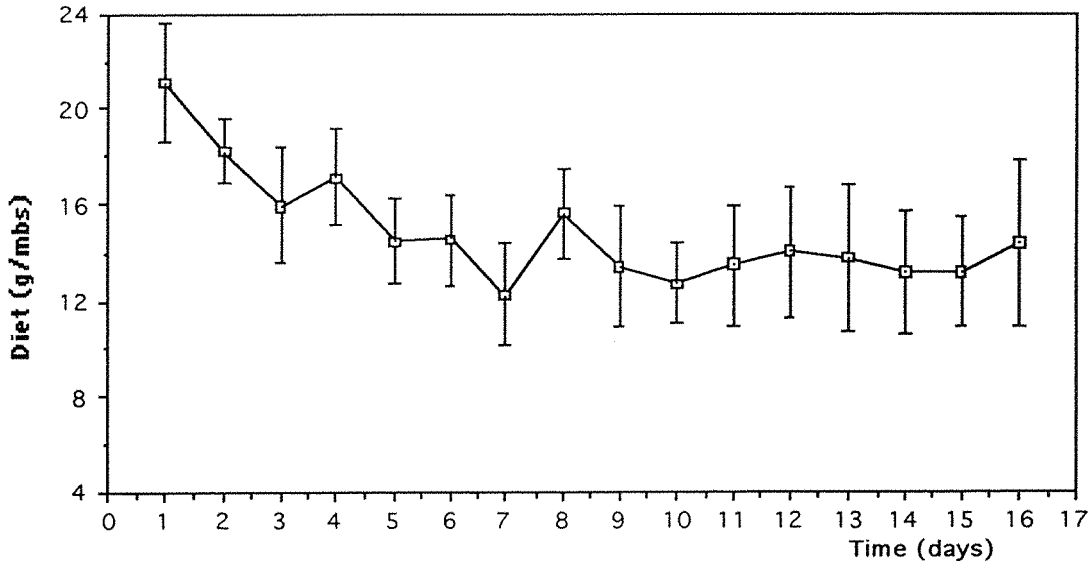

Figure 3.6a : Mean daily amount of protein diet eaten by the group expressed as g/metabolic body size (±SEM)

Due to the differing amounts of diet eaten on each day, the rats' total energy consumed also varied over the study period and this is expressed in figure 3.7 per kg body weight and in figure 3.7a per metabolic body size.

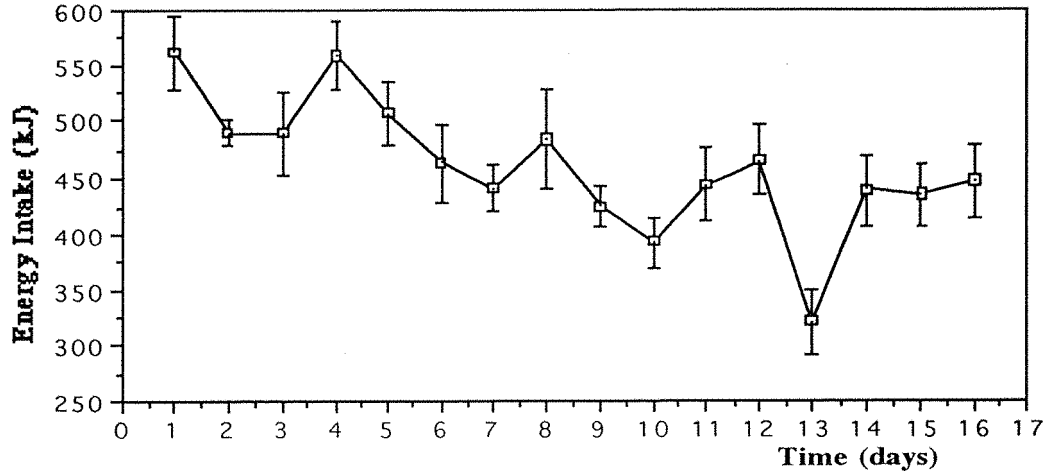

Figure 3.7 : Mean daily amount of energy consumed by the group expressed as kJ/kg body weight

Total metabolisable energy intake declined from the beginning of the study up to day 12, with a further sudden decrease at day 13. Over the last three days of the study however, the total energy consumed sharply increased to reach a value near to that of the level eaten on day one. It can also be seen from figure 3.7 that the variation within the group was relatively small. This is mirrored in figure 3.7a when expressed as per metabolic body size.

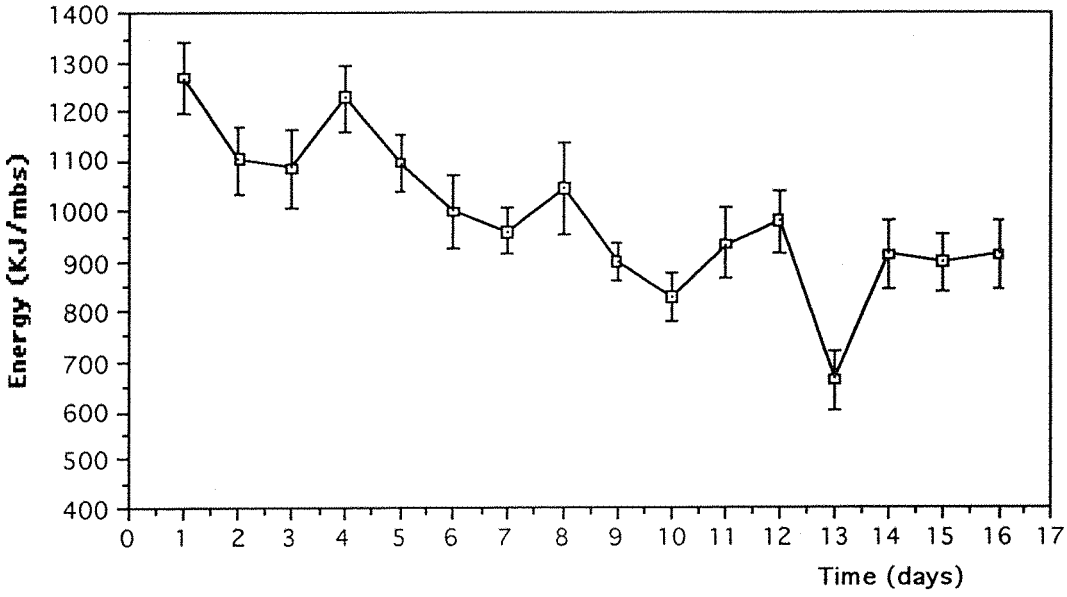

Figure 3.7a : Mean daily amount of energy consumed by the group expressed as kJ/metabolic body size

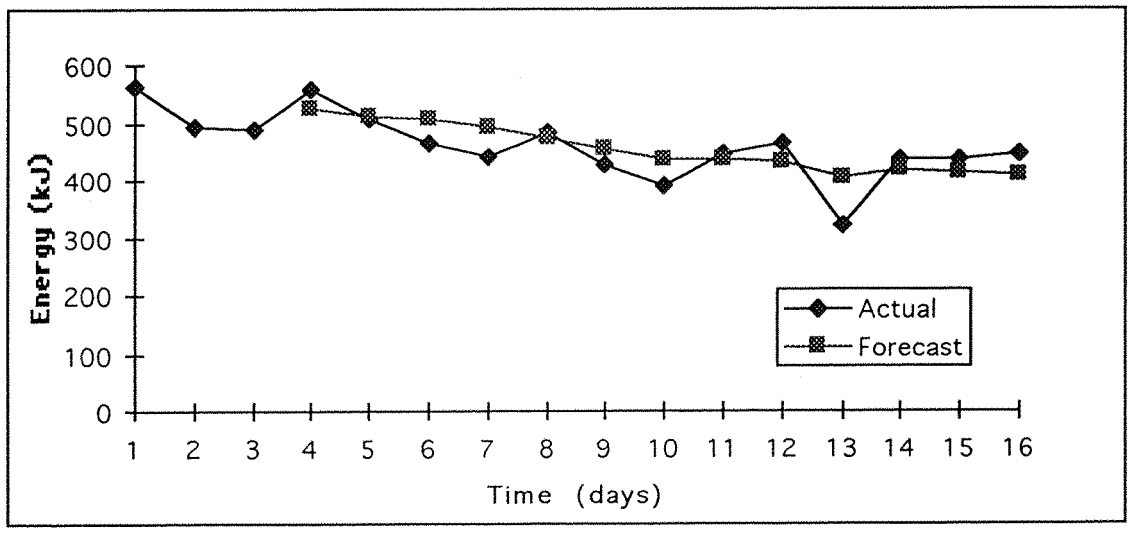

Figure 3.8 : Rolling average (cycle 4 days) for mean energy intake expressed as kJ

Figure 3.8 gives the rolling average for the energy intake expressed in the same format as the macronutrients in that the interval cycle is 4 days. If the intake is expressed in this way it can be seen the energy consumption is relatively constant over the whole study period and that where in the original graph day 13 had a large variation which as it is a single day may be considered a measurement artefact, the rolling averages graph eliminates this variability.

Finally, for the validation of the adults' intake, as the fat diet was more energy dense than the other two diets, 33.9kJ/g compared to 15.1kJ/g, figure 3.9 expresses the amount of each diet eaten as a percentage of the total metabolisable energy consumed. This graph shows that the protein diet, irrespective of its variation of grams eaten contributed a consistent 25% of the total metabolisable energy consumed over the whole of the study period. The fat and carbohydrate diets therefore mirrored each other in the percentage of energy they contributed, with the fat diet having the largest percentage of the energy intake. This was particularly evident after day 7 when the proportion of fat eaten increased sharply with a corresponding fall in the proportion of carbohydrate.

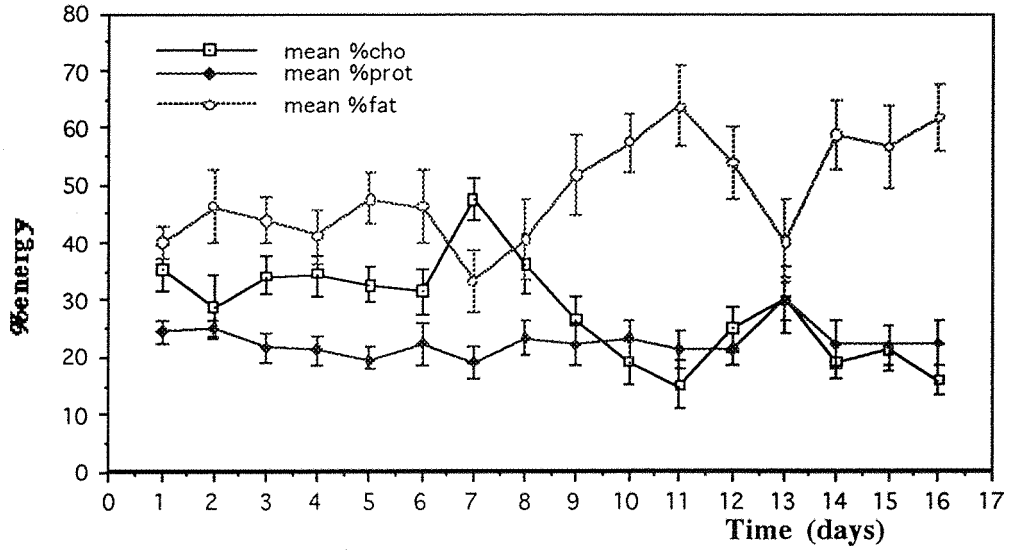

Figure 3.9 : Group means for each of the diets eaten expressed as % of total energy intake

3.3.2 Weanling Rats

Figure 3.10 shows that offspring of both sexes followed a normal growth curve when maintained on the macronutrient self-selection diet although there seems to be an adjustment period upto age 28 days where the curve is slightly flatter.

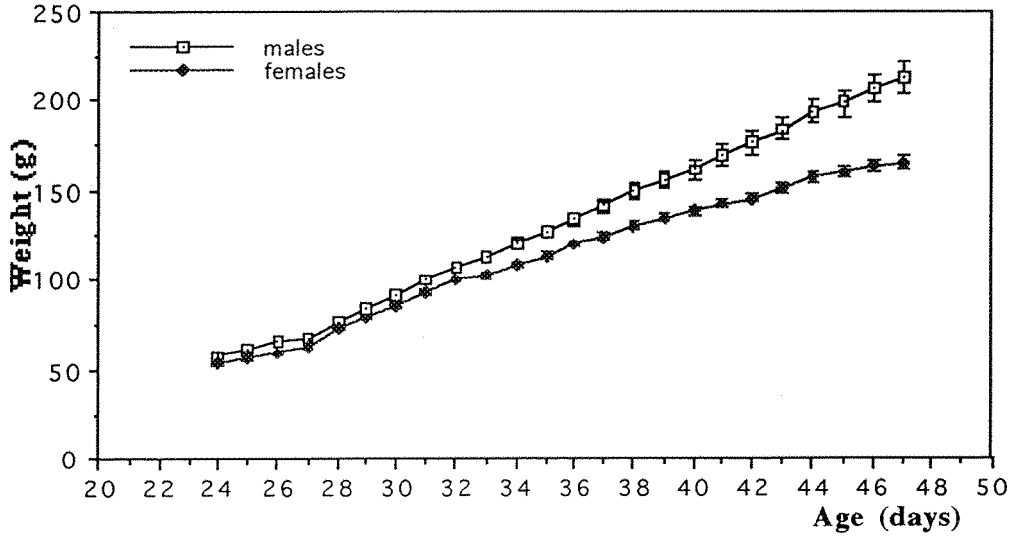

Figure 3.10 Growth Curve for male and female rats on the macronutrient self-selection diet

The intakes of each macronutrient expressed as grams of diet eaten for the males and the females are shown in figure 3.11 and 3.12 respectively. If the male rats are considered first, it can be seen that fat intake increased steadily throughout the study such that at the beginning of the study mean intake of the group was 1.7g±0.3g whereas at the end the value has risen to 5.2g±1.2g. The day-to-day differences were very small. Carbohydrate intake increased sharply over the first few days from 4.8g±0.8g to 9.5g±1.4g which then reached a plateau and was maintained upto 38 days. Following this, carbohydrate intake fell, reaching a new plateau at 8.5g±1.8g for the remainder of the study.

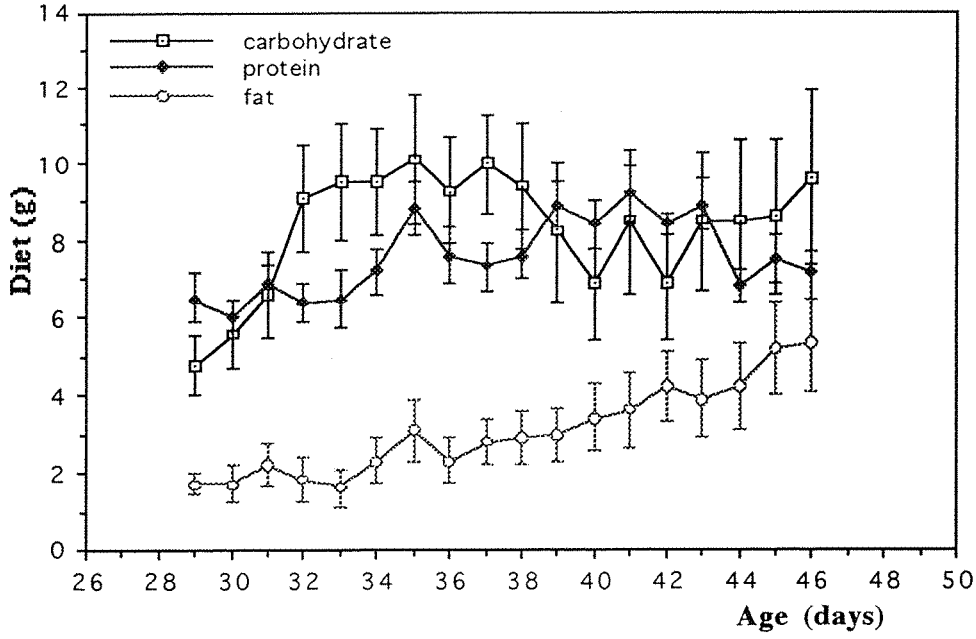

Figure 3.11 : Macronutrient intake in males expressed as grams of diet eaten

The protein intake remained relatively constant at 7.0g±0.6g/day until the rats were aged 36 days at which point there was an increase which is maintained until 44 days. The variation between rats at each timepoint was relatively small, with the smallest variance occurring in the protein intake and the largest with the carbohydrate diet as shown by the error bars on the graph.

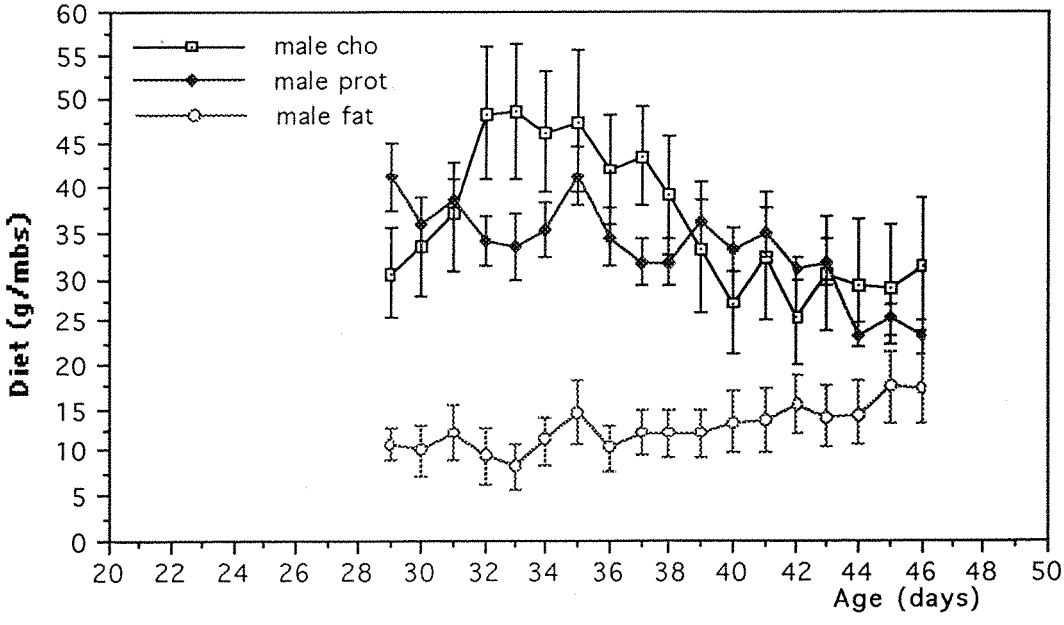

Figure 3.11a : Macronutrient intake in males expressed as g/metabolic body size

Figure 3.11a expresses the weaning intake data per metabolic body size, and as the pattern is similar to that when expressed as per grams as in figure 3.11, it indicates that there was little variability between each rat on a particular day.

The pattern of intakes was similar in the female group with respect to all three macronutrients however there was a much smaller increase in total fat intake over time in the females. This is also true when expressed as per metabolic body size as in figure 3.12a.

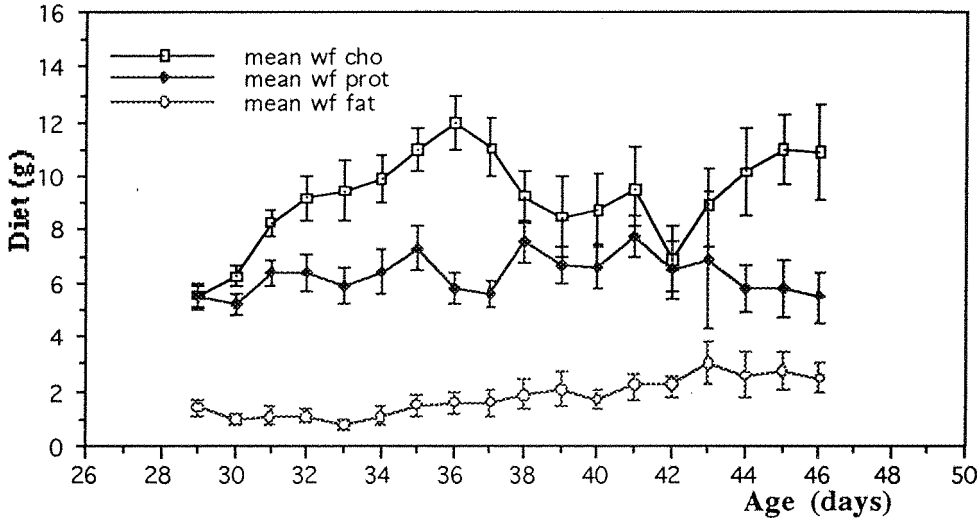

Figure 3.12 : Macronutrient intake in females expressed as grams of diet eaten

Protein intake was also relatively constant at around 6g/day although there was a slight increase over 38 to 43 days of age. The carbohydrate intake showed the greatest daily variability with a steady increase from 5.5g±0.4g at 39 days upto 12.0g±1.0g at 36 days. The intake then decreased to 6.8g±1.2g at 42 days before sharply increasing to the original peak.

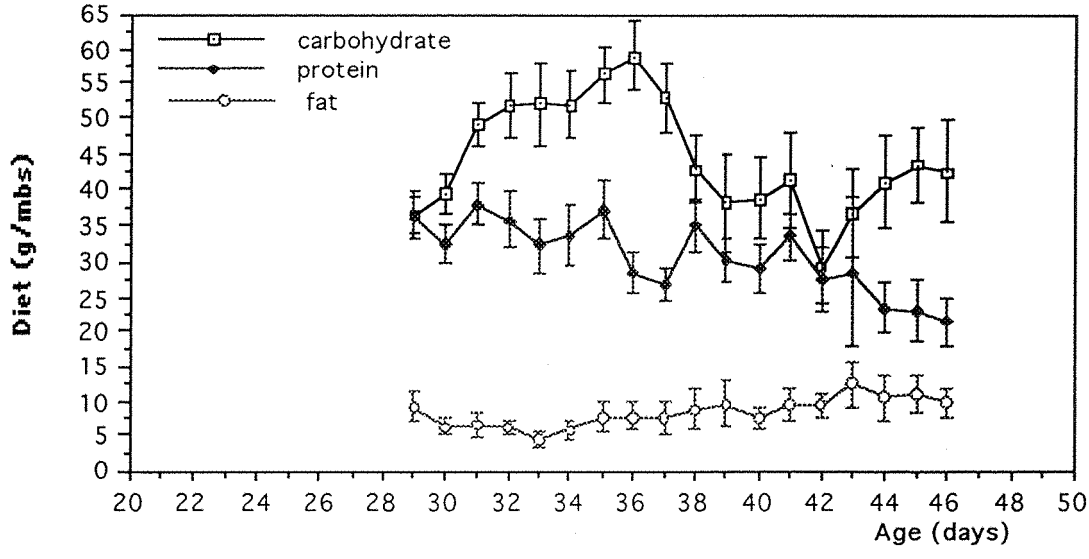

Figure 3.12a : Macronutrient intake in females expressed as g/metabolic body size

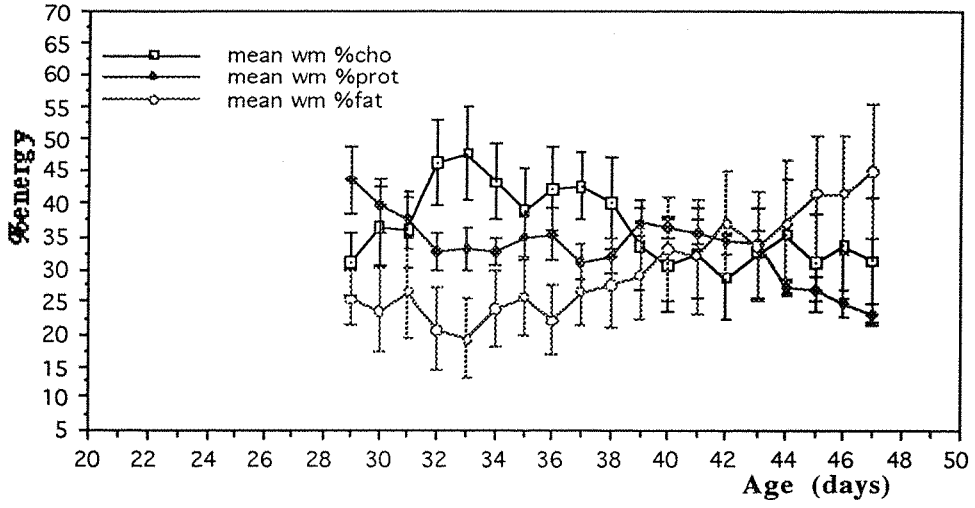

Figure 3.13 Macronutrient intake in males expressed as %energy

As with the males the largest variability between the rats on an individual day was in the carbohydrate intake. The individual macronutrient intakes expressed as a percentage of total metabolisable energy intake eliminated the variability in the amount eaten due to growth. The results for the males are shown in figure 3.13.

The variability in intake for a particular macronutrient is increased especially for the fat intake when expressed in this format. The absolute increase in fat intake over the study resulted in it changing from contributing the least amount of energy to the greatest. The percentage of energy from protein remained fairly stable with the percentage from carbohydrate decreasing as the percentage derived from fat rose.

The females rats (figure 3.14) demonstrated a much larger initial difference between the separate macronutrients than the males, with the carbohydrate intake providing the greatest proportion of metabolisable energy throughout the whole study The percentage of energy derived from fat increased in a similar manner to the males, although the protein decreased slightly and then more pronounced after 42 days.

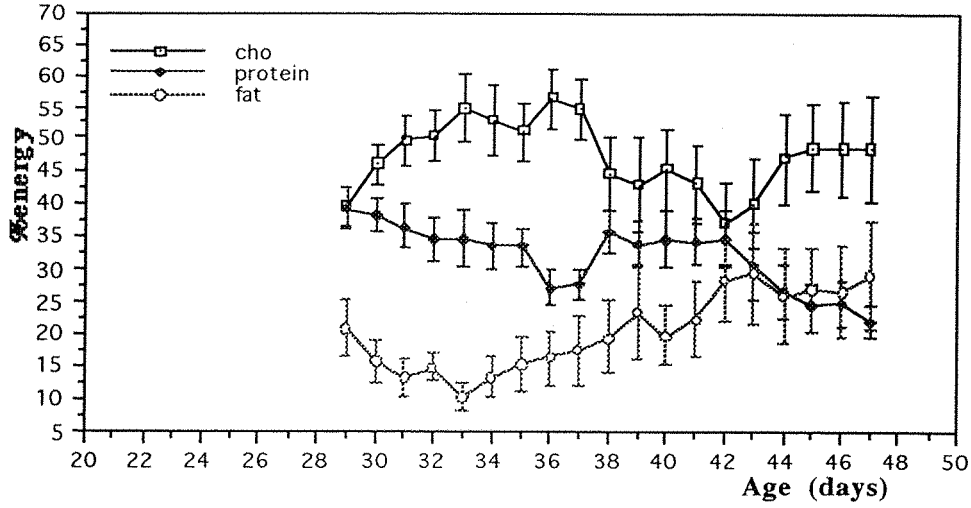

Figure 3.14 Macronutrient intake in females expressed as %energy

3.3.2.1 Carbohydrate intake

The results for the male rats are shown in figure 3.15. The largest daily variation in intake occurred with the dry uncooked batch which appeared as a sharp trough in the figure. This resulted in a corresponding peak in percentage energy for both the fat and protein.

The greatest variability within the group on a single day was with the dry cooked diet as shown by the error bars. The smallest variability occurred with the dry ground diet. It was over this time period that the fat and protein intakes were most stable.

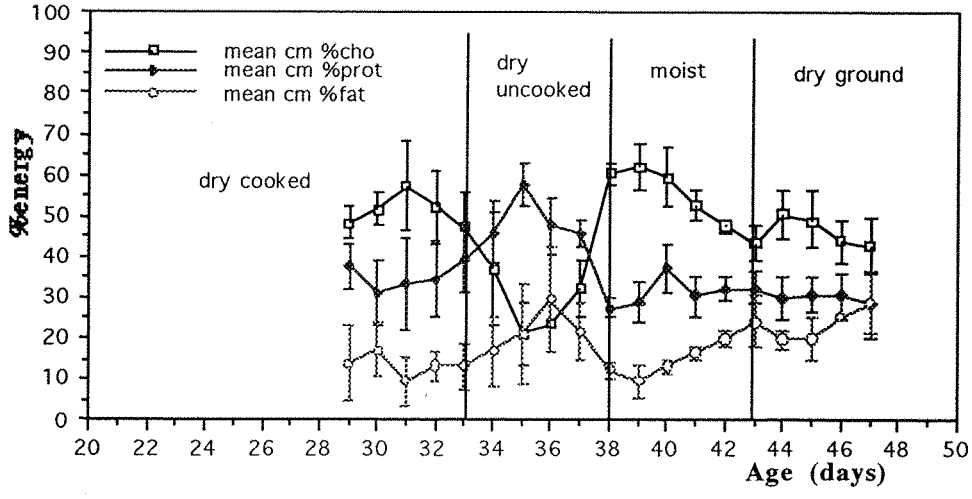

Figure 3.15 Differences in %cho using different batch consistencies in males

The pattern of carbohydrate intake in the females was similar to that of the males with the variability of the group being greatest with the dry cooked diet. A sharp increase in the contribution of carbohydrate to the total energy intake occurred when the moist diet was offered. However, with the dry uncooked batch, a plateau with this diet in the females was seen whereas the dry ground diet has a slight decrease with a corresponding increase in the fat percentage.

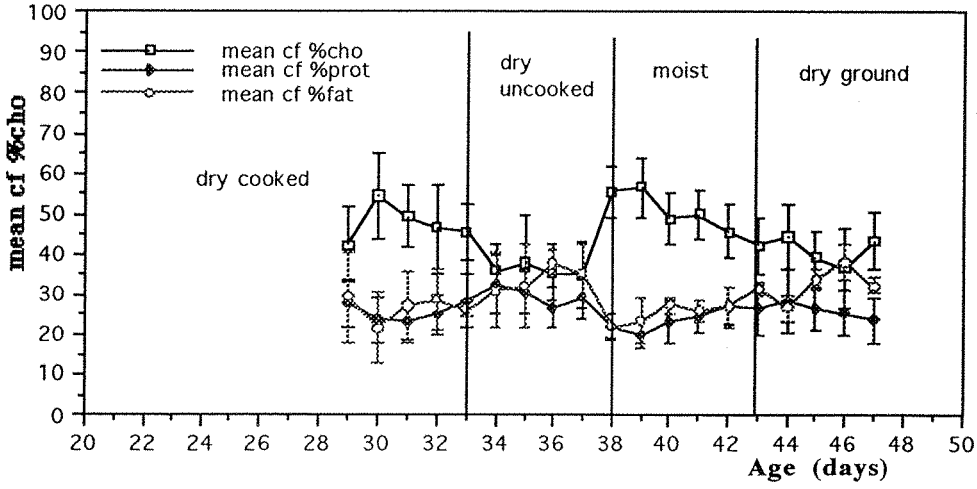

Figure 3.16 Differences in %cho using different batch consistencies in females

3.3.2.2 Protein Intakes

The male rats consumed a constant intake for all three macronutrients throughout the whole study (figure 3.17). The carbohydrate showed greatest variability when the protein diet was given in block form.

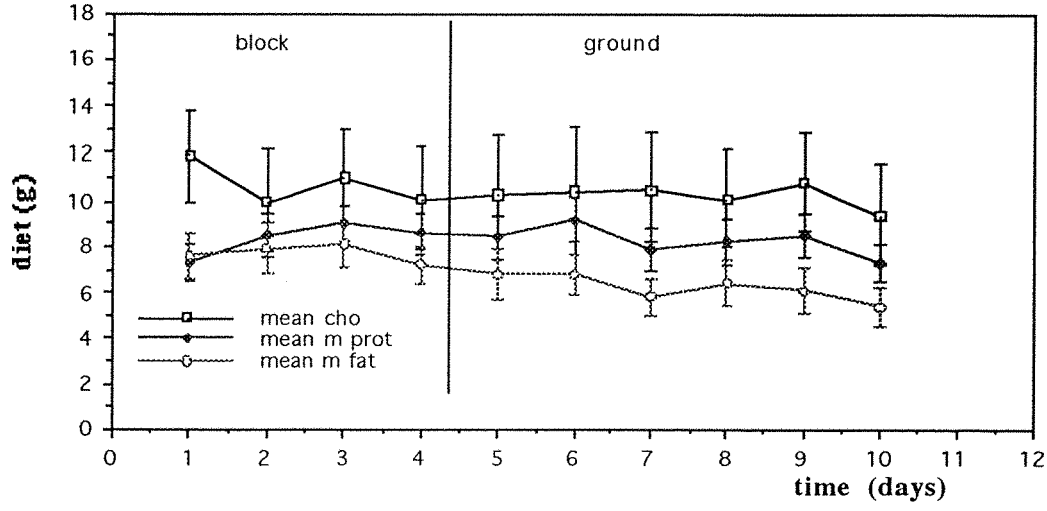

Figure 3.17 Macronutrient intakes during different physical form of protein diet in male rats

Figure 3.18 shows results from the same study carried out in the females, and like the males, the intakes were very stable with the most variability occurring in the carbohydrate intake when the rats were offered the protein diet as a block rather than in a ground form. The low variation within the group indicated by a smaller error was more evident in the females than the males.

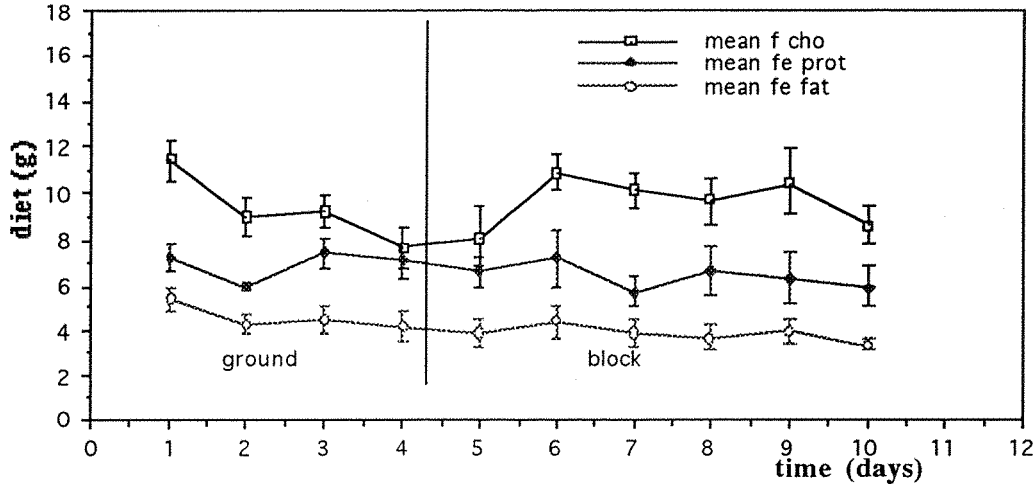

Figure 3.18 : Differences of intake for different batches of protein diet for females

3.4 Discussion

The validation studies for adult females and weanlings will be discussed separately as one study arose from the other. In the adult study, the rats had essentially stopped growing, whereas in the weaning rats there was weight gain along the normal trajectory.

It can be seen from the results of the adult study that there was both wide intra- and intervariation in macronutrient intake in these rats. The intervariation was not unexpected and confirmed findings of previous studies (Leibowitz et al 1991). All the adult rats followed the expected growth trajectory over the period of the study suggesting they were able to select the required amounts and proportions of macronutrients from the separate diets supplied. The groups of rats used were all adult rats aged 30 weeks therefore as they were females they had reached the plateaux of their growth curve (Leibowitz et al 1991) and hence their need for protein remains relatively constant. This was demonstrated in this study particularly in the percentage of energy provided by protein. The initial drop seen in the intake when expressed as g/kg body weight could be due to the rats adapting to the macronutrient self-selection regimen, therefore a week of feeding the self-selection diet, before any measurements are made, should be allowed to give time for adaptation.

There was a very small variation within the group when intake was expressed as actual amount eaten on a weight basis which increased rather than decreased when the protein intake was expressed as g/kg body weight. This could have been due to the fact that the amount of protein an adult rat requires is similar irrespective of its body weight as weight variation could be due to a larger fat mass rather than a greater lean body mass. To try to help reduce variation in body size surface area correlation can be used, however this can be hard to determine accurately. Therefore a metabolic body size was used which is a linear relationship with the slope of the graph being 0.75, the metabolic body size is therefore calculated by raising the body weight to the power of 0.75 (body weight $(kg)^{0.75}$). These calculations were performed in both the adult and weaning studies, however the metabolic body size results were very similar to those expressed as g/kg, indicating that there was little difference in body size between these animals and it was not this accounting for differences in dietary intake.

The intake of the carbohydrate and fat diets did not however, show this regular pattern. There was a large variation both within an individual and between rats within the group. This was particularly evident for the carbohydrate diet. Some of the variation in the carbohydrate intake may be explained by the fact a different batch was used from day 7 of the study. This diet appeared drier than the previous one and may explain the sudden decline in the amount of diet eaten as it has been shown previously that the more moist the diet the more the rats eat (Kanarek 1985). Also with respect to diet consistency, there was no inter-batch variation in the protein and fat diets, whereas the carbohydrate diet, although prepared from an identical recipe (including water content) and cooked at the same temperature and for the same length of time, there was still a variation in the consistency. Although the diet was kept in airtight containers at 4°C, the diet tended to dry out over time, which is one reason why the diets were changed on a daily basis. The amount of weight the moist diet lost overnight in drying out was minimal (unpublished observations) and cannot account for such a large decrease in the carbohydrate intake. This may explain why there was such a large %CV, particularly with this macronutrient. One other reason may be that the %CV were calculated over a period of 16 days, which even in adult female rats which have reached their growth plateaux, includes some weight gain and therefore an increased demand for macronutrients and energy.

The fat intake mirrored that of the carbohydrate intake whereby when the carbohydrate intake peaked there was a reciprocal fall in the fat intake such that total energy intake was unaffected. It is not known whether it was the fat intake which was variable with the carbohydrate being manipulated to keep the energy intake relatively constant or visa versa, but from the known change in the carbohydrate, it is most likely it was the fat intake being manipulated to counter balance the change in the carbohydrate intake.

The carbohydrate intake demonstrated a four-day cycle of increasing and decreasing intake. This was possibly due to the changing levels of gonadal hormones in the oestrous

cycle although it had been shown previously in one study that oestrous cycle does not affect macronutrient intake (Abadie et al 1993). A validation study in males would have helped answer this question if such a cycle was not seen.

The very low weights of carbohydrate eaten by both rat 2 and rat 7 on day 11 was due to the fact that fresh diet was not placed in the cage as there was an excess from the day before. Over the period, the diet had dried to a consistency that the rats were unable to physically bite into the block.

From the determination of the rolling averages, it was concluded that four days intake would be used to determine the energy intake of each rat, as if more days were included into the rolling average, the mean value would not change significantly.

It can be seen from the validation of weanling intakes, all rats gained weight over the period of the study again suggesting they were able to select the necessary amounts and proportions of macronutrients from the separate diets supplied.

Immediately post-weaning (29-32 days old), the consumption of carbohydrate increases sharply, to meet the high demand of the weanling rats for energy to support growth. The protein intake was slightly surprising in that the amount consumed remained constant and therefore the percentage of energy derived from this macronutrient decreased. It may have been expected that the protein intake would increase alongside the increase in weight, as rats require large amounts of amino acids and nitrogen to sustain growth. Therefore the results possibly suggest that there is a change in the efficiency of handling this macronutrient. Fat intakes were low in all rats and one possible explanation for this was that in the young rat effort was concentrated on the ingestion of nutrients which most easily support the requirements for growth and depositing carcass protein. (Leibowitz 1991), together with a physiological drive to consume carbohydrate.

Although there was considerable variation in the intake data between rats, there appeared to be a change in the pattern of macronutrients consumed in both sexes at around 38 days. This was at the time of onset of puberty in the rat and the changes in intakes may have reflected the changes in circulating hormones. Fluctuations in carbohydrate intake have been linked to changes in gonadalsteroids (Leibowitz 1992) and both groups of rats here showed the greatest change in carbohydrate intake at this time. Protein intake increased alongside the growth spurt seen at puberty, particularly in the males.

Although there was variation in the dietary intakes between individual rats, different patterns in macronutrient intakes between male and female rats were shown, especially in the percentage of energy each macronutrient contributed. The variability within each sex was relatively small suggesting that differences between groups would be able to be determined.

From the validation of carbohydrate intake, it can be seen that the physical form in which the diet was given was also very important. The different batches of diet not only

affected the carbohydrate intake, but also they were reflected in changes in the other macronutrients, particularly the fat. The increased intake of moist diet may not be due to more energy being consumed but the fact the water had diluted the energy content slightly so the rats had to consume more to obtain the same amount of energy. Some of the changes, particularly for the dry uncooked diet, may have been due to the response to the previous diet. The major problem in offering the diet in this form was that it had a very 'dusty' texture and it tended to be distributed around the cage and rat. The diet which had the least spillage and variation in intake was the dry ground diet, so on this basis this was the form chosen for the following studies.

Finally the validation of protein intake showed very regular intakes. This may have been due to the fact the rats that were used were now 8 weeks old and so were reaching the plateau in their growth curves. Again the ground diet gave the lower spillage and variability compared to the block diet so it was decided that the protein diet would also be given in a ground form. These forms were now the same as those used by Leibowitz who reported very small variation in her studies.

Therefore based on the findings from this study the protein and carbohydrate diets within the macronutrient self-selection regimen were be offered as the dry ground form, such that the diet would be mixed with water, dried in an oven overnight and then ground.

Chapter 4.0

Body composition analysis of rats exposed to a low-protein diet in utero and fed a macronutrient self-selection diet

4.1 Introduction

Epidemiological studies have demonstrated that poor intrauterine growth is linked to non-communicable diseases in adulthood, such as type II diabetes, hypertension and coronary heart disease (Barker et al 1990, Barker et al 1992, Hales et al 1991, Phipps et al 1993, Goldberg & Prentice 1994). Insulin and insulin resistance are known to be very important in type II diabetes (NIDDM) but it is also hypothesised that insulin is involved in the manifestation of essential hypertension particularly seen in the obese (Landsberg 1986, 1990, 1992, Daly & Landsberg 1991). We have previously shown that rats fed a macronutrient self-selection diet from weaning having been exposed to a low-protein diet in utero exhibit alterations in the pattern of macronutrient intake and regional adipose accumulation.(McCarthy et al 1994). Furthermore, gender differences were also observed. The low-protein exposed females became significantly heavier than the control group and developed significantly increased regional adipose masses especially at the omental and intestinal sites. The male offspring from the low-protein regimen however were not significantly heavier than the controls, although the omental and intestinal fat pads were significantly heavier in the low-protein offspring. Additionally it has been shown previously that offspring from this model display altered glucose disposal (Langley et al 1994a). This study however lacked measurements of insulin and the rats were fed on standard laboratory chow. The purpose of this study was therefore to extend the metabolic studies on the group of animals described in the publication by McCarthy et al 1994, to examine body composition and glucose tolerance.

4.2 Method

Offspring were produced from rat dams who had been fed the standard maternal dietary regimen (see 2.1). 24 rats were used in total, six males and six females in both the 9% and 18% casein groups. They were fed for 20 weeks from weaning the macronutrient self-selection diet (see 2.4). After this time they were fasted overnight prior to an intravenous glucose tolerance test (IVGTT), see section 2.7 for details. Plasma insulin and glucose concentrations were then determined. The insulin assay is outlined in section 2.9. The glucose measurements are included with acknowledgement to Richard Browne.

Total carcass composition was then carried out according to the method in 2.12 with the addition of anhydrous sodium sulphate to absorb the fat from the carcass during drying. The sulphate was added in a ratio of 2:1 of the rat's weight.

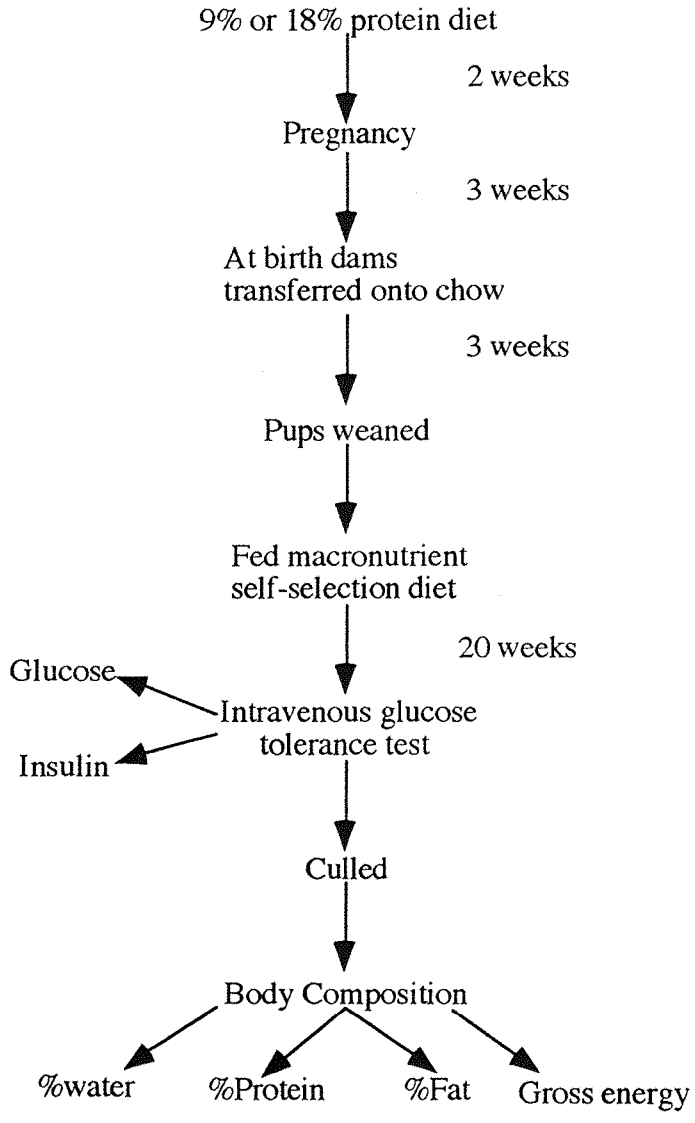

Figure 4.1 Flow chart to indicate method design

Similar equations to those found in section 2.12 were used to determine body composition, but as the sulphate was contributing to the weight of the sample, the equations were modified in the following manner :

Equation 1: $$\frac{test - blank * 0.1 * 14.008}{(weight / 5)} * 6.25 = \frac{mgProtein / gRat}{100} = \%protein(dry)$$

Equation 2: $$\%protein(wet) = \%prot(dry) * \frac{\%drywt}{100}$$

Equation 3: $$\frac{beforewt - afterwt}{samplewt / 5} * 100 = \%fat(dry)$$

Equation 4: $$\%fat(wet) = \%fat(dry) * \frac{\%drywt}{100}$$

Equation 5: $$Energy\ kJ / g\ (wet) = Deflection * energy\ in\ 1\ deflection * 5 * \frac{\%dry\ wt}{100}$$

Equation 6: $$Total\ Energy = Energy(kJ / g) * Body\ wt$$

4.3 Results

4.3.1 Plasma Glucose and Insulin Concentrations

The mean plasma glucose concentration at each time point for the two groups of female offspring is shown in figure 4.2. Fasting glucose concentration was significantly higher in the 9% casein group compared to the 18% casein group, at 6.0±0.5mmol/l and 4.6±0.1mmol/l respectively (P=0.02).

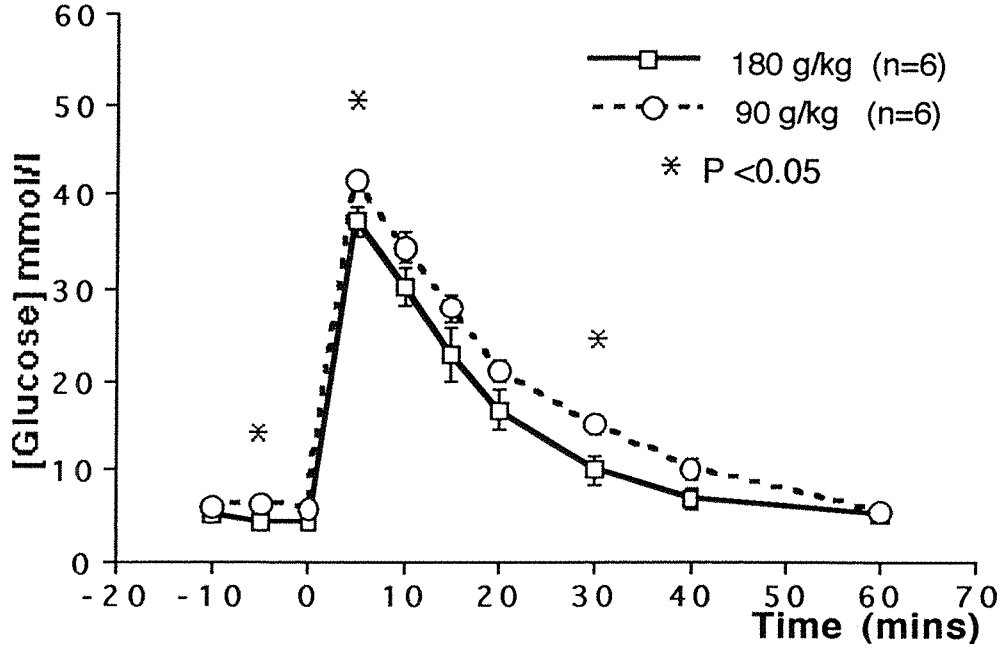

Figure 4.2 Mean plasma glucose concentrations for 9% and 18% females following a glucose load administered at time zero

The peak glucose concentration was also significantly higher in the 9% group, 42.2±0.6mmol/l versus 37.2±1.8mmol/l in the 18% rats (P≤ 0.05). Although the absolute glucose concentrations in the 9% group were higher, the pattern of response was similar in all the rats with values in both prenatal groups returning to baseline in the 60 minute time period. The area under the curve in the offspring in the 9% group was similar to the 18% females, 711.5 units and 580 units respectively (P=NS).

The glucose tolerance test curve in the male offspring is shown in figure 4.3. On this occasion neither the baseline (9%=7.3±0.9mmol/l, 18%=6.0±0.2mmol/l) nor the peak glucose concentrations (9%=51.9±1.9mmol/l, 18%=49.7±1.8mmol/l) were significantly different between the two prenatal groups. The response is similar to that of the females in that both groups glucose curves again return towards baseline although the 9% group is shifted to the right. The area under curve was not significantly different between the two groups, although the 9% male curve had a larger area of 1253 units compared to 894 units for the 18% male glucose curve.

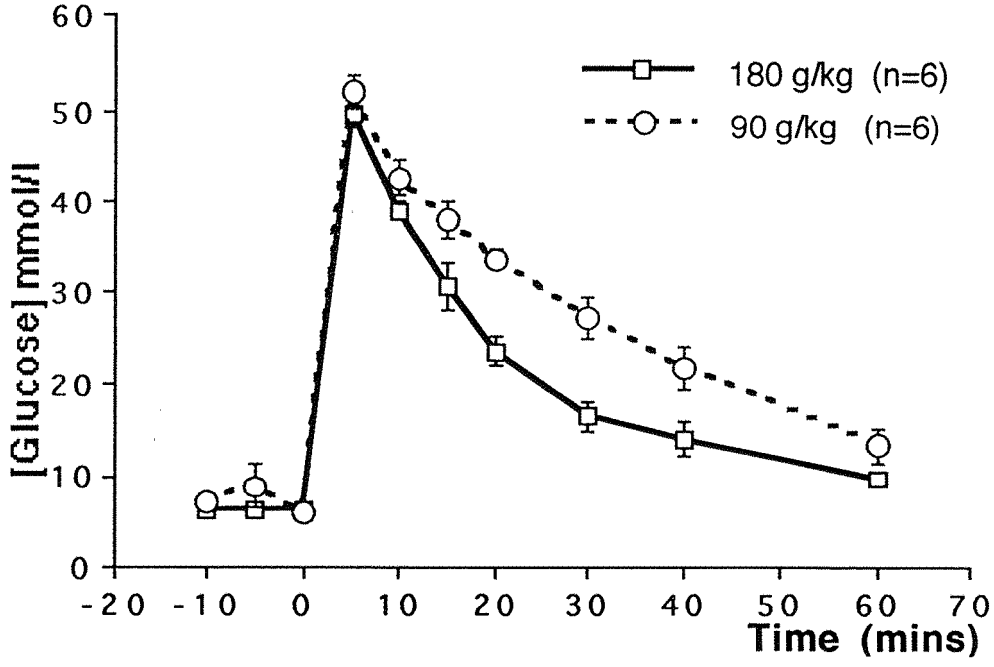

Figure 4.3 Mean plasma glucose concentrations for 9% and 18% males following a glucose load administered at time zero

The plasma insulin responses are shown in figures 4.4 and 4.5 for the females and males respectively. Clear differences in the pattern of release between the prenatal 9% and 18% groups are evident but females and males tended to behave similarly.

Figure 4.4 shows the insulin concentrations in females. The fasting insulin level was significantly higher in the 9% group (1.9±0.4 ng/ml) versus 0.8±0.1 ng/ml in the 18% group, P < 0.01.

The initial peak insulin response was however lower in the 9% group, although this was not statistically significant, (7.9±1.5 ng/ml vs 11.4±3.1 ng/ml) but in the 9% rats a second peak in plasma insulin concentration occurred after 20 minutes, such that after 60 minutes, the insulin concentration was markedly increased when compared to the 18% rats (81% increase). The 60min insulin values were 9.4±2.8 ng/ml for the 9% group and 5.2±3.3 ng/ml for the 18% group. This did not reach significance due to the large standard error seen at the final timepoint, due to the insulin concentration increasing five fold over the final 20 minutes in one rat in the 18% group. Statistical significance was reached at the 30 and 40 minute timepoints (P≤ 0.05). The values for the mean area under the curve were 307.4±47.9 units and 269.1±45.0 units for the 9% and 18% insulin curves respectively (P=NS).

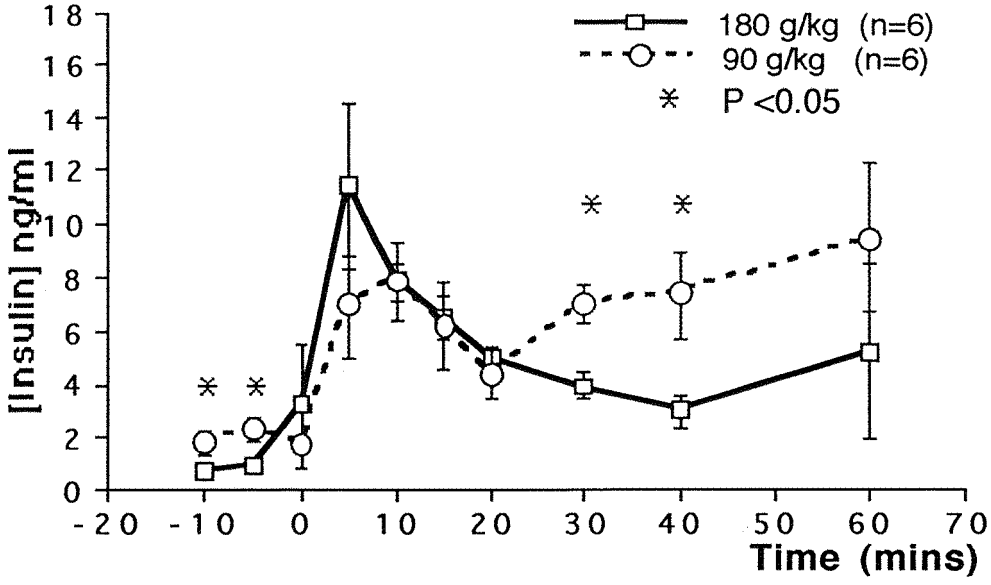

Figure 4.4 Mean plasma insulin concentrations for 9% and 18% females following a glucose load administered at time zero

The insulin response in males is shown in figure 4.5. The pattern of response is similar to that in the females. The initial insulin peak is lower in the 9% group (7.8±1.8 ng/ml compared to 10.6±1.6 ng/ml in the 18% rats). This was achieved however from a lower fasting level which was not observed in the females. The 9% group had a fasting insulin level of 2.4±0.2 ng/ml which was significantly lower than the 18% group value of 3.4±0.4 ng/ml (P= 0.03).

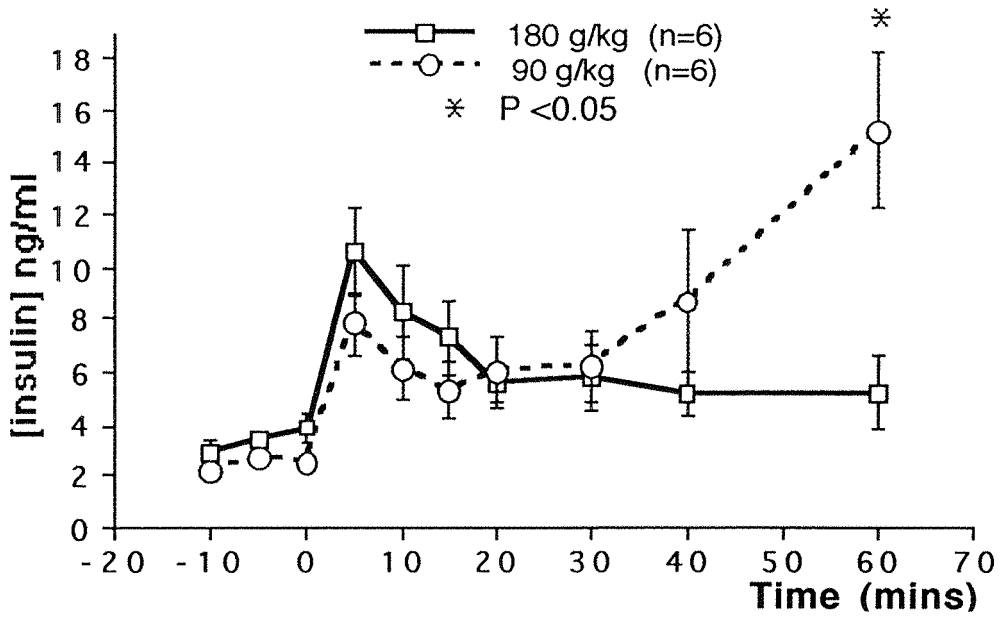

Figure 4.5 Mean plasma insulin concentrations for 9% and 18% males following a glucose load administered at time zero

Again a second rise in plasma insulin concentration occurred after 20 minutes, which this time lead to significantly higher levels at 60 minutes in the 9% rats (15.2±3.0 ng/ml vs 5.2±1.4 ng/ml, $P < 0.05$). The area under the curve in the 9% male offspring was 357.0±99.7 units whereas that in the 18% group was 205.4± 32.9 units (P=NS). These changes in insulin concentration lead to an increase in the area under the curve of 17% for the females and 43% for the males ($P < 0.05$)

4.3.2 Body composition

Percentage water, protein and fat and the gross energy content of the carasses are shown in table 4.1. The values for fat were unusually high and resulted in the value for the body composition to total over 100%. This was without including a value for carcass ash. As the rats were fasted overnight they were assumed to have negligible carbohydrate content so it was assumed that all the energy was derived from the protein and lipid components of the carcass. The percentage carass fat was therefore re-calculated by difference from the energy content of the whole carcass and percentage protein (hence protein energy) values using the following equations.

Equation 7: $Energy\ from\ fat = Total\ energy - (body\ wt * \frac{\%prot}{100}) * 22.9$

Equation 8: $\%fat = (\frac{energy\ from\ fat}{39.1}) / body\ wt * 100$

	Body wt (g)	% Water	% Protein	% Fat - Soxhlet	% Fat by difference	Total Energy (kJ)	Energy kJ/g
9% Male	500.0 ±16.5	49.4 ±1.0	18.9 ±0.8	37.9 ±1.8	26.6 ±2.1	7396 ±563	14.7 ±0.8
18% Male	478.9 ±12.4	52.2 ±1.1	18.0 ±0.5	32.4 ±2.3	25.0 ±1.8	6673 ±481	13.9 ±0.7
9% Female	306.2 ±20.8*	48.5 ±1.3	18.7 ±0.6	40.5 ±3.9	30.6 ±2.2*	4965 ±421*	16.2 ±0.9*
18% Female	269.6 ±5.8*	50.4 ±1.1	18.7 ±0.8	36.6 ±2.0	24.6 ±1.4*	3754 ±200*	13.9 ±0.6*

* 9% group significantly different from 18% group $P < 0.03$

Table 4.1 Body compositions expressed as % of wet weight (Mean ±SEM)

Table 4.1 shows the body compositions for the two prenatal groups of rats in both sexes. No significant differences in any chemical component were observed between the 9% and 18% males, although there was a tendancy for a higher protein and fat in the 9% group. Carcass fat calculated by difference was significantly lower in both prenatal groups compared to the percentage fat obtained by Soxhlet extraction.

In females, significant differences in both total carcass energy and energy (kJ/g) were observed, both being greater in the 9% group. The percentage fat when derived from Soxhlet extraction was not significantly different between the prenatal groups, but when derived by difference, the 9% group had a significantly greater percentage of body weight from fat. The %water and %protein were not significantly different in both between groups or sexes.

Following the initial discrepancy in the fat content obtained via the Soxhlets, the method of fat extraction was revised. In subsequent studies the solvent was washed in 2% calcium chloride solution prior to evaporation and then the lipid residue weighed.

4.4 Discussion

The results showed that although the glucose tolerance curves were similar between the control rats and the rats exposed to a low protein diet *in utero*, in both sexes, this was as a result of strikingly different patterns insulin secretion. The rapid clearance of an IV glucose load in the 9% casein group agreed with previous results (Langley et al 1994a), in rats which had been fed standard laboratory chow post-natally. In that study, the glucose load was cleared more quickly in the 9% group. Plasma insulin concentrations were not measured in that study so these can not be compared.

It has been reported that insulin is secreted in two stages in direct response to increasing blood glucose levels. Firstly there is the release of stored insulin which is held in the ß-cells of the pancreas. There is then a secondary response which requires the de novo synthesis of insulin (Ganong 1991). This could partially explain why there is an insulin peak which declines before the secondary increase in the 9% group as opposed to a direct increase from the first peak, since there would be a time delay as the insulin was being synthesised until its release. The reduced initial peak seen in the 9% rats of both sexes may indicate a lower level of stored insulin. This could be due to a difference in the structure of the pancreas in these rats compared to the 18% group although this was not investigated here. Previous studies have demonstrated changes in pancreatic structure as a result of exposure to a low-protein diet *in utero* (Snoeck et al 1990). A corresponding pattern of a secondary peak has also been demonstrated in the genetically obese Zucker rat (Fletcher et al 1986).

The fact that there is a large secondary insulin secretion in this study suggests there may be some degree of insulin resistance in the 9% group, as these rats are requiring a greater concentration of insulin to clear the glucose at the same rate as the 18% group. A feature of insulin resistance is down regulation of insulin receptors in target tissues, such as skeletal muscle and liver, as a response to increased levels of insulin present in the blood (Ganong 1991). This correlates with the fasting insulin levels in females which are significantly higher in the 9% group. It was therefore surprising that the findings in males were in fact reversed, with the 9% values being significantly lower than the control 18% casein rats.

In the females the increased plasma glucose concentration observed in the 9% group above that of the control group, together with a normal plasma clearance agrees with previous results shown in the genetically obese Zucker rat compared to their lean littermates (Fletcher et al 1986). It was concluded in that study, that the abnormal insulin levels may be influential in the abnormal growth of the obese Zucker rat as insulin stimulates lipid deposition. This may also be the case in the present study as the females in the 9% group had significantly increased adipose tissue masses at the time of measurement (McCarthy et al 1994).

The lower basal plasma insulin concentration in the 9% males compared to the 18% rats may indicate why these animals did not become obese, even though they showed a similar response to the glucose load as the females.

The explanation underlying the observation that offspring which have been exposed to a low-protein diet *in utero* are insulin resistant and relatively hyperinsulinaemic has yet to be established and is an area for future work.

The animals in the present study were maintained on a macronutrient self-selection diet. Their intake consisted of a relatively low carbohydrate and higher fat consumption (McCarthy et al 1994), especially when compared to rats consuming standard laboratory

chow (SLC). This selection is similar to the proportions eaten by obese Zucker rats on a similar regimen (Castonguay et al 1992). It would be therefore interesting to measure the insulin response in rats which have been exposed to a low-protein diet in utero but maintained on a high carbohydrate, low fat diet such as standard laboratory chow.

It appears that feeding a low-protein diet during pregnancy has no significant effect on the body composition of the offspring in males, despite a small but non-significant increase in body weight and gross energy. The increase in carcass energy content can be explained by the significant increase in omental and intestinal fat pads weights (McCarthy et al 1994) in the 9% male offspring. These results agree with similar studies by Jones et al (1984) & Anguita et al (1993). In both of these studies total food intake of the pregnant dams was restricted and their offspring accumulated more body fat mass than the offspring from the dams which had not been restricted. This can also be correlated with the findings in humans shown by Ravelli et al (1976) where a restricted diet due to the Dutch hunger winter lead to an increase in obesity, if the restriction was in the first two trimesters of pregnancy.

The significant increase in body weight in the 9% females was due to an increase in fat mass as this was the parameter which was also significantly increased. This would also agree with the significant increase in the total energy and the energy per gram of rat. In the study by McCarthy et al (1994) all regional adipose masses were significantly increased in the 9% females. However, this increase was observed only when the carcass fat was determined by difference and not by Soxhlet extraction. The percentage carcass fat content appeared high when compared to a study by Ballor et al (1990) which examined rats of the same age. In that study, carcass protein was similar to that in the rats in the present study. The water content was slightly low, 50% as opposed to 55-60%. This was most likely due to the fact that frozen carcass weights were used as not all weights at time of sacrifice were available. As the carcass energy content was significantly higher in the 9% group, a difference in the extractable lipid content would have been expected. The 'by difference' results therefore seem to be more appropriate. Usually the Soxhlet extraction method under estimates the true fat content. Pilot studies in this laboratory showed that when lard (100% extractable fat) was solvent extracted using this method only 80-90% of the initial fat weight would be accounted for. One possibility for the difference in the carcass extraction could have been the addition of sodium sulphate during the drying process, and although no extraction occured when sodium sulphate was refluxed alone, some binding of the sodium sulphate to the fat may have occurred during the drying process which caused it the be extracted along with the fat. The simple assumption of a change in ratio of the sulphate weight to rat weight, from 2:1 to 4:1 may be inaccurate during the drying process. The addition of sodium sulphate was therefore excluded from further studies.

The intake of macronutrients was very different both between prenatal groups and sexes, but the fact that there were only small changes in body composition in the males and the only difference in the female offspring which reached statistical significance was the percentage carcass fat, suggests that the metabolic handling of these macronutrients is different in those rats exposed to a low-protein diet *in utero* compared to the control.

Chapter 5.0

Influence of a macronutrient self-selection diet upon systolic blood pressure in rats with hypertension induced in utero

5.1 Introduction

Previous work in this department had shown that rats exposed to a dietary protein restriction *in utero* were hypertensive when measured at weaning and this hypertension persisted throughout adult life, when the offspring were maintained on standard laboratory chow (Langley & Jackson 1993). This prenatal model can also develop an 'obese-like' appearance in female offspring when they are allowed to self-select macronutrients, but not when fed on standard laboratory chow from weaning (McCarthy et al 1994). The major difference between the two types of diet is the amount of fat consumed, this being 3% in chow compared with typically 30-40% in the self-selection regimen when expressed as percentage weight of diet consumed. Percentage energy consumed from each macronutrient is also different.

The purpose of this study was to examine whether feeding a macronutrient self-selection diet influenced the expression of hypertension observed in rats following exposure to a low-protein diet in utero.

5.2 Method

Offspring were produced from rat dams which had been fed either 18% or 9% casein by weight as outlined in section 2.1. Thirty-two rats in each of the 9% casein and 18% casein offspring groups, 16 of each sex were used. At age 24 days they were weaned onto standard laboratory chow for one week until mean body weight reached 70g. Baseline systolic blood pressure was then measured as described in section 2.6.

Animals were housed individually and fed the macronutrient self-selection diet (section 2.4.1). After ten days on this diet systolic blood pressure (SBP) was remeasured and all the rats transferred onto standard laboratory chow for two weeks. After this time the SBP were again measured and the procedure of alternating diets repeated over the ensuing five weeks.

During the study the body weights of the rats were recorded every second day and food intake recorded as an average over a four day period when on the self-selection regimen (section 2.5).

5.3 Results

5.3.1 Weight Gain and Macronutrient Intake

The growth curves for the female offspring are shown in figure 5.1. There was no significant difference in body weight between the 9% and 18% casein groups at any timepoint throughout the study and alternating the diets did not appear to affect the growth trajectory.

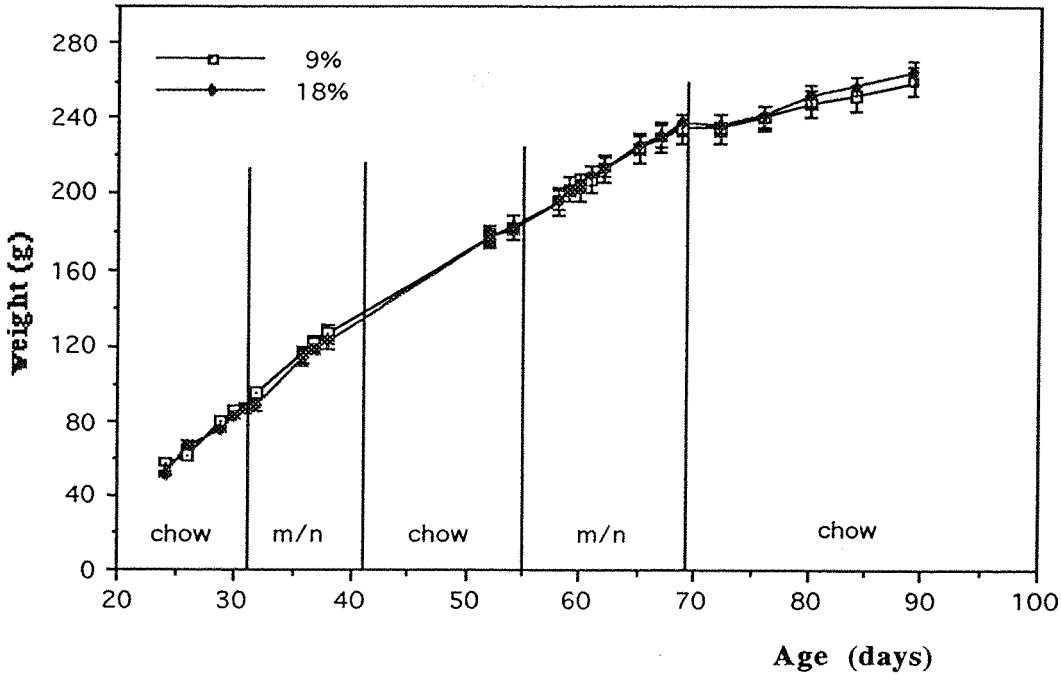

Figure 5.1 Growth curve for female offspring

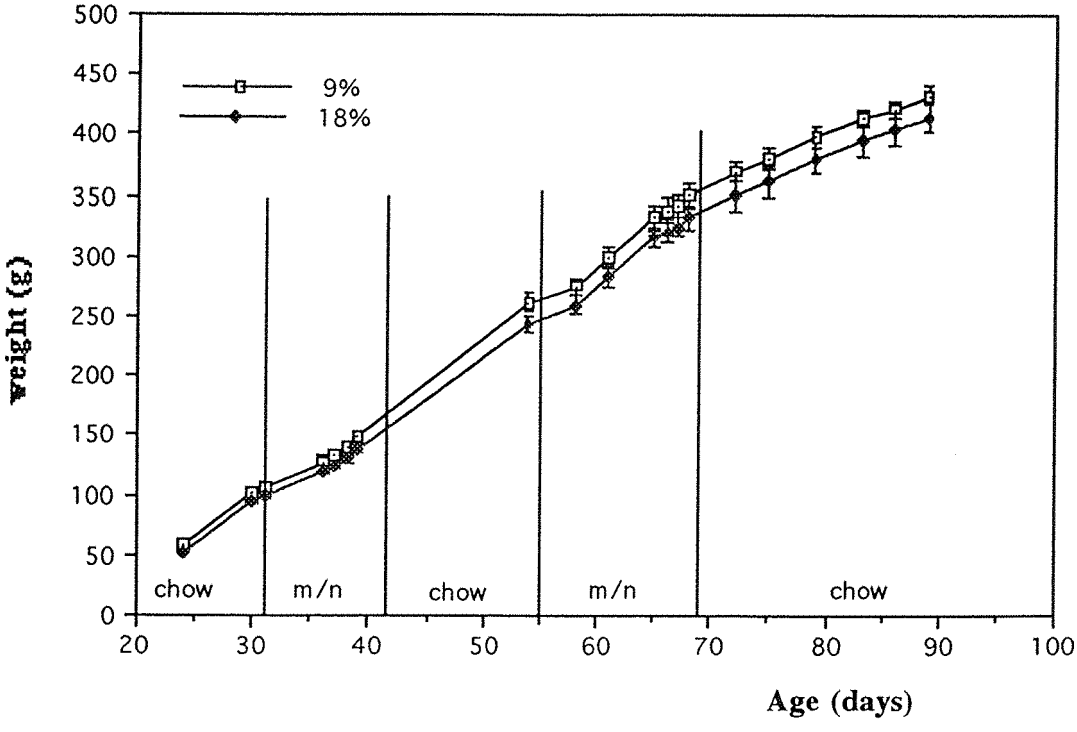

Figure 5.2 Growth curve for male offspring

A similar pattern of weight gain was seen in the male rats from the 9% and 18% casein group (figure 5.2). The 9% rats were however heavier than the 18% on this occasion although this did not reach statistical significance at any timepoint.

Intakes of macronutrients are shown in tables 5.1 and 5.2, for the females and males respectively, which give the mean intake of macronutrients for four consecutive days at age 4 weeks and at age 9 weeks. The table indicates the weight of diet eaten and although each separate diet is predominantly of one macronutrient they are all balanced for fibre and micronutrients therefore these values do not indicate directly to the amount actual macronutrient being eaten.

Age		Weight of Diet Eaten (g)			Total Energy
		Carbohydrate	Protein	Fat	(kJ)
4 weeks	9%	2.6 ± 0.6	7.1 ± 0.8	3.3 ± 0.5	257 ± 12
	18%	2.3 ± 0.5	8.2 ± 1.1	2.9 ± 0.6	255 ± 3
9 weeks	9%	5.8 ± 1.1	7.2 ± 1.3	5.0 ± 0.7	362 ± 22
	18%	5.1 ± 1.0	8.7 ± 1.0	4.7 ± 0.6	366 ± 20

Table 5.1 Female macronutrient intake expressed as grams of diet eaten (mean±SEM)

Age		Amount of Diet Eaten (g)			Total Energy
		Carbohydrate	Protein	Fat	(kJ)
4 weeks	9%	5.7 ± 0.9	7.6 ± 0.8	4.1 ± 0.6	339 ± 18
	18%	4.6 ± 0.9	7.5 ± 0.8	3.9 ± 0.5	313 ± 10
9 weeks	9%	7.9 ± 1.2	12.1 ± 1.6	6.2 ± 0.9	510 ± 23
	18%	8.5 ± 1.5	11.8 ± 1.2	6.0 ± 0.8	507 ± 20

Table 5.2 Male macronutrient intake expressed as grams of diet eaten (mean±SEM)

In both sexes of both prenatal groups, the amount of diet and hence the total metabolisable energy intake increased with increasing age, although this increase was modest for protein intake in the female 9% and 18% groups. In general the females of the 9% group consumed more carbohydrate and fat, but less protein than the 18% group although this resulted in similar energy intakes. However none of these differences in intake were significant at the 0.05 level, therefore it is not clear whether these are real differences between the two groups, but this could be addressed with larger number of rats. This was also true for the males except that the 9% group consumed more protein.

Figure 5.3 shows the percentage of total metabolisable energy intake from each macronutrient. This shows that although the fat diet was the least eaten in terms of grams consumed, this macronutrient contributed the highest percentage of energy intake. It

should be noted that there is a discrepancy in the 18% female group at age four weeks, in that the total value obtained is over 100%, and is due to the large standard error on this measurement.

This figure also shows that although the total metabolisable energy intake increased over the five weeks, the percentage derived from each of the macronutrients remained essentially unchanged.

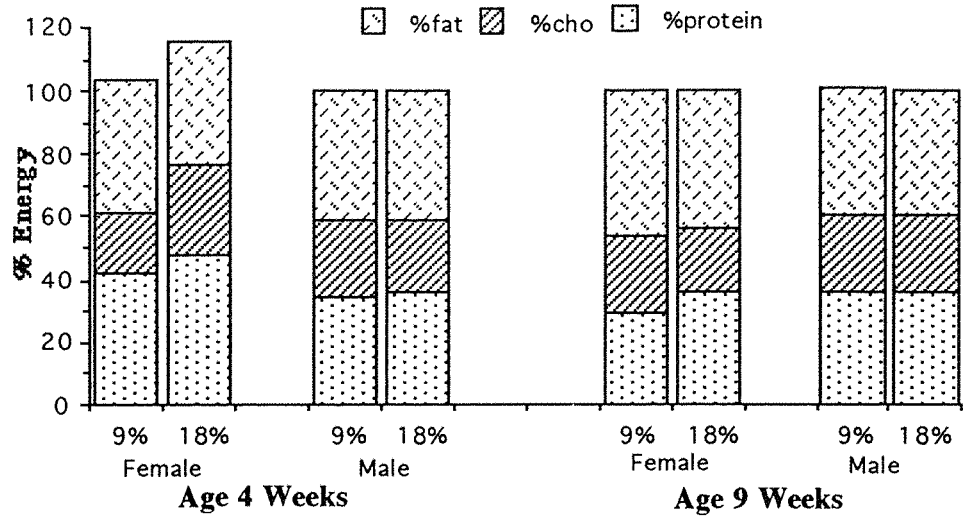

Figure 5.3 Percentage energy intake for each of the macronutrient diets

5.3.2 Systolic blood pressure

Figure 5.4 shows the systolic blood pressures (SBP) in the female rats at various timepoints. At weaning SBP was significantly higher in the 9% group ($P < 0.05$). After feeding the macronutrient self-selection diet (MSS) for two weeks this significant difference disappeared and SBP of the 9% group fell below that of the 18% rats.

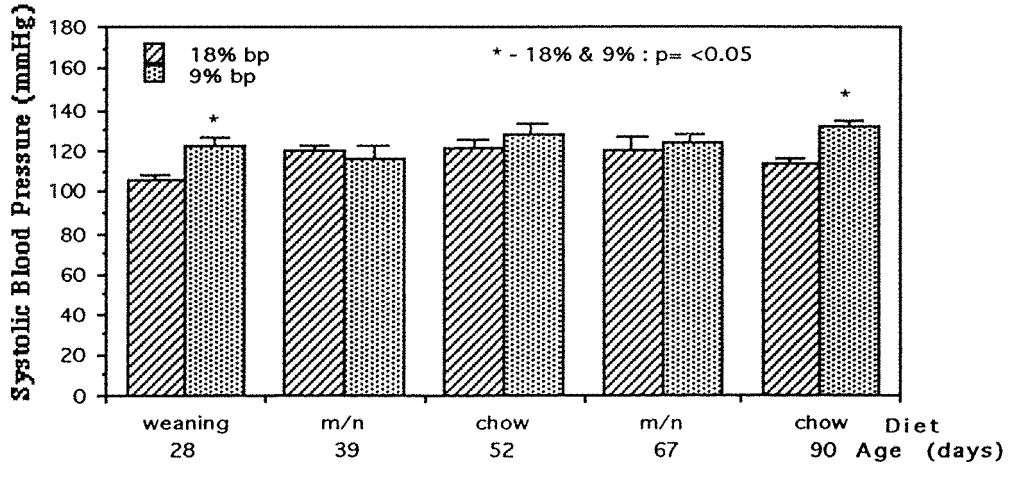

Figure 5.4 Systolic blood pressure in the female offspring

The similarity in SBP was only present however when the animals were fed the MSS diet. When refed standard laboratory chow for two weeks, SBP increased in the 9% group, whilst that in the 18% group remained stable. This resulted in a significant difference at age 90 days, with the SBP in the 9% group again increased by 20mmHg ($P<0.05$).

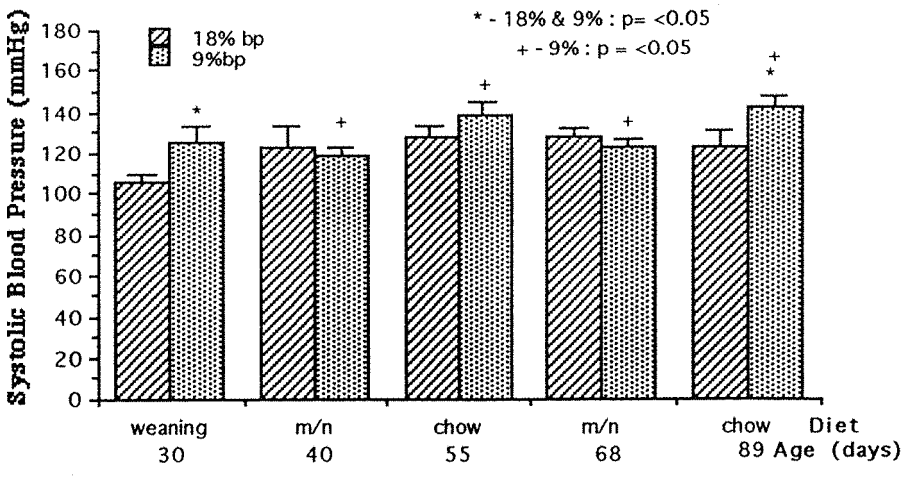

Figure 5.5 Systolic blood pressure in the male offspring

The results for the male offspring are presented in figure 5.5. Similarly, the mean SBP was significantly higher in the 9% group at weaning ($P<0.05$), and this increase was sustained when the rats were maintained on standard laboratory chow. Following two weeks of MSS feeding, the SBP in rats from the 9% group decreased whereas that in the 18% group was unaltered. The relative hypertension of the 9% group was expressed again following re-introduction of standard laboratory chow.

5.4 Discussion

These results demonstrate that the type of diet consumed post-weaning influences the systolic blood pressure of rats which have been exposed to a low-protein diet *in utero*. It can be seen that for both males and females of both prenatal dietary groups, the macronutrient self-selection regimen has little effect, if any, on growth, indicating that the rats were able to select an adequate proportion of each macronutrient to sustain the rate of growth, as displayed by the rats fed standard laboratory chow.

The weight gain for both groups (9% and 18%) is also very similar, which agrees with previous results for the males but not the females where those in the 9% group were shown to be significantly heavier at weaning and at all timepoints throughout the study (McCarthy et al 1994). One possible reason for this discrepancy could be that the rats were only on the macronutrient self-selection (MSS) diet for a short period of time before

being maintained on chow, although differences in the previous study were seen almost immediately following weaning (McCarthy et al 1994). Another possibility is that these animals were not weaned directly on to MSS since they had to achieve a body weight of 70g before the first blood pressure measurement could be made, and this could have affected future growth and partitioning of energy. Furthermore, whereas in the previous study, consistent and significant group differences in macronutrient intakes were observed no such differences were evident in this study. The reason for this cannot be explained at this stage.

The proportion of macronutrients consumed were also similar at four and nine weeks of age with the metabolisable energy intake increasing which is consistent with the increasing energy required by the growing and larger rat, together with the protein intake increasing in the male rats. This occurs to meet the presumed high requirement in the growing rat for amino acids and nitrogen to be able to support maximal growth. This pattern of intake is consistent with previous results (Leibowitz et al 1991). If however the macronutrients consumed are expressed as a percentage of metabolisable energy intake, the protein intake in the males remains constant over the 5 weeks at 35%.

The systolic blood pressure (SBP) at weaning is significantly higher in both the male and female 9% rats compared to their 18% counterparts, which is consistent with previous results from rats which have been produced from a similar maternal protocol (Langley-Evans & Jackson 1993, 1995). This difference is abolished after feeding the MSS diet in either the males or the females. This is partly due to an age-related increase in the 18% SBP although the MSS diet caused the SBP in the 9% group to actually decrease. The increase seen in the 18% group is part of the normal development in the rat as shown in previous studies (Langley & Jackson 1993, Langley et al 1994b), and is possibly due to the maturing of the kidney in the first few weeks of post-natal life. The fact that the SBP in the 18% group remains very stable over the ensuing 5 weeks further suggests that the feeding of the MSS diet has no effect on the SBP of the 18% group. If the rats are maintained on chow throughout their adulthood then the significant difference seen at weaning is maintained (Langley-Evans et al 1993). This is not the case for the 9% group whose SBP increased when fed standard laboratory chow, and then decreased upon MSS feeding. This important observation cannot be fully explained at this stage, but taken together with findings in other studies, notably those in the spontaneously hypertensive rat, it is possilbe to suggest an explanation. When the spontaneously hypertensive rat (SHR) was fed a high-fat diet from weaning, the hypertension is abolished.(Wexler 1981). It was suggested that hypopituitarism in this strain was caused by the high-fat diet which prevented the increase in SBP since significantly smaller pituitaries were found in the high-fat fed group. However other factors may also account for this inhibition of hypertension such as changes in insulin sensitivity.

The rats in the 9% group were selecting 40% of their metabolisable energy intake as fat which is very much higher than that in chow (~6%). Therefore it is possible that this change in the ratio of carbohydrate to fat consumed is one contributory factor to the changes seen in SBP, and that the 9% group has some adaptation in the handling of these macronutrients. The SBP in the 18% group which also consumed these proportions in the MSS was not affected. Although there is some variation in intake on the MSS regimen, what remains at all times is a significant difference of the proportion of fat to carbohydrate between the MSS and chow feeding.

The effect is more prominent in the male offspring with each change of diet producing a significantly different SBP from the previous diet, although the trend is also seen in the females. Body weight does affect systolic blood pressure which may be one reason why this effect is seen more clearly in the males as there is a bigger difference between the two groups in body weight compared to the females, However the difference in body weight between the male 9% and 18% rats is small and does not reach significance so this cannot explain the differences seen especially as they were present early on in the study when the weights were nearly identical. Therefore the stronger relationship seen in the males may be due to two factors. Firstly it has been shown in the SHR that the hypertension is androgen dependent (Chen & Meng 1994) and these rats are showing a similar response to a higher fat diet as the SHR. It is possible therefore that rats in the 9% group have androgen dependent hypertension and the SBP is decreased by the same mechanisms as in the SHR. The other factor could be the effect of the oestrus cycle in females as blood pressure changes over the course of this cycle (Takezawar 1994) and these small changes may cloud the effect in the females.

Because of this important finding of the effect of macronutrient self-selection feeding upon SBP in the 9% group, and our hypothesis that such an effect is due to the change in the proportion of macronutrients consumed. A change in the proportion of fat to carbohydrate in the diet will cause a major effect on the insulin response, with a lower response with a greater proportion of fat in the diet. Insulin resistance has been shown in studies with the spontaneously hypertensive rat (Swislocki & Tsuzuki 1993) and a strain of transgenic hypertensive rat (Vettor et al 1994). Insulin levels have also been shown to correlate with blood pressure in clinical and population-based studies in humans (Landsburg et al 1991). The following experiments were performed to further elucidate the mediators of this hypotensive effect and to test our hypothesis.

Chapter 6.0
Comparison of Macronutrient Intake in Spontaneously Hypertensive Rats (SHR) and Hypertensive Rats of Fetal Origin

6.1 Introduction

The SHR has long been used as a model for human hypertension. The genetic mutation has still yet to be identified although much has been learned about the origins of the hypertension in this model. One important observation has been changes in insulin action and glucose metabolism suggestive of insulin resistance (Hulman et al 1993, Swislocki & Tsuzuki 1993, Mondon & Reaven 1988). It had been recently observed that some metabolic and physiological similarities occur between rats which had been exposed to a low-protein diet in utero and the spontaneously hypertensive rat. For example, both models become hypertensive when fed on standard laboratory chow but normotensive when fed a high-fat diet (Wexler 1981, Chapter 5). Also both demonstrate similar altered insulin responses to a glucose load when maintained on standard laboratory chow (Mondon & Reaven 1988, Chapter 4). Furthermore recent investigations have shown urinary excretion of PGE_2 was similar between the two models (Sherman & Langley-Evans 1998).

In view of these important similarites, the purpose of the present study was to examine whether these likenesses extended to macronutrient choice which could possibly give some further insight into the mechanisms for the observed changes seen in the low-protein exposed rats.

6.2 Method

Offspring exposed to a low-protein diet in utero were produced from the maternal protocol in section 2.1. These rats were of the Wistar strain, the SHR were originally derived from the same strain. The Wistar rats maintained on 18% casein diet were used as controls. The SHR were obtained from the University of Southampton's breeding colony. Both sets of offspring were maintained on the MSS regimen (section 2.4) from age 4 weeks until the age of 16 weeks. There were eight offspring in each group of males and females as summarised in table 6.1. Energy intake was measured at age 5, 8, and 10 weeks. Weighed portions of each macronutrient were placed in the cage each day and removed and reweighed at the same time each following day as outlined in section 2.5.

9% casein		18% Casein		SHR	
M	F	M	F	M	F
8	8	8	8	8	8

Table 6.1 Summary of number of offspring in each group

6.3 Results

6.3.1 Pre-weaning weights

SHR are known for having a high risk of losing their pups if disturbed shortly after birth (unpublished observations), so the first body weights were recorded at age 10 days to minimise the stress imposed upon the mother. These body weights were compared to the Wistar rats from both the 9% and 18% exposed groups. From figure 6.1 it can been seen that the SHR pups were significantly lighter at all timepoints post-natally at which they were weighed, and this significance increased as the pups aged. When the pups were weaned, the SHR were on average 20g lighter than the all the Wistar rats, irrespective of which diet they experienced prenatally.

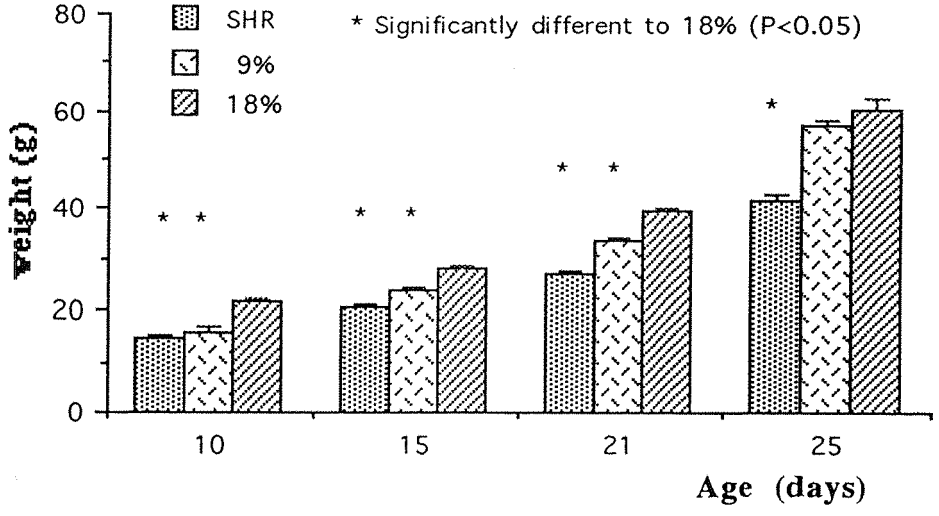

Figure 6.1 Preweaning weights of SHR and Wistar pups (Mean ±SEM)

6.3.2 Female offspring

The SHR remained significantly lighter than both other groups of Wistar rats from weaning throughout adult life. The weight difference between the SHR and Wistar rats at 12 weeks of age was approximately 60g, at this age the SHR were culled. The Wistar rats were similar in weight irrespective of which diet they were exposed to in utero, although

the control (18%) animals on average were slightly heavier than the 9% group (P=NS), as seen in figure 6.2.

The growth curves are compared to their energy intake in figure 6.3 overleaf. This chart shows the total energy the three groups were consuming at three time points. The results are expressed as the mean of four days intake at each timepoint. At five weeks of age it can be seen that the SHR were already significantly lighter than both the Wistar groups (figure 6.2), however this weight difference cannot be explained by differences in their food intake as it can be seen in figure 6.3 that at this time all groups were eating a similar quantity of energy.

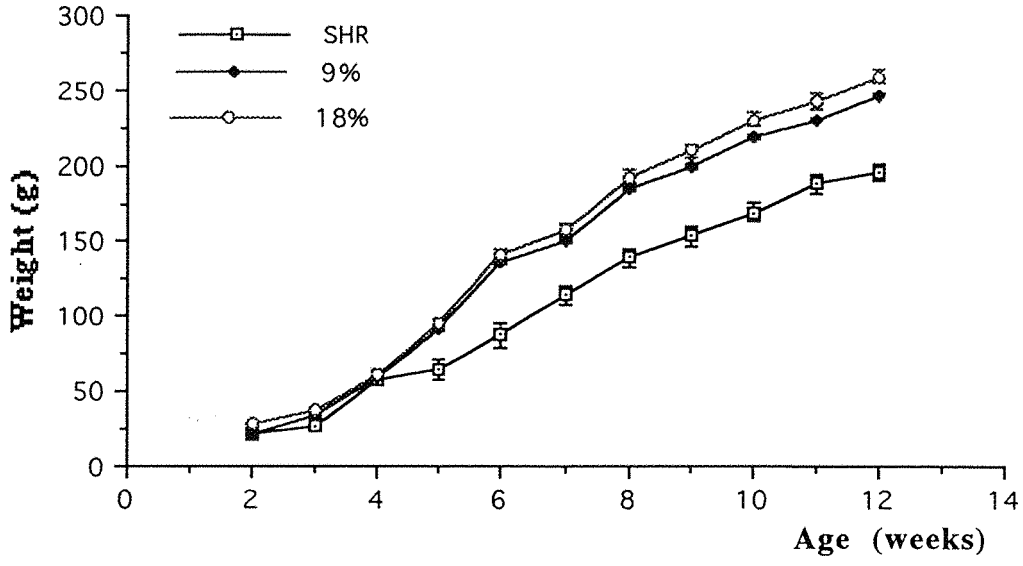

Figure 6.2 Female growth curve

The different proportions of macronutrients may be a determinant in the differences in growth. At all times during the study the SHR were eating less protein than the other two groups, this was significantly different to the 9% group at age 5 and 10 weeks. The proportion of energy dervived from fat increased with age in all groups. The SHR had a signifcantly less proportion of energy derived from fat at age 8 weeks compared to the 18% rats. The total energy consumed increased sharply from 5 weeks to 8 weeks of age and then a much smaller increase to age 10 weeks. Although many comparisions have been made between the SHR and 9% rats, with respect to macronutrient intake, there appears to be little similarity between the SHR group and the *in utero* low-protein exposed group.

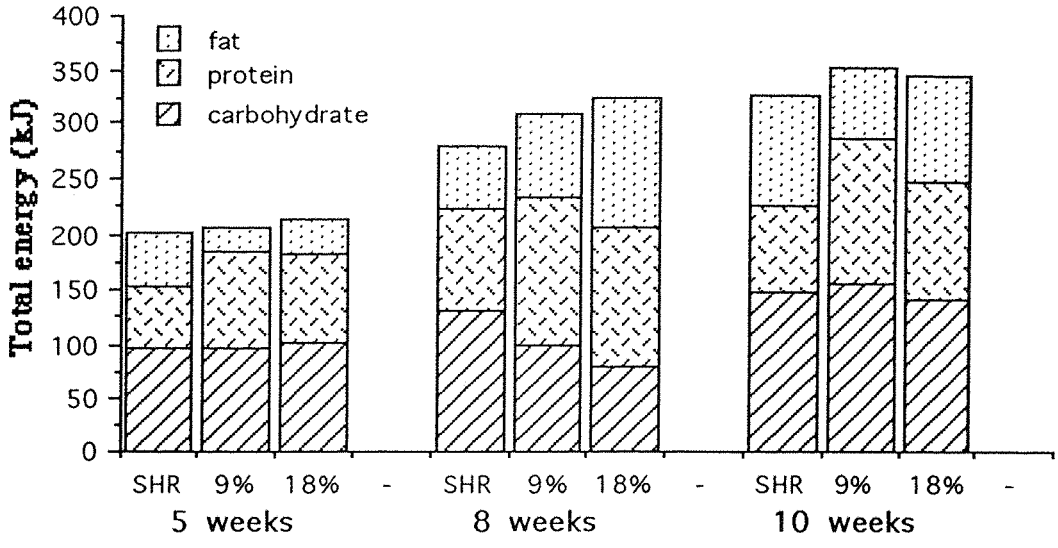

Figure 6.3 Mean energy and macronutrient intakes of the female SHR and Wistar 9% and 18% groups (kJ)

The mean percentage energy for each of the three macronutrients over the same three timepoints was also calculated and the results can be seen in figure 6.4. The contribution of carbohydrate to total energy in the SHR group remained remarkably similar over time at approximately 47%. The 9% and 18% groups had similar percentage energy from carbohydrate at 5 weeks this dropped sharply such that at 8 weeks of age the values had fallen to 32% and 25% respectively. The fall in proportion of energy from carbohydrate was replaced by an equivalent increase in the proportion of fat, with protein remaining stable.

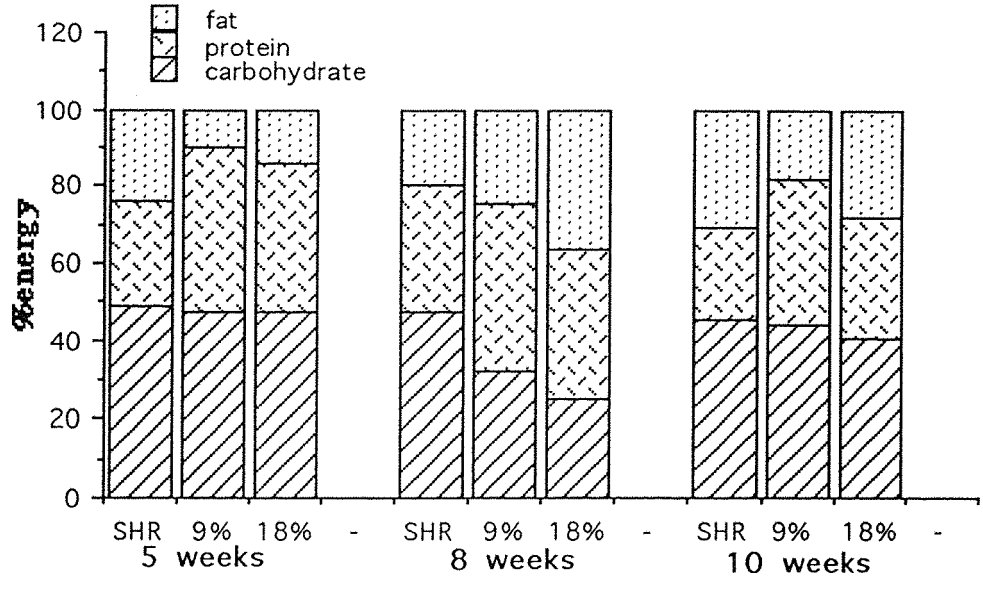

Figure 6.4 Female energy intake expressed as a percentage

At age 10 weeks the carbohydrate levels were similar to those as the start of the study for all groups, however the percentage fat remained higher and hence there is a relative decrease in the proportion of protein contributing to the total energy consumed.

6.3.3 Male offspring

The growth curves for the male rats are shown in figure 6.5. The general findings were similar to the females in that the SHR group was significantly lighter than the other two groups. However at age 5 weeks there appeared to be a clear point where the SHR animals were unable to sustain the same rate of growth as the Wistar rats. The 9% and 18% animals had a similar pattern of growth, with the 18% being slightly heavier than the 9% protein-exposed group. This reached significance at the final timepoint of the study at 12 weeks of age ($P<0.05$). The SHR reached a plateau of growth at age 11 weeks, whereas the Wistar groups remained on an upward trend.

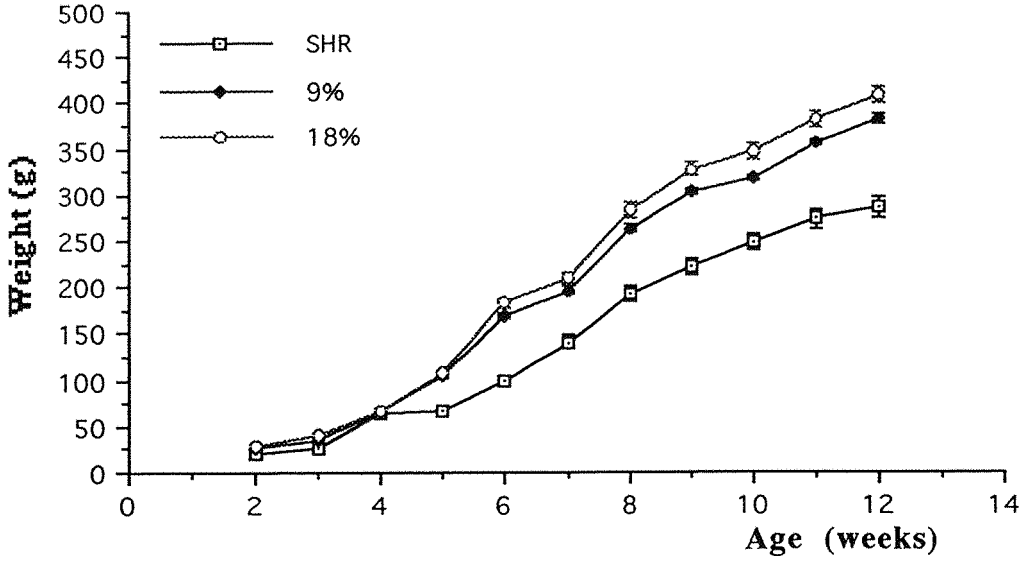

Figure 6.5 Male growth curve

Figure 6.6 indicates that the SHR group consumed a similar quantity of energy to the Wistar rats at age five weeks but this was not maintained at 8 or 10 weeks of age. SHR energy intake was significantly lower than the 18% group at age 10 weeks and both the 9% and 18% groups at age 8 weeks. The SHR group did not increase their amount of protein consumed in the manner that the 9% and 18% rats did, such that at 10 weeks of age the SHR were only eating on average 91kJ whereas the 9% animals were consuming 179kJ and the 18% animals 159kJ from protein.

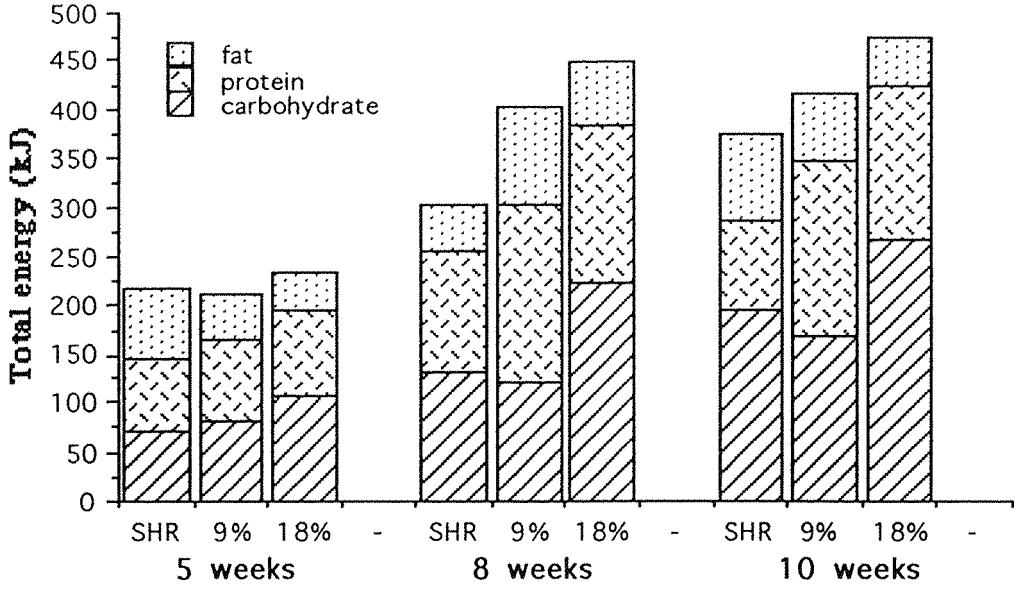

Figure 6.6 Mean energy and macronutrient intakes of the male SHR and Wistar 9% and 18% groups (kJ)

This difference in the consumption of macronutrients is also reflected in the percentage of energy from each nutrient as seen in figure 6.7. At both 5 weeks and 10 weeks of age, the proportion of energy derived from fat was the same across the three groups. The SHR had the greatest percentage and the 18% rats the least. This was not the case at 8 weeks where the 9% animals had the largest percentage from the three groups.

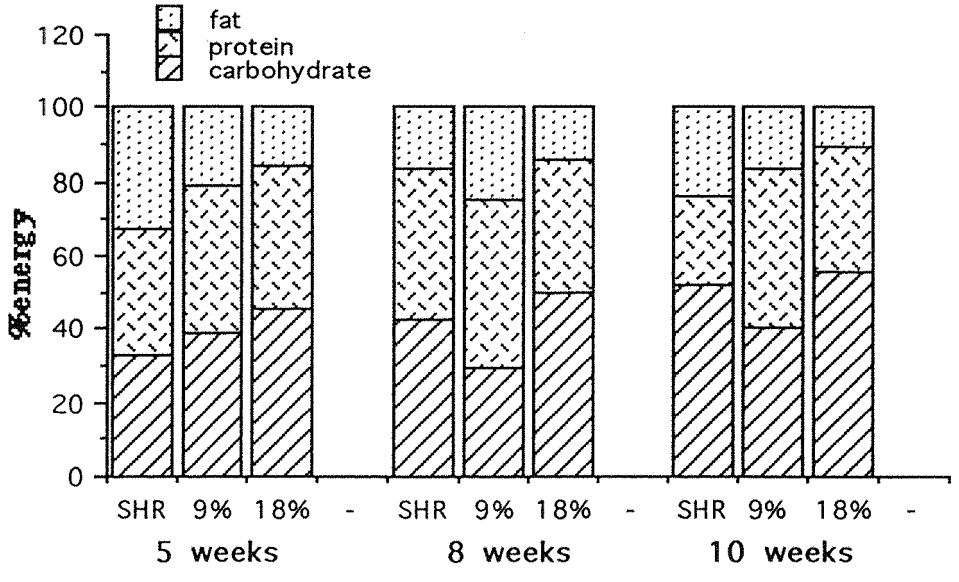

Figure 6.7 Male energy intake expressed as a percentage

The percentage of energy coming from carbohydrate steadily increased over time in both the 18% and SHR groups. Carbohydrate consumption in the 9% group was

lower than the 18% group at all timepoints but this only reached significance at 8 and 10 weeks of age ($P<0.05$).

6.4 Discussion

The purpose of this study was to investigate whether energy and macronutrient intakes were similar in different models of hypertension which have also been shown to posess a number of similar characteristics. The results indicated no similarities between the SHR group and those exposed to the low protein diet in utero. At all timepoints in the study for both sexes the SHR were consuming significantly less protein that both the 9% and 18% Wistar groups. At age 8 weeks, again in both sexes there was a stepwise decrease in consumption of carbohydrate across the SHR, 9% and 18% groups respectively.

If their growth is considered, it was shown that in both the male and the female groups, the SHR were significantly lighter throughout the study than the 18% control Wistar group. From weaning they are also significantly lighter than the 9% group, however this group were also significantly lighter than the 18% animals until aged 25 days. It appears in this group of animals that the exposure to low-protein in utero may have had some effect on weight but the offspring were able to show catch-up growth and so by weaning there was no significant difference between the 9% and 18% Wistar groups. The SHR weight difference was first observed at a very early age, it suggests that it is not just that the animals are born lighter, but most likely they are genetically smaller rats. It has been shown that SHR dams produce less protein in their milk compared with other strains such as the Wistar, and it may be this which is contributing in part to the lower weight gain (Rose & McCarty 1994).

The SHR offspring may be 'programmed' into handling a certain proportion of protein and hence when fed the macronutrient self-selection regimen reflected this in a lower proportion of their energy intake contributed by protein compared to the other two groups. This may result in changes in protein turnover, partitioning of energy as protein and reflected in linear growth. The 9% groups although exposed to a lower protein level *in utero*, as the dams are transferred at birth onto standard laboratory chow the offspring during the majority of the lactation period would be receiving the same amount of protein compared to that of the controls, although there would be a short 'run-in' period of a couple of days when the maternal body would be readjusting to the different protein concentration.

The males of the SHR group had a greater difference in weight from the Wistar groups to that of the females and this was probably due to the fact that there was a greater rate of growth in male Wistar rats to that of female which the male SHR were not able to sustain.

The intake of the SHR females at 5 weeks is similar to that of the Wistar rats and is not significantly less at the other measurements, however a similar weight gain is not maintained. This suggests that the SHR are not as efficient at converting food energy into lean and fat tissue as the Wistar rats, and that their energy expenditure is higher. This was not measured in these animals. One would speculate that SHR demonstrate greater basal metabolic rate (BMR), higher levels of physical activity or increased brown adipose tissue activity. The fact that macronutrient intakes were not similar was surprising,bearing in mind the number of other similarities they appear to have, such as reduction of blood pressure when fed a high-fat diet (Wexler 1981, Chapter 5), altered insulin responses to a glucose load (Mondon & Reaven 1988, Chapter 4), and similar urinary excretion of PGE_2 (Sherman & Langley-Evans 1998).

The results for the male offspring are perhaps slightly simpler to explain as the SHR group do have a significantly lower intake of energy than the Wistar groups at 8 and 10 weeks of age which would explain the lower growth rate as the Wistar rats. Again however there was little correlation between the SHR group and the 9% Wistar group.

Chapter 7.0
The effect of feeding composite diets of differing macronutrient composition on metabolic parameters

7.1 Introduction

In chapter 4 it was shown that rats exposed to a protein restricted diet *in utero* have altered insulin responses to intravenous glucose loads. This confirmed findings in previous studies (Phillips et al 1994, Smith et al 1975, Tse et al 1995), and is suggestive of insulin resistance. Additionally we have shown that rats from dams fed a 9% casein diet during pregnancy develop increased regional adipose masses compared with the 18% animals, when fed on a macronutrient self-selection diet (McCarthy et al 1994). Furthermore previous studies demonstrate changes in specific organ weights (Langley-Evans et al 1996a). This is in addition to an elevation of systolic blood pressure when fed on standard laboratory chow postnatally (Langley & Jackson 1993). Similar to the previous chapter, when the systolic blood pressure was measured from *in utero* low-protein exposed rats which had been fed a macronutrient self-selection diet since weaning, at 20 weeks they did not demonstrate an elevation in systolic blood pressure (unpublished observations McCarthy & Pickard).

As previously noted the major difference between these studies examining blood pressure is the amount of dietary fat ingested, this being 3% of metabolisable energy in chow compared with 35% in the self-selection study. Wexler et al (1981) showed that if spontaneously hypertensive rats were fed a high fat diet from weaning this prevented the manifestation of hypertension. Additionally the hypertension observed in the 9% offspring is reversed by feeding a macronutrient self-selection diet and we hypothesed that this might be due to a greater ratio of fat : carbohydrate ingested as it is re-established on refeeding of standard laboratory chow (Chapter 5). The similarity between the findings of Wexler and those in this study is notable and suggests a similar mode of action. It is also possible that some other unknown dietary factor could account for the differences in hypertension in these models.

Therefore the purpose of this study was to examine the influence of feeding diets, rich in one macronutrient, upon systolic blood pressure, glucose tolerance and body composition in rats exposed *in utero* to a maternal 9% protein diet. Here it could be seen whether the specific dietary stress influenced any of these parameters.

7.2 Method

24 female virgin Wistar rats were fed the maternal diets as described in section 2.1. From these dams a total of 120 offspring were used in the study. These comprised of 8 of each sex from each prenatal dietary group in one of four postnatal dietary regimens namely high-protein, high-fat, high-carbohydrate and standard laboratory chow. The compositions of these diets are detailed in section 2.4.2. Table 7.1 summarises the number of offspring in each group.

Maternal Diet	9% casein								18% casein							
Post-weaning diet	h/fat		h/prot		h/cho		chow		h/fat		h/prot		h/cho		chow	
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
No.	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8

Table 7.1 : Summary of number of offspring in each group

At four weeks of age, systolic blood pressure of the offspring was measured using the tail-cuff method (section 2.6) and they were then housed individually according to the standard protocol outlined in section 2.3. The postweaning diets were fed for 8 weeks during which time systolic blood pressure and food intake were measured at two week intervals. Weight gain was measured every second day. At age 12 weeks an intravenous glucose tolerance test was carried out as described in section 2.7 and the samples were frozen and later assayed for glucose and insulin (sections 2.8 and 2.9 respectively). The animals were then killed by cervical dislocation and exanguination and regional adipose masses, organ and muscles were weighed (section 2.11). The rats were frozen at -20°C for later body composition analysis (section 2.12). Due to the large numbers of offspring involved in this study, the protocol was carried out in two parts, the high-fat and high-protein carried out together and then the high-carbohydrate and chow groups. Only the female offspring were analysed for glucose, insulin and muscle fibre type.

7.3 Results

7.3.1 Maternal Data

Figure 7.1 shows the mean growth curves for both dietary groups prior to and throughout pregnancy. There was no difference in initial body weights or pre-pregnancy weight gain in the two dietary groups, but the 18% casein group gained a mean of 25g more weight during pregnancy, although this did not reach significance at any timepoint.

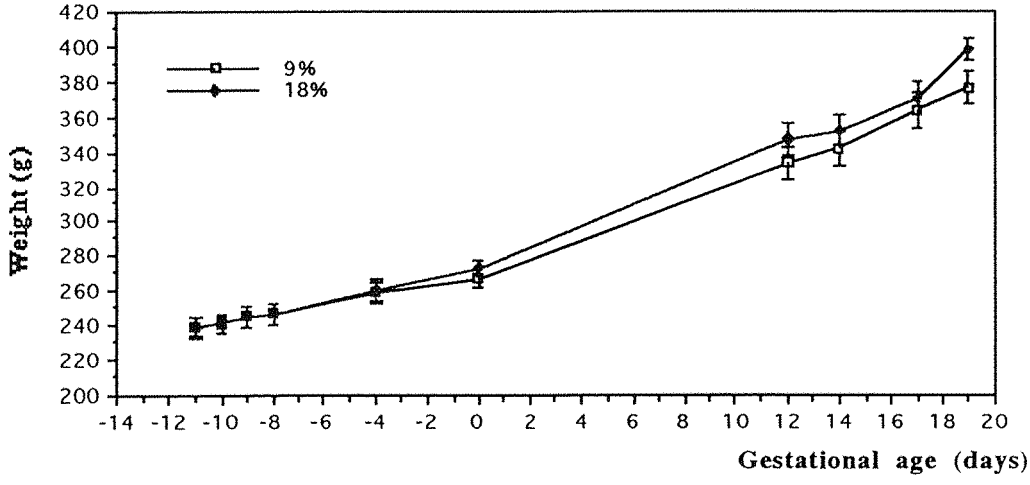

Figure 7.1 : Maternal growth curves

The maternal dietary intake is shown in figure 7.2. It can be seen that, at pre-pregnancy, there is no difference in food intake between dietary groups, and although both groups significantly increased their intake in the 2nd trimester of pregnancy ($P<0.05$), the 18% group increased their intake by 10g to 30g±0.8g, which was significantly greater than the 9% dams.

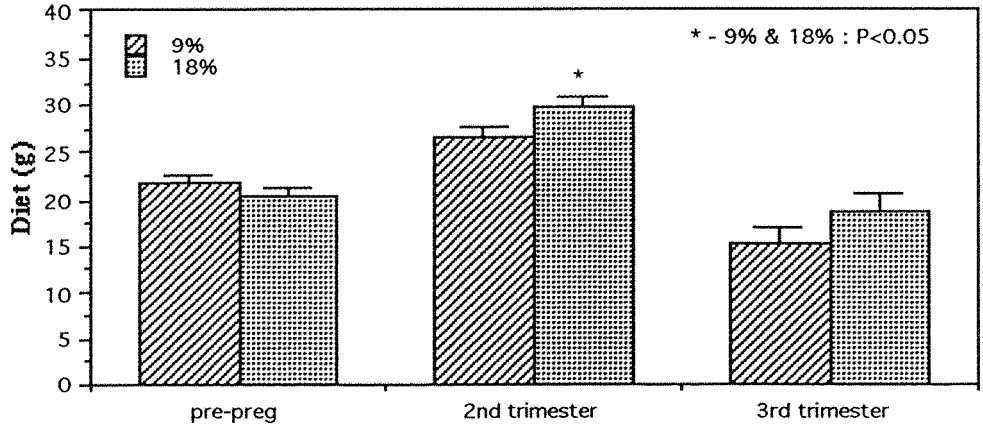

Figure 7.2 : Maternal dietary intake

During the 3rd trimester there was no difference in intake between the 9% and 18% groups and food intake was significantly reduced in both groups to below that of the pre-pregnancy levels ($P<0.05$).

There was no significant difference in either litter size (13, 13), total litter weight (61.1g±5.2, 62.3g±4.6) or birth weights (4.91g±0.1g, 4.87g±0.1g) between the 9% and 18% mothers respectively.

7.3.2 Offspring Data

7.3.2.1 Preweaning growth

Figure 7.3 shows body weights from birth to weaning for the 9% and 18% offspring. The 9% offspring were significantly lighter than the 18% rats at weaning (age 31 days) ($P<0.05$).

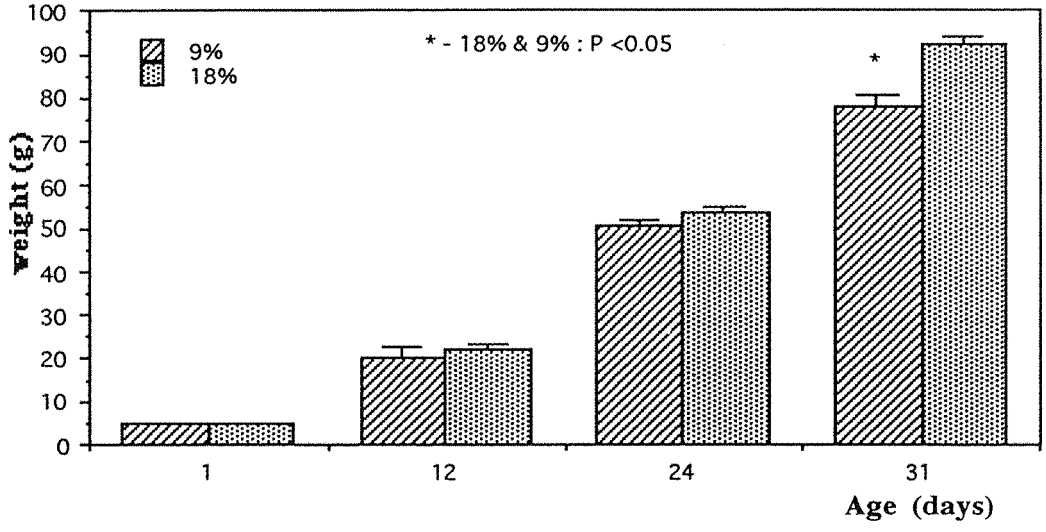

Figure 7.3 : Pre-weaning offspring growth

At age 31 days the pups were divided by sex and fed one of the four different postnatal diets, i.e. high-fat, high-protein, high-carbohydrate or chow. Due to the differences in body weight already present the groups could not be weight matched and so were divided randomly.

7.4 Female Offspring

7.4.1 Growth Curves and Dietary Intakes

The growth curves show weight gain as opposed to actual weight due to differences in preweaning weight between the 9% and 18% groups. Figure 7.4 shows the growth curves for rats in all the postnatal dietary groups. The weight gains were similar between the 9% and 18% offspring within the same postnatal dietary group. There were, however differences between the four postnatal dietary groups. Females gained more weight on the standard laboratory chow diet than on the other diets, with the animals on the high-carbohydrate diet having almost a similar gain. This is particularly evident in the 9% group. The rats in the high-fat groups gained on average 20g less than those on the former diets and those on the high-protein diet gained the least weight, 42g±1.8g less compared to the chow fed group. 2-way ANOVA analysis for the 9% and 18% female offspring fed four postnatal diets at each different timepoint indicated there were significant differences ($P<0.01$) between the female offspring at every timepoint.

Food intake expressed as metabolisable energy for the 9% and 18% offspring for all the postnatal diets are shown in figures 7.5 and 7.6. There were no significant differences between the 9% and 18% rats within each postnatal diet so they have been displayed on separate graphs. This enables the differences between the postnatal diets to be seen more clearly.

In Figure 7.4, it can be seen that the 9% animals on all the diets followed a similar pattern of weight gain over the study period with a steady increase up to age 9 weeks and then a slight reduction at age 11 weeks.

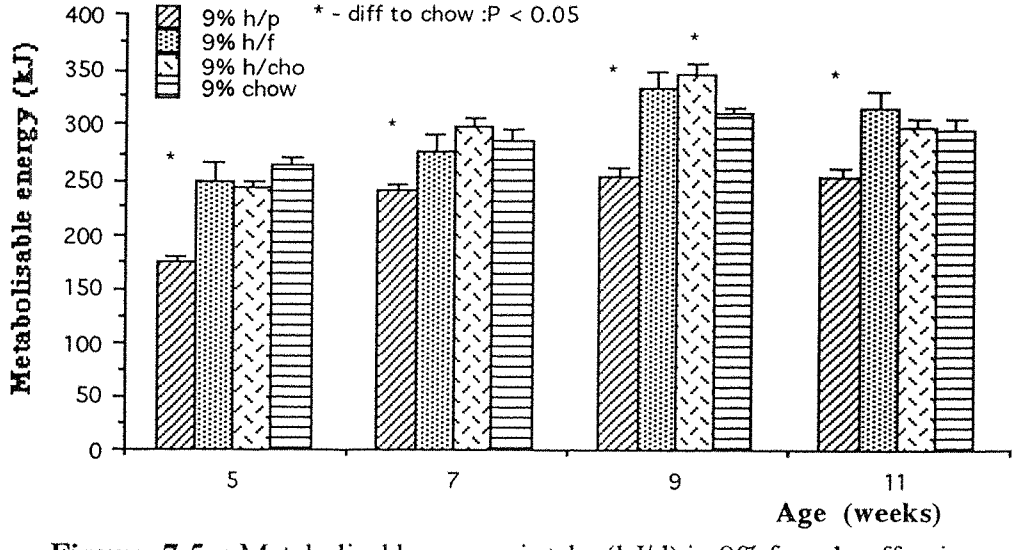

Figure 7.5 : Metabolisable energy intake (kJ/d) in 9% female offspring at ages 5 to 11 weeks

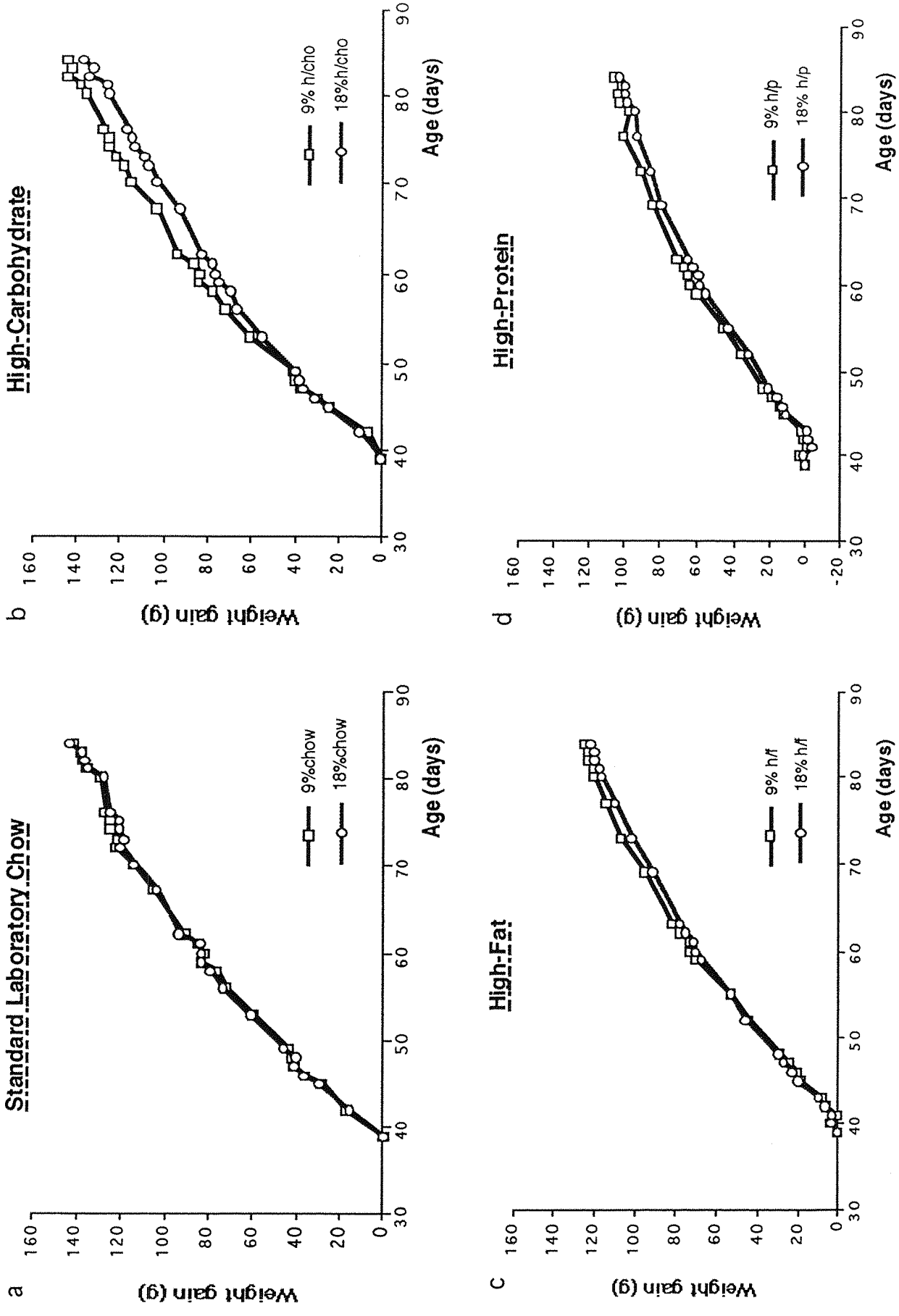

Figure 7.4 : Female growth curves

The high-protein-fed group consistently consumed significantly less energy than those fed chow ($P<0.05$), but the high-fat and high-carbohydrate-fed rats consumed a similar energy intake to that of those fed chow. However at age 7 weeks the high-carbohydrate fed group ate a significantly higher energy intake than the chow-fed animals ($P<0.05$).

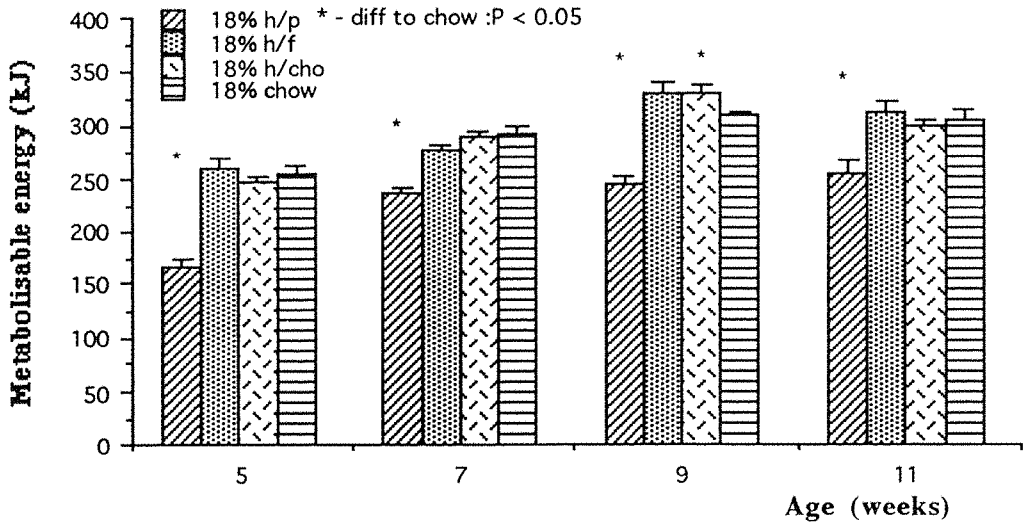

Figure 7.6 : Metabolisable energy intake (kJ/d) in 18% female offspring at ages 5 to 11 weeks

As shown by figure 7.6, the 18% group behaved similarly to the 9% group. The high-protein animals again consumed a significantly lower energy intake than those fed standard laboratory chow throughout the study period with the greatest difference occurring at age five weeks. The only significant difference between chow and any of the other diets was at age 9 weeks in the high-carbohydrate group, which consumed significantly more energy ($P<0.05$).

7.4.2. Regional Adipose Tissue, Skeletal Muscle & Organ Weights

A 2-way ANOVA test for the hind-leg muscle weights in the female offspring was carried out. Differences occurred between the anterior tibularis, extensor digitorum longus and soleus ($P<0.05$), however there were no significant differences in the gastrocnemius muscle between the different dietary groups, although further statistical analysis (Student's t-test) revealed that the 9% rats fed a high-protein diet post-natally had significantly lighter gastrocnemius muscles compared to those fed chow or high-carbohydrate, shown in table 7.2

Table 7.2 shows the mean weights for the two of the four hind-leg muscles, for both the 9% and 18% female offspring. A complete table of analyses for all muscles can be found in appendix F. As significant differences were mainly found only in the extensor digitorum longus and soleus these are the only data presented in this chapter. Generally muscles were lighter in the 9% offspring apart from the group fed the high-carbohydrate diet whereby the reverse was generally the case. These differences did not reach statistical significance except for the high-carbohydrate group where the mean weight of the extensor digitorum longus (e.d.l.) in the 9% group was significantly heavier than that of the 18% rats ($P<0.05$).

Muscles were lighter in the high-protein fed group compared to those in both the chow and high-carbohydrate groups, in the 9% animals ($P<0.05$). The high-fat animals also had significantly lighter e.d.l. and soleus muscles when compared to the same two groups

There were fewer significant differences between the postnatal diets in the 18% rats with the only differences occurring in the soleus muscle. The soleus muscles from the high-fat and high-protein animals were significantly lighter than in both the high-carbohydrate and chow groups.

		Muscle (mg)			
Prenatal diet	Postnatal diet	Extensor digitorum longus		Soleus	
		Mean	SE	Mean	SE
9%	h/fat	94.9c	3.0	165.0bc	22
	h/prot	94.0c	3.1	182.0bc	8.7
	h/cho	108.4a	3.0	208.4	9.4
	chow	102.0	2.5	203.1	4.9
18%	h/fat	101.8	3.8	150.1bc	21.4
	h/prot	100.4	2.5	165.0bc	14.9
	h/cho	100.0	2.3	194.4	4.8
	chow	103.6	4.2	207.6	6.9

Table 7.2 : Muscle weights for female offspring (Mean ± SE). a : significantly different to 18%, b : significantly different to chow, c : significantly different to high-carbohydrate. $P<0.05$

As for the muscle data, the adipose site data has been displayed in an edited form in this chapter to highlight the main differences, however significant differences as calculated by 2-way ANOVA were found between all adipose sties. A full table of weights is shown in appendix F. Therefore Table 7.3 shows the data for the two of the eight regional adipose sites dissected. There were few significant differences in weights between the 9% and the 18% rats within the same postnatal dietary group. Notable differences occurred in the interscapular BAT and retroperitoneal sites in the high-protein fed animals, where the 9% rats were significantly lighter than the 18% rats. Interscapular BAT fat pads were significantly heavier the 9% rats in the high-carbohydrate group compared to the 18% group. The fat pads were generally lighter in the high-protein fed group for both 9% and 18% animals than in the other three postnatal dietary groups.

		Regional adipose site weight (g)			
Prenatal diet	Postnatal diet	Interscapular BAT Mean	SE	Intestinal Mean	SE
9%	high-fat	0.557bd	0.05	2.956d	0.37
	high-protein	0.299abc	0.02	1.979bc	0.16
	high-cho	0.579ab	0.04	2.788	0.13
	chow	0.405	0.02	2.857	0.20
18%	high-fat	0.708bcd	0.08	3.539	0.47
	high-protein	0.387c	0.03	2.299b	0.19
	high-cho	0.470	0.02	2.789	0.21
	chow	0.410	0.03	3.193	0.23

Table 7.3 Weights of adipose sites for female offspring a: sig diff to 18%, b: sig diff to chow, c: sig diff to h/cho, d: sig diff to h/protein ($P<0.05$) BAT = brown adipose tissue

For the 18% rats in the high-protein fed group, fat pads were significantly lighter than those in the high-fat fed group at all sites. The interscapular site in the 18% high-protein animals was also significantly lighter compared with those high-carbohydrate-fed animals. The inguinal, popliteal, I-WAT, I-BAT, parametrial and retroperitoneal fat pads were significantly heavier in the 18% high-fat-fed rats compared with all other postnatal groups. The I-BAT was significantly heavier compared with both chow and high-protein fed animals. Overall the interscapular BAT showed the most differences whereas the inguinal had the least.

Table 7.4 shows the mean weights of the various organs in the female offspring for all dietary groups. A range of significance levels were observed and full analysis can be found in appendix F. The liver and pituitary weights did not reach significance when analysed using 2-way ANOVA, although there are significant differences between the postnatal groups for the heart, adrenal and kidney. There tended to be little difference between prenatal groups and this was the case with all organs and diets except for the brain in the 9% high-fat and high-protein groups which was significantly lighter than the corresponding 18% animals ($P<0.05$). In addition the mean adrenal weight in the 9% high-carbohydrate group was significantly lighter than that in the 18% group ($P<0.05$).

		Organ weight (g)					
Prenatal	Postnatal	Kidney		Adrenal		Heart	
diet	diet	Mean	SE	Mean	SE	Mean	SE
9%	high-fat	1.830bd	0.08	0.092c	0.01	1.038bc	0.04
	high-protein	2.216bc	0.05	0.092c	0.01	0.983b	0.07
	high-cho	1.959b	0.07	0.068ab	0.01	0.924b	0.03
	chow	1.594	0.08	0.080	0.01	0.816	0.02
18%	high-fat	1.742cd	0.04	0.098	0.01	0.954b	0.04
	high-protein	2.116bc	0.06	0.103bc	0.01	0.867	0.04
	high-cho	1.836b	0.02	0.081	0.01	0.917b	0.02
	chow	1.621	0.05	0.079	0.01	0.829	0.03

Table 7.4 Weight of organs for female offspring. a: sig diff to 18%, b: sig diff to chow, c: sig diff to high-cho, d: sig diff to high-protein ($P< 0.05$)

In the pre-natal 9% group, liver, kidney and heart were all heavier in the high-fat, high-carbohydrate and high-protein groups compared with the chow group, although this did not reach significance in the liver. The adrenals from the rats in the 9% high-fat and high-protein groups were significantly heavier than those from the high-carbohydrate-fed rats. These in turn were significantly lighter than those for the chow-fed animals. The hearts from the high-fat group were also significantly heavier than those for the high-carbohydrate-fed rats. The kidney and heart weights also varied with postnatal diet in the 18% group ($P<0.01$). Kidneys were significantly lighter in the high-fat-fed rats than the high-protein and high-carbohydrate-fed rats and although this did not reach statistical significance. Mean kidney weight was also heavier than the chow-fed animals. The kidneys were significantly heavier in the high-protein-fed animals ($P<0.05$) compared to both those of the chow and high-carbohydrate-fed groups.

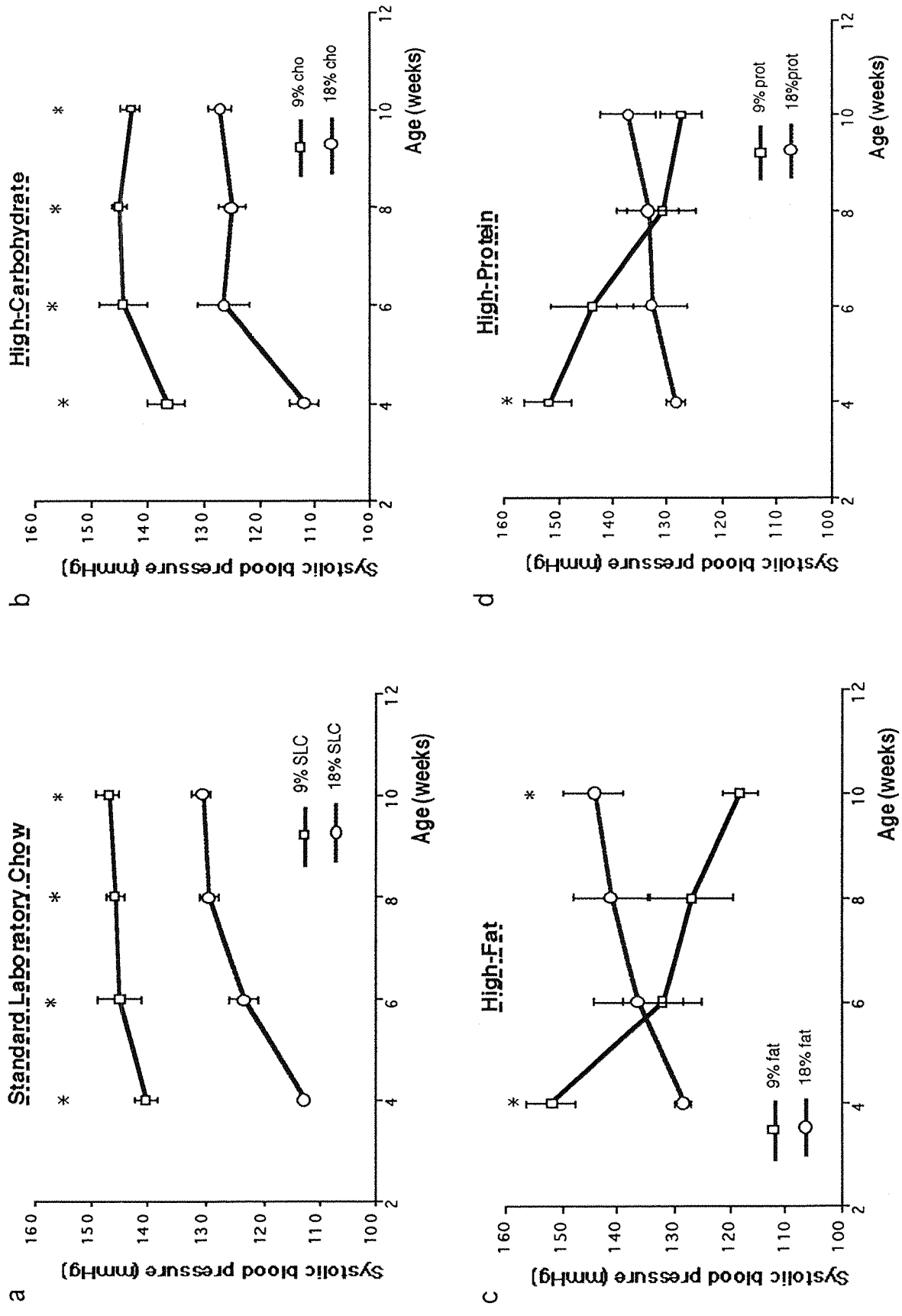

Figure 7.7: Female systolic blood pressures. * = P<0.05

7.4.3 Systolic Blood Pressure

Figure 7.7 shows the systolic blood pressure measurements for both prenatal and postnatal dietary groups. If the chow-fed groups are considered first it can be seen that the 9% group had a significantly higher blood pressure at weaning compared to the 18% rats($P<0.01$). This difference was maintained throughout the study so at age 10 weeks the systolic blood pressure of the 9% group was still significantly higher than that of the 18% animals. The high-carbohydrate-fed groups showed a similar pattern with the 9% animals having a significantly higher blood pressure at every timepoint throughout the study compared to the 18% group. The 9% animals maintained at plateau at around 140mmHg for both of these diets. The blood pressure increased in the 18% group over the first two of weeks from 110mmHg to between 120 and 125mmHg in both postnatal dietary groups. This was a significant increase compared with the baseline measurement.

In the high-fat-fed group, the 9% offspring had significantly higher blood pressure at weaning from the 18% rats but this fell from 151.9±4.3mmHg to 132.0±7.0mmHg over the two weeks of the study and continued to fall so by the final reading at age 10 weeks the blood pressure in the 9% group was 118.3±3.3mmHg. All values following the weaning value were significantly different from the weaning value. The 18% animals however had a similar response to that seen in the chow animals in that there was a steady increase of their blood pressure over the study period from 128.4 to 144.3mmHg. The blood pressure measurements at age 8 and 10 weeks were significantly higher than those of the weaning value. Therefore by the end of the study the 18% rats had a significantly higher systolic blood pressure than those of the 9% group.

Finally the high-protein-fed rats followed a similar pattern to the high-fat-fed animals. The 9% animals had a significantly higher blood pressure at weaning than the 18% group which gradually fell by age 8 weeks. As systolic blood pressure in the 18% group increased over the study 9% final blood pressure measurement was lower than the 18% animals, although not significantly. The 18% increase was significant at 10 weeks.

7.4.4 Glucose and Insulin Tolerance Test

Figures 7.8 and 7.9 show both the plasma glucose and insulin responses to a glucose load administered at time zero in the prenatal 9% and 18% groups for the four postnatal diets respectively.

The glucose responses of the 9% and 18% rats were similar within each postnatal dietary group. If the 9% rats are considered first, all the postnatal groups showed a response to the glucose load administered at time zero by an increase in the plasma glucose and insulin concentrations. The increase in insulin was greatest in the high-

protein group and least in the high-fat group, however the high-fat animals had the highest fasting levels of plasma insulin. All the 9% animals were able to clear the glucose and return the plasma levels to baseline within the 60 minutes. However, in the high-fat group this was at the expense of a continued increase in insulin levels (figure 7.8c).

The peak glucose concentrations were similar at approximately 22mmol/l for the chow, high-carbohydrate and high-protein postnatal groups, whereas the high-fat fed animals demonstrated a higher response to the injected glucose nearer 28mmol/l. This is not an indication of lower body weight in that groups as the dose was administered per kg body weight.

The 18% animals were also able to clear the glucose load within 60 minutes although the insulin concentrations remained elevated in both the chow and high-fat fed groups (figures 7.9a, c). The insulin concentration in the 18% chow-fed animals was significantly higher than that in the 9% animals after 60 minutes. The 18% high-carbohydrate-fed rats showed a similar increase in plasma insulin to the chow-fed animals at 5 minutes but then the concentration decreased over the following 15 minutes. A plateau was then reached at 0.9ng/ml until the 60 minute timepoint (figure 7.9b).

In the high-fat-fed groups, the 18% animals showed a similar pattern of insulin response to the 18% chow-fed rats, but with significantly higher fasting levels of both plasma insulin and glucose in the high-fat group. The high-protein-fed group showed a similar pattern of insulin response over the time period to that of the 18% high-carbohydrate-fed group, with an initial peak falling until 20 minutes when there was a slight increase in plasma levels before falling again back to baseline levels (figure 7.9b,d).

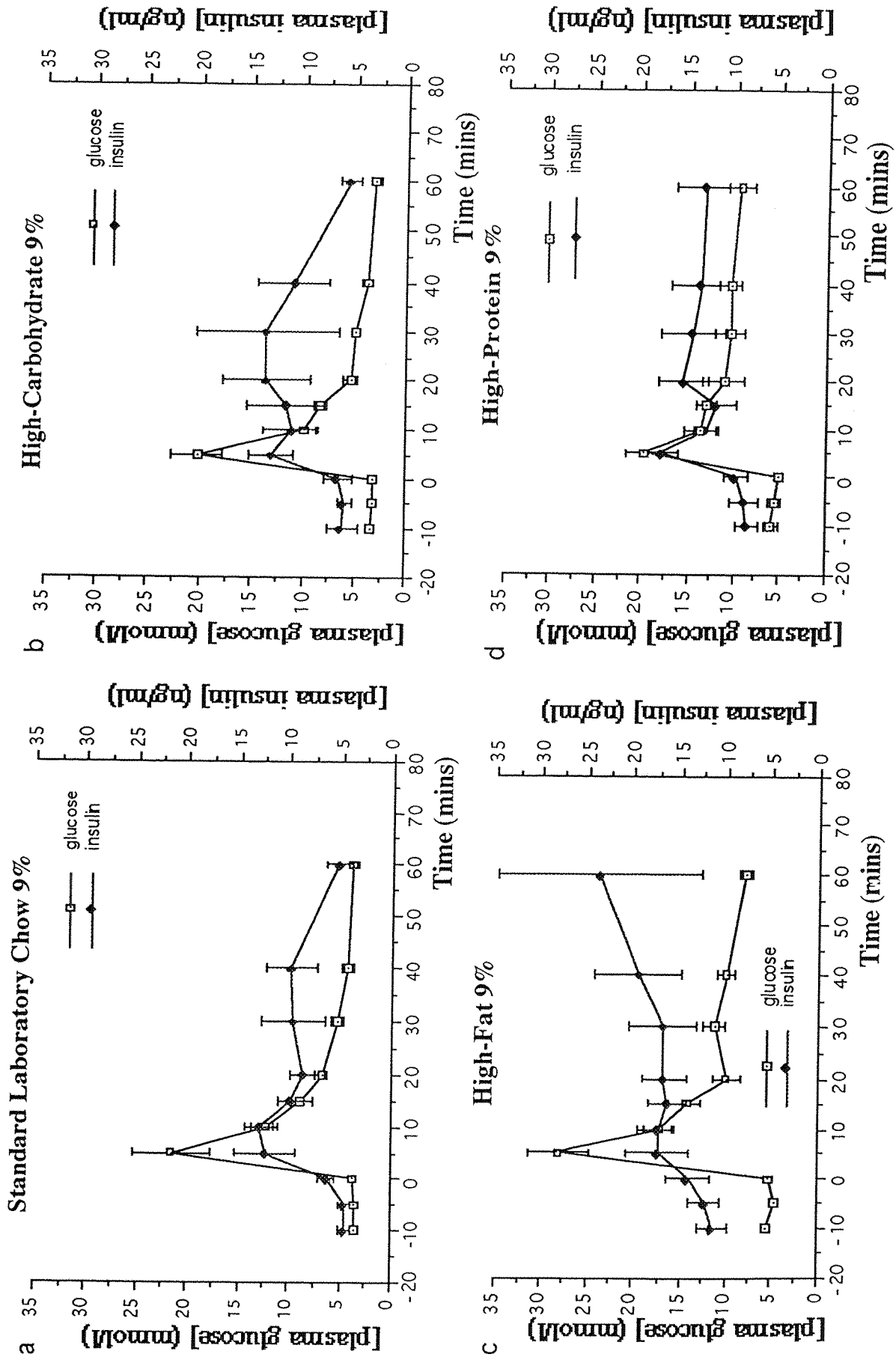

Figure 7.8: Plasma glucose and insulin concentration of 9% group in response to a glucose load administered at time zero * = P<0.05

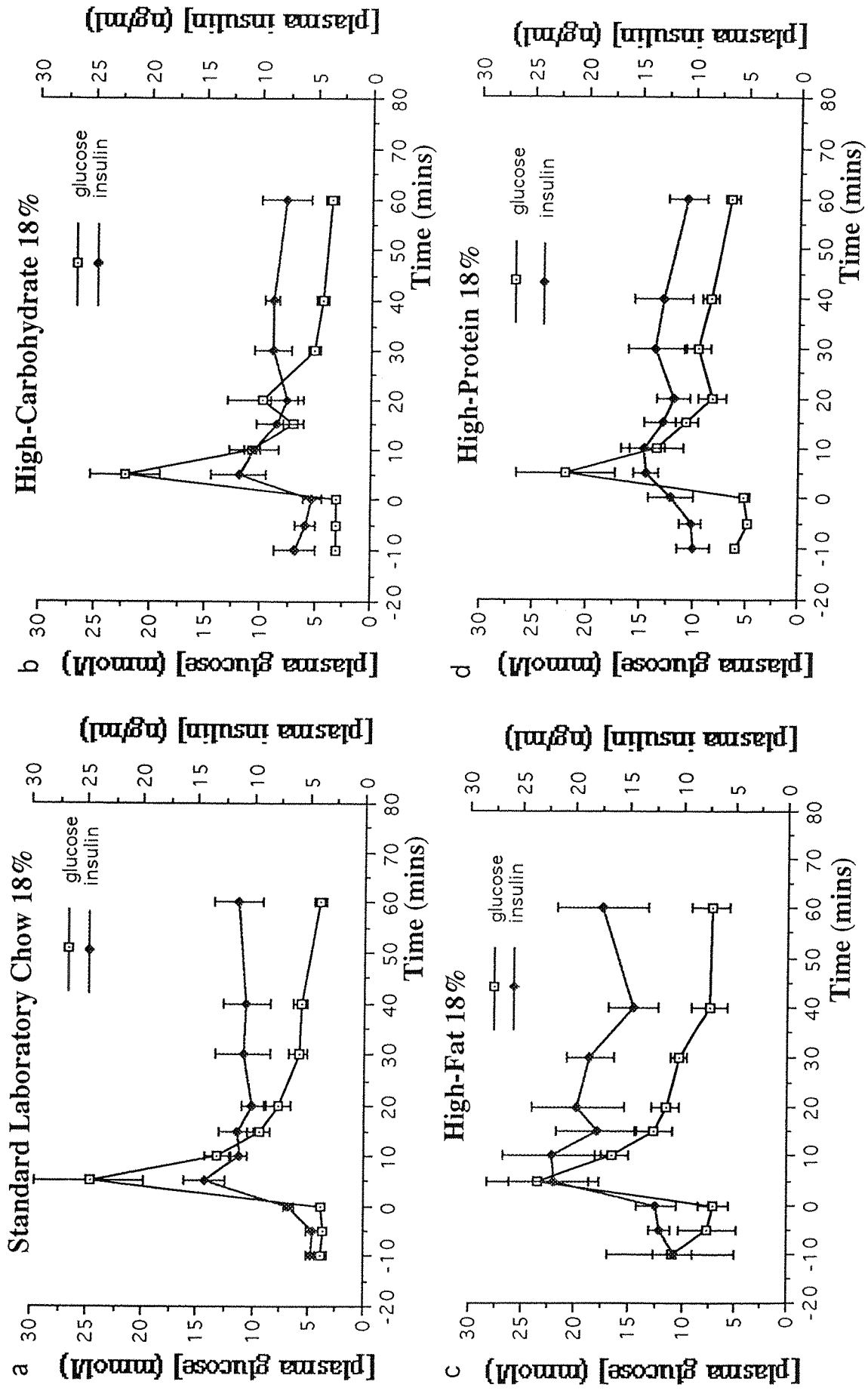

Figure 7.9 : Plasma glucose and insulin concentration of 18% group in response to a glucose load administered at time zero * = P<0.05

7.4.5 Body Composition Analysis

The chemical analyses of body composition are shown in table 7.5. Percentage water and percentage protein were also determined but there were no significant differences (P=NS) in these figures between any of the dietary groups either pre- or postnatal. Therefore these results have been excluded from the table for clarity and can be found in appendix F. Mean %Water = 61%±0.6 and %Protein = 19.5%±0.4.

		Body Composition					
Prenatal diet	Postnatal diet	Final body weight (g)		%Fat		Energy/g body weight (kJ)	
		Mean	SE	Mean	SE	Mean	SE
9%	h/fat	233.3	12.4	8.5b	0.3	31.3	1.4
	h/protein	216.1b	6.3	5.7a	0.3	25.4b	0.9
	h/cho	243.8	6.2	6.5	1.0	25.7b	0.3
	chow	245.6	7.3	6.0	0.7	28.9	0.7
18%	h/fat	244.6	7.1	9.8b	1.1	34.3b	0.8
	h/protein	223.8b	6.5	6.7	0.3	24.3	0.9
	h/cho	237.6b	2.6	5.5	0.4	26.5	0.4
	chow	250.3	7.1	5.9	0.3	26.9	1.0

Table 7.5 Body composition for female offspring. a: sig diff to 18%, b: sig diff to chow, ($P < 0.05$)

Although lighter in body weight than the chow-fed animals (control group), the high-fat fed animals from both pre-natal groups had a significantly higher percentage body fat, this was reflected in a significantly greater energy content per gram of body weight in the 18% group ($P<0.05$). Both high-carbohydrate-fed and high-protein-fed groups in the 9% animals had significantly lower energy content per gram body weight than both the chow and high-fat fed animals within the same pre-natal group. However this was not the case in the 18% rats.

7.4.6 Skeletal Muscle Fibre Types

Three muscles of the hind-limb were analysed for muscle fibre type. Significant differences between prenatal groups were only observed in the extensor digitorum longus (e.d.l.), shown in table 7.6. A significant move towards an increase in the proportion of type II fibres was observed as a result of high-protein and high-fat feeding in the 9% group, which was not seen in the pre-natal 18% group. This increase

in the proportion of type II fibres in the high-protein fed animals resulted in a significant difference between the two prenatal groups (P<0.05).

Prenatal diet	Postnatal diet	Extensor digitorum longus				Soleus			
		Type I		Type II		Type I		Type II	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	89.0b	0.8	11.1b	0.8	83.9	2.5	16.1	2.5
	h/protein	88.0ab	1.4	12.0ab	1.4	82.5	2.7	17.6	2.7
	h/cho	90.3	1.0	9.7	1.0	78.4	1.9	21.6	1.9
	chow	92.9	1.1	7.1	1.1	79.7	1.6	20.3	1.6
18%	h/fat	87.9	2.4	12.1	2.4	82.4	2.7	17.6	2.7
	h/protein	93.5	1.1	6.5	1.1	80.4	3.2	19.6	3.2
	h/cho	92.6	1.0	7.4	1.0	84.4	2.7	15.6	2.7
	chow	91.9	1.5	8.1	1.5	79.5	1.1	20.5	1.1

Table 7.6 Skeletal muscle fibre composition of extensor digitorum longus and soleus from female offspring a: sig diff to 18%, b: sig diff to chow, (P< 0.05). Values are expressed as a percentage

7.5 Male Offspring

7.5.1 Growth Curves and Dietary Intakes

The growth curves for all the male offspring are shown in figure 7.10. The graphs are presented in the same format as for the females in that they show weight gain as opposed to actual weight. Within each postnatal diet, the prenatal 9% and 18% groups displayed a similar mean body weight gain, with a trend for the 9% animals gaining slightly more weight than the 18% offspring, although this did not reach statistical significance (P=NS). This was opposite to that of the females where the 18% rats gained slightly more weight. The chow-fed animals gained the most weight with the high-carbohydrate-fed animals gaining a similar amount. The high-fat-fed animals gained approximately 30g less than these two dietary groups whereas the high-protein-fed rats gained the least amount of weight, an average of 200g as opposed to 280g for the chow-fed group.

Figure 7.11 shows the mean metabolisable energy intake for the 9% male offspring on the four postnatal diets at intervals of two weeks. There were highly significant differences up to age 9 weeks (P<0.001) but at age 11 weeks the energy intake only just reached statistical significance (P<0.05).

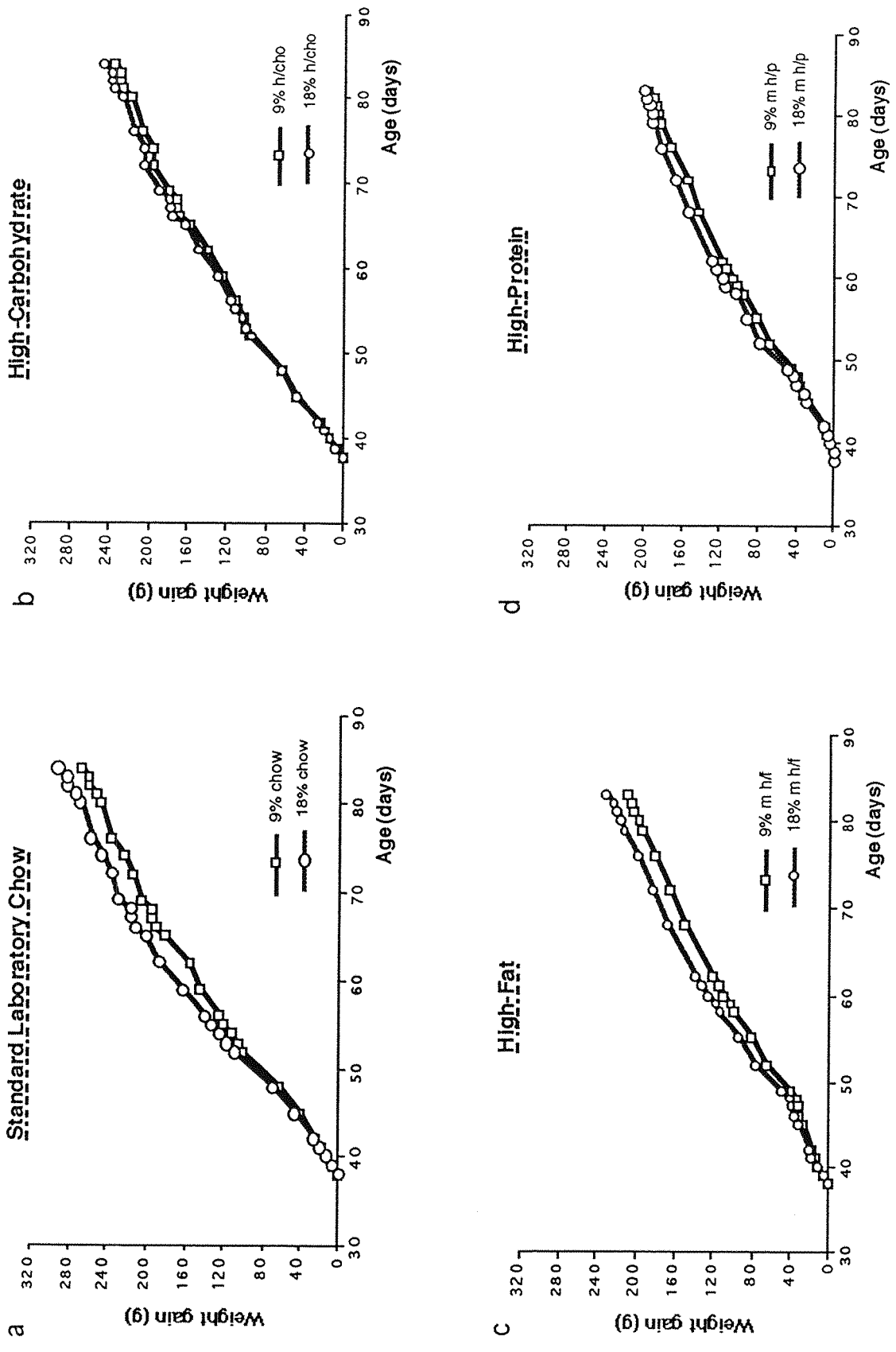

Figure 7.10: Male growth curves

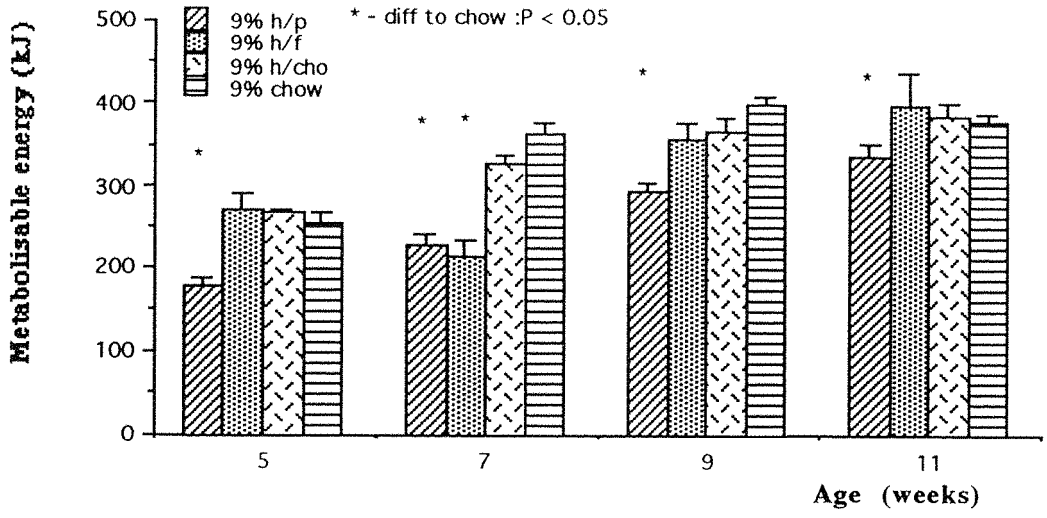

Figure 7.11 : 9% males energy intake expressed as kJ

At all time points the high-protein-fed animals consumed significantly less diet when expressed as metabolisable energy than those maintained on chow ($P<0.05$). The high-carbohydrate-fed rats consumed a similar level of intake to those fed chow whereas the high-fat-fed animals at age 5 weeks had a higher intake. By age 7 weeks, the intake of the high-fat-fed rats was significantly less that the high-carbohydrate and chow-fed animals. At age 9 weeks, intake of the high-fat-fed animals was still lower than that of the chow-fed rats but this was not statistically significant. At the final timepoint the high-fat intake was once again greater than that of the chow group (P=NS).

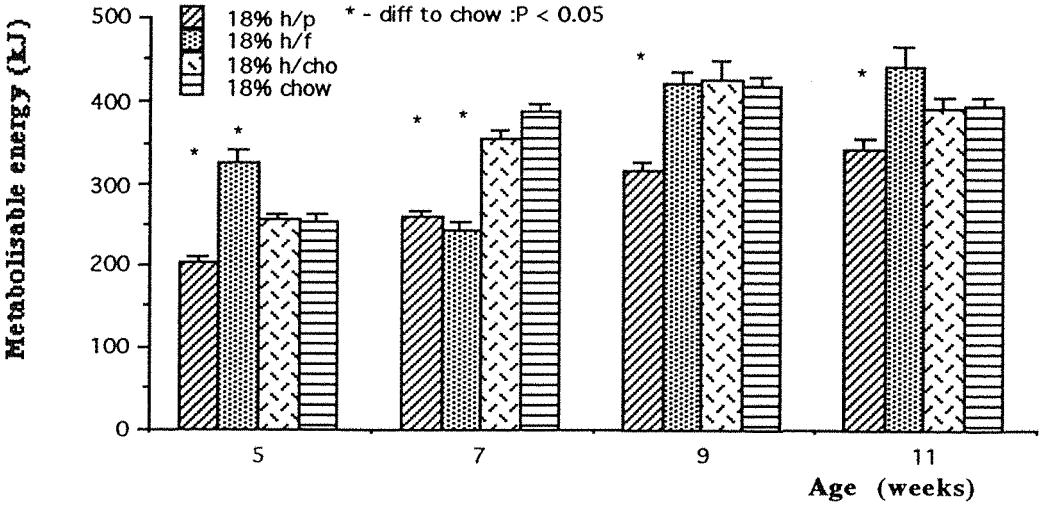

Figure 7.12 : 18% males energy intake expressed as kJ

The metabolisable energy intake for the 18% males is shown in figure 7.12 in the same format as that for the 9% males. A similar pattern is seen in that the high-protein-fed rats demonstrating a significantly lower intake than those animals fed chow throughout the whole study. The high-fat-fed animals also had the same pattern to those in the 9% group where their energy intake was significantly higher than those fed chow at age 5 weeks. Again by 7 weeks of age, intake was significantly lower. At age 11 weeks this difference was no longer present as the intake of the high-fat rats was higher than that of the chow although it did not reach statistical significance. Intake of the high-carbohydrate diet was very similar to the chow in terms of energy intake throughout the study period. There were no significant differences within postnatal dietary groups between the prenatal 9% and 18% groups (P=NS)

7.5.2 Regional Adipose Tissue, Skeletal Muscle & Organ Weights

Skeletal muscle, regional fat pads and organ weights are shown in tables 7.7, 7.8 and 7.9 respectively. If the weight of the muscles are examined first, it can be seen that there were very few differences between the prenatal 9% and 18% males within each postnatal diet. The only difference reaching significance was that of the anterior tibularis in the chow-fed group where the 9% animals had a significantly lighter weight to that of the 18% group ($P<0.05$).

Observations between the postnatal diets in the 9% group showed the majority of differences occurred between the high-fat and chow-fed groups as all the muscles for the high-fat group were significantly lighter than the corresponding muscles in the chow animals. Both the high-fat and high-protein-fed groups demonstrated significantly lighter weights than the high-carbohydrate group for the anterior tibularis, extensor digitorum longus (e.d.l) and soleus muscles.

In the prenatal 18% group the anterior tibularis, e.d.l and gastocnemius muscles of the chow group are all significantly heavier than all the other three postnatal dietary groups. The soleus muscle is also significantly heavier than that in the high-protein group. The only other difference of significant value is that the high-protein anterior tibularis is also lighter than the chow group.

Prenatal diet	Postnatal diet	Muscle (mg) Anterior tibularis		Extensor digitorum longus		Gastrocnemius		Soleus	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	533.9bc	26.9	125.7bc	5.1	1408b	80	265.4bc	15.3
	h/prot	523.0bc	7.0	133.1bc	5.4	1562	80	236.8bc	26.7
	h/cho	634	10.0	152.0	3.0	1509	60	309.0	8.0
	chow	631.9a	17.1	151.4	4.4	1656	60	314.5	12.4
18%	h/fat	592.6b	25.5	143.1b	5.8	1549b	70	280.1	27.7
	h/prot	581.9bc	23.4	143.0b	5.2	1455b	70	254.8b	20.9
	h/cho	638.0b	10.0	148.0b	3.6	1573b	50	304.8	11.1
	chow	674.9	10.1	159.6	3.1	1749	30	290.4	44.6

Table 7.7 : Weight of four muscles for male offspring (Mean ± SE). a : significantly different to 18%, b : sig diff to chow, c : sig diff to high-carbohydrate ($P<0.05$)

Table 7.8 shows the regional adipose site weights in the same format as in table 7.7 for the skeletal muscles, with a complete table in appendix F. As for the skeletal muscle weights, there were few significant differences between the 9% and 18% rats within each postnatal dietary group. The 9% high-fat-fed animals had significantly lighter interscapular WAT and BAT masses compared to the 18% animals. In addition, the 9% chow-fed rats had significantly lighter intestinal fat pads than the 18% chow-fed group.

Prenatal diet	Postnatal diet	Regional Adipose Site Weight (g) Interscapular WAT		Interscapular BAT		Epididymal		Intestinal	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	0.529a	0.07	0.666a	0.05	8.153	2.07	4.165b	0.71
	h/prot	0.511b	0.04	0.515b	0.03	5.261b	0.55	3.187b	0.27
	h/cho	0.379b	0.04	0.651	0.06	5.303b	0.64	3.235b	0.38
	chow	0.609	0.03	0.731	0.04	10.090	0.43	5.660a	0.27
18%	h/fat	0.752b	0.07	0.932b	0.05	11.010	1.41	5.480	0.60
	h/prot	0.591	0.04	0.567	0.06	6.182b	0.53	3.319b	0.26
	h/cho	0.499	0.05	0.770	0.07	6.318b	0.65	3.645b	0.37
	chow	0.523	0.04	0.713	0.09	10.660	0.44	6.350	0.20

Table 7.8 Weight of regional adipose sites for male offspring. a: sig diff to 18%, b: sig diff to chow, ($P< 0.05$)

There were further differences within each prenatal group, but these differences varied. In the 9% group, although all the high-fat-fed rats had lighter fat pads compared with the chow-fed animals, this only reached a significant difference for the intestinal site. This differed to that of the females where the high-fat-fed group had heavier fat pads compared with those fed chow. All animals on the high-protein diet had fat pads which were significantly lighter than those fed chow. This was the case for those fed the high-carbohydrate diet apart from the interscapular BAT where the differences did not reach significance.

If the 18% group is now considered, the high-fat-fed rats had heavier regional adipose sites than all the other groups although this only reached statistical significance against the chow-fed group at the interscapular sites. All adipose sites were significantly heavier compared with those fed the high-protein and high-carbohydrate diets. The high-protein-fed group had significantly lighter fat pads than the chow group except for the interscapular pads, as the BAT was only significantly lighter compared to high-carbohydrate-fed group.

		Organ weight (g)					
Prenatal	Postnatal	Kidney		Adrenal		Heart	
diet	diet	Mean	SE	Mean	SE	Mean	SE
9%	high-fat	2.439d	0.14	0.079c	0.01	1.545b	0.18
	high-protein	3.069bc	0.13	0.078c	0.01	1.308b	0.05
	high-cho	2.608	0.12	0.055b	0.02	1.240b	0.04
	chow	2.466a	0.09	0.067	0.04	1.125	0.02
18%	high-fat	2.778	0.14	0.089bc	0.01	1.513bcd	0.06
	high-protein	3.086bc	0.15	0.092bc	0.01	1.240	0.05
	high-cho	2.634	0.08	0.060	0.04	1.308b	0.04
	chow	2.710	0.05	0.069	0.03	1.219	0.02

Table 7.9 Weight of organs for male offspring. a: sig diff to 18%, b: sig diff to chow, c: sig diff to high-cho, d: sig diff to high-protein ($P < 0.05$)

Organ weights compared in table 7.9. It can be seen that no differences between any of the groups either prenatally or postnatally occurred for the brain weights. The only significant difference between the 9% rats and the 18% rats was for the kidney where in the chow-fed animals they were significantly lighter in the 9% group. In the 9% group there were no significant differences between postnatal dietary groups for the liver, whereas for the heart, all the postnatal dietary groups were significantly heavier than the chow-fed rats. In addition, adrenal gland weight was significantly heavier in the high-fat-fed rats compared with the high-carbohydrate rats. Their mean kidney weight was also

significantly lighter than that of the high-protein-fed group. The kidneys of the high-protein-fed rats were significantly heavier than all the other postnatal groups, and the adrenal weights were heavier than those of the high-carbohydrate-fed group, which in turn were significantly lighter than in the chow-fed group.

The differences were more diverse in the 18% group. The adrenal and heart weights were significantly heavier in the high-fat-fed group compared to the chow-fed group. Organs, except the heart, were significantly lighter in high-protein-fed rats compared to chow-fed rats.

7.5.3 Systolic Blood Pressure

For the initial post-weaning systolic blood pressure (SBP) measurement at 4 weeks of age, the temperature of the experimental room in which the measurements were recorded, was incorrectly set at 36°C, due to a fault at that time in the air conditioning. This had a confounding effect on the blood pressures at this time. For the ensuing measurements and for all the measurements in females, the temperature was controlled at the correct level of 28°C. Furthermore, it was critical that blood pressure measurements were taken at age four weeks.

The results were similar to those of the females where the SBP in the prenatal 9% rats was significantly higher in the high-carbohydrate and chow-fed groups at weaning at age four weeks, which were maintained throughout the study compared to the prenatal 18% rats. The SBP in the 18% rats increased throughout the study, especially over the first two weeks so by age 10 weeks, the SBP was significantly higher than the weaning measurement (figure 7.13).

Although the SBP in the high-fat-fed group was increased at age 4 weeks, the mean SBP of the 9% rats was still significantly higher than in the 18% rats. After two weeks of feeding the high-fat and high-protein diets a significant reduction in the systolic blood pressure was observed in the 9% animals which continued to fall throughout the period of the study. The SBP in the 18% high-fat-fed group also fell significantly from the weaning value, but then increased gradually and by age 8 weeks SBP in the 9% group was significantly lower than in the 18% group.

As the rats were divided into the dietary groups after the blood pressure measurements at weaning were taken, the measurements are the same for both the high-fat and high-protein-fed rats for the first recording. As with the high-fat-fed group, the SBP was significantly lower in the 9% high-protein-fed after being maintained on the high-protein diet for two weeks. However this did not continue to fall but reached a plateau for the remainder of the study. The 18% rats fed the high-protein diet had no significant changes in their blood pressure.

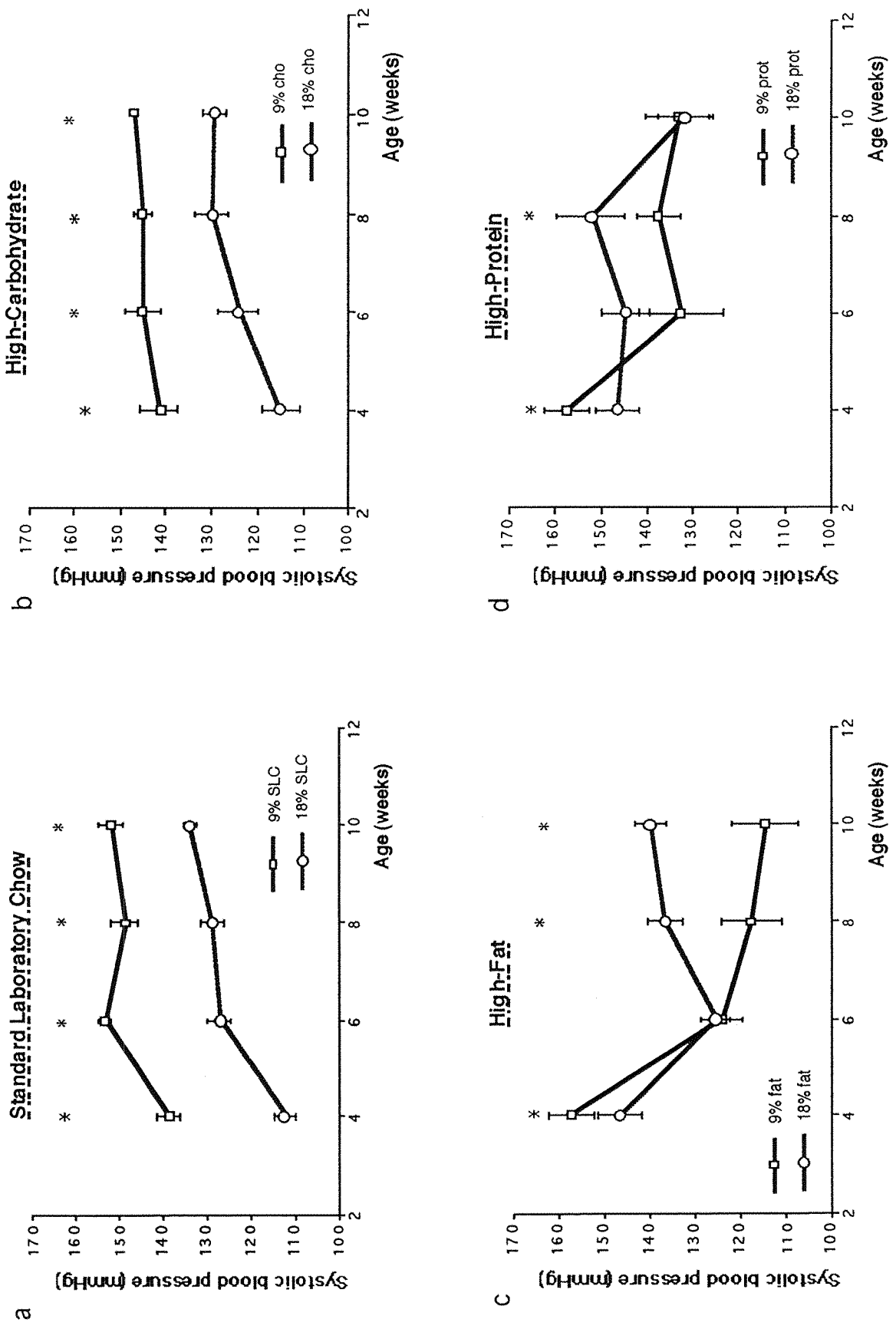

Figure 7.13 : Male systolic blood pressures *= P<0.05

7.5.4 Body Composition

Table 7.10 gives the significant differences in body composition which were observed for the male offspring. A full table of analysis can be found in appendix F.

		Body Composition					
Prenatal diet	Postnatal diet	Final body weight (g)		%Fat		Energy/g body weight (kJ)	
		Mean	SE	Mean	SE	Mean	SE
9%	h/fat	307.7b	19.9	7.0	1.2	33.0b	0.8
	h/protein	291.4b	9.6	4.9ab	0.3	26.9	1.0
	h/cho	344.0	10.7	4.2b	0.6	29.7	0.5
	chow	371.5	13.3	6.8	0.3	27.7	0.6
18%	h/fat	349.8b	14.1	7.4	0.8	32.6b	0.8
	h/protein	308.3b	9.7	6.0	0.6	25.3b	0.6
	h/cho	350.5b	7.3	5.1	0.6	29.6	0.5
	chow	394.5	5.5	6.7	0.6	27.5	0.6

Table 7.10 Body composition for male offspring. a: sig diff to 18%, b: sig diff to chow, ($P < 0.05$)

It can be seen that although the high-fat fed animals in both prenatal groups were significantly lighter than the chow fed group, there was no significant difference in the percentage body fat between these animals. However, a significant increase in energy per gram of body weight in the high-fat fed group was observed. The percentage of body fat in the high-carbohydrate and high-protein groups was lower than that of the chow-fed group although this only reached significance ($P<0.05$) in the 9% prenatal group.

This data differed from the females where percentage body fat was higher in the high-fat-fed group compared to any other group, in both 9% and 18% prenatal groups.

7.6 Discussion

The results of this study show subtle differences in various parameters between those animals which had been exposed to a 9% casein diet *in utero* and those exposed to a control 18% casein diet.

The fact that the 9% mothers were able to maintain a similar weight gain compared to the controls prior to pregnancy suggests that the protein restriction they were receiving was not too severe and that they were able to accommodate the restriction of protein, which enabled the system to support normal growth. However when the extra metabolic demand of pregnancy was added, this ability was lost and weight gain fell behind that of the control group. Although the offspring appeared normal as shown by the similarities in both litter size and birth weight, there may have been irreversible changes that occurred in the fetus, the so-called 'programming effect' and the differences observed postweaning.

When examining at the food intake of the mothers, it can be seen that the 18% group increased their intake during pregnancy significantly more than the 9% rats. As the diets were matched for energy content this also resulted in an increase in energy intake in the 18% group. It may be possible that the 9% mothers reached a ceiling in intake in that if they had increased their food intake further to try to compensate for the reduction in protein content in the diet, the metabolic cost of handling this extra energy would be too much for the system. These findings agree with previous studies (Rose 1938, Langley & Jackson 1993) but also contradict findings in other studies where the 9% mothers had a higher intake of food during pregnancy compared with the 18% group. (Levy & Jackson 1993, Pickard & McCarthy unpublished observations). The fall in food intake in both groups just before parturition to a level below that of prepregnancy is a finding previously observed (Menaker & Navia 1973)

Although there was no difference in birth weight between prenatal groups, the 9% offspring gained less weight up to the point of weaning so that they were significantly lighter than the 18% offspring. One possible reason for this could be a decrease in the protein content of the milk, as although the dams were transferred on to standard laboratory chow at birth there could have been a period when the protein level was still below normal as the animal accommodated to the new level of protein. The milk in these animals has not been measured but there has been found a decrease of protein in the milk of spontaneously hypertensive rats (Rose & McCarty 1994). The SHR displays many similarities to this model, such as fetal growth retardation and increased placental weight (Johnson 1995). Also their hypertension is normalised by feeding a high-fat diet or energy-restricted diet (Wexler 1981) and they also demonstrate an altered insulin response to a glucose load (Iwase 1994, Plato 1994)

Postweaning, in both the male and female offspring, the 9% and 18% exposed rats gained similar weight within a postnatal dietary group. However as the 9% offspring gained less weight prior to weaning, the final body weight was significantly heavier in the 18% chow-fed male rats compared to the 9% group. The high-fat-fed rats did not gain as much weight as the high-carbohydrate or chow-fed rats despite having a similar energy intake. The high-protein-fed animals gained the least weight but this corresponded with a significantly reduced energy intake. The high-protein females lost weight over the first week of intake. One reason for this could be an immature kidney at this age being unable to handle the large amounts of nitrogen. Furthermore it is possible that the high-carbohydrate and chow diets may provide a more readily available energy source which is required by the offspring for rapid growth.

There were greater differences in the muscle weights from both the male and female offspring as a result of postnatal diet than prenatal diet suggesting that it was the postnatal environment which appeared more important in determining the weight of the hind-leg muscles than the prenatal diet in this model. The female offspring appeared to be more strongly affected by the prenatal diet than the male offspring as there were fewer statistical significant differences between postnatal dietary groups in the 18% offspring. The anterior tibularis comprises mainly type I muscle fibres which are slow twitch oxidative fibres which use glucose, free fatty acids and amino acids as substrate. The extensor digitorum longus (e.d.l). is comprised mainly of type II muscle fibres which are fast twitch and rely primarily on glycolysis for their contraction. The gastrocnemius and soleus muscles have a mixture of both these muscle types. In the 9% female offspring the e.d.l. and the soleus were significantly lighter in weight in the high-fat and high-protein-fed groups compared to the high-carbohydrate-fed group. This could simply be due to them being smaller animals or due to differences in substrate availability and metabolism and hence changes in specific fibre types. The results from the muscle fibre analysis indicated that there was a trend towards an increase in the proportion of type II fibres in the e.d.l. in the high-fat and high-protein-fed groups in those animals exposed to a low-protein diet *in utero*. This is some what surprising as these diets were both low in carbohydrate but it is the type II fibres which predominately use carbohydrate as a substrate. However it has been indicated before that the 9% animals seem to share some of the metabolic and physiological characteristics of the spontaneously hypertensive rat (SHR). This is also true for skeletal muscle fibre types as there is also a higher proportion of type II fibres in skeletal muscle of the SHR (Lewis et al 1994). Interestingly there has also been shown to be a negative correlation between obesity and the proportion of type I fibres in skeletal muscle (Hickey et al 1995, Lillioja et al 1987). Although the rats in this study were not obese as a result of the high-fat diet, they did have an increased percentage body fat and total carcass energy compared to the chow-fed offspring.

Within the male offspring the statistical differences were more evenly spread between the four muscles. All the muscle weights in the high-fat-fed animals were significantly lighter than the chow-fed group, which again could be the result of the same causes as in the females.

All the regional adipose tissue sites of the high-fat-fed female offspring from both prenatal dietary groups were heavier than the chow-fed animals, whereas in the male offspring, all the 9% high-fat-fed animals had lighter fat masses compared with the chow-fed group. In the 18% high-fat-fed male offspring the visceral fat was lighter than the chow-fed group but the other adipose sites were heavier. These findings suggest a sexual dimorphism of the effect of postnatal diet on the regional adipose tissue accumulation. This dimorphism has been demonstrated before in a similar model where the 9% and 18% offspring were maintained on a macronutrient self-selection regimen (McCarthy et al 1994). One that occasion, the 9% female offspring had significantly heavier fat masses at all sites whereas the 9% male offspring only had statistical significant differences at the intestinal and omental sites. The heavier adipose site weights in the high-fat-fed group compared to the chow-fed group in the present study occurred although the former group had lighter body weights. This suggests a change in total body composition probably as a result of differences in the partitioning of energy between the lean body mass and fat mass. There were significant changes in mass in both the interscapular white and brown adipose tissue (WAT/BAT). The causes and significance of this are unknown but could possibly reflect a change in the conversion of BAT into WAT.

In both the male and female offspring there was a disproportionate growth of specific organs. The female offspring demonstrated no statistically significant changes in pituitary weight between or within either the prenatal or postnatal dietary groups, and the only difference reaching significance was in the liver weight between the high-fat-fed and chow-fed 18% offspring, where the former group had significantly heavier mean liver weight. However there were several changes observed within the kidney, adrenal and heart weights. The kidney and heart weights of the high-fat, high-protein and high-carbohydrate-fed groups were all heavier than in the chow-fed group and reached statistical significance in the prenatal 9% exposed offspring. Similarly 9% male offspring fed the high-protein diet, high-fat and high-carbohydrate had increased heart weight. This could be due to an increased mechanical stress on the heart related to hypertension which caused the heart muscle to hypertrophy. This stress can be caused by high blood pressure, although the SBPs were normal in this case, it is thought that there could be changes in the composition of the blood vessels and vascular compliance, however these changes were not measured in these animals. Changes in specific organs have also been shown by Langley-Evans et al (1996a) in 20 day fetuses, birth and weanling rats.

Both males and females in the high-fat-fed group had decreased pituitary weight compared with those in the chow-fed group, which reached significance in the male offspring. This could reflect a decrease in pituitary function, which could have wide-ranging effects as the pituitary secretes many different hormones regulating the endocrine activities of other glands. TSH, ACTH and growth hormone are all important in diabetes in that they are all diabetogenic in their actions. It has been previously shown that rats exposed to a low protein diet *in utero* have altered insulin responses to a glucose load (Chapter 4, Smith et al 1975, Phillips et al 1994, Tse et al 1995) and in humans those of low-birth weight are at a greater risk of NIDDM in later life (Barker et al 1993). It could be that changes in pituitary function are responsible, at least in part, for these outcomes. Furthermore Langley-Evans et al (1995) have shown alterations in ACTH secretion in 9% offspring in that they have changes from the usual diurnal pattern. Vasopressin, secreted from the posterior pituitary has actions on the kidney for the retention of water, and can also strongly affect blood pressure as it is a powerful vasoconstrictor. Interestingly the reduction in pituitary weight was also seen in the SHR when fed a high-fat diet (Wexler 1981). These animals became normotensive and it was suggested that this hyopituitarism could be responsible for this effect. The high-protein-fed animals showed increased kidney weight which could relate to an increase in function as when a high-protein diet is consumed there is both an increase in urea formation and its handling by the kidney.

The changes in organ, muscle and fat masses in the animals in this study give an indication of disproportionate growth which is a key issue in the 'fetal origins of adult disease' hypothesis (Langley-Evans et al 1996a). Furthermore, the pre and postnatal diets could have affected the levels of critical hormones. For instance, the high-fat diet could particularly affect the steroid hormones, such as glucocorticoids, mineralocorticoids and gonadalsteroids which have all been implicated in one way or another in the fetal programming hypothesis. Secondly, insulin release and action could also be affected, thus influencing overall growth and possibly growth of specific organs.

Finally if the blood pressure changes are considered, it can be seen that the 9% rats responded very differently to the postnatal diet, compared with the 18% offspring, although the changes were similar in both sexes. The changes in the 18% females was similar irrespective of post-natal dietary treatment. At weaning the female 9% rats had a significantly higher systolic blood pressure (SBP) than their 18% counterparts and it is hypothesised that the high-fat and high-protein-fed male offspring would have also shown a significant difference had the temperature of the room been at the correct level. This suggestion was based on previous observations in the department. The significantly raised systolic blood pressure at weaning in the low-protein exposed group is consistent with an increasing number of studies (Chapter 5, Langley & Jackson 1993, Langley et al 1994, Langley-Evans & Jackson 1995a &b 1996, Langley-Evans et al 1996). Over the

ensuing 6 weeks, the 9% offspring fed chow maintained a significantly higher systolic blood pressure than the 18% offspring. The chow results confirm those which have been previously reported in that the hypertension was maintained into adulthood in the 9% group (Langley & Jackson 1993, Langley et al 1994, Langley-Evans & Jackson 1995a &b 1996, Langley-Evans et al 1996). The high-carbohydrate-fed offspring also had significantly increased systolic blood pressure (SBP) in the 9% group indicating that as this was a semi-synthetic diet there does not appear to be a dietary component specific to chow which caused the increase in blood pressure. The key finding in this study was that the 9% high-fat and high-protein-fed rats demonstrated a significant fall in SBP, with the high-fat effect being more immediate and greater in magnitude. However, the 18% offspring fed these two diets had a increase in SBP above that of the normal age-related increase seen over the first two weeks in the chow and high-carbohydrate-fed rats, which could be a weight-related phenomenon. The data from the high-fat diet confirmed our hypothesis in that it is an increase in the proportion of metabolisable energy from fat which was responsible for the lowered blood pressure in the 9% group. However as the high-protein diet had a similar hypotensive effect it is now proposed that it is not simply an increase in fat but more likely a decrease in the proportion of carbohydrate which is critical in reducing blood pressure in this model.

The reasons why blood pressure falls in the 9% offspring on these diets are not yet entirely clear, yet it is possible to propose some ideas. A similar effect has been demonstrated when the spontaneously hypertensive rat (SHR) was fed a high-fat diet (Wexler 1981). Here the spontaneous hypertension which occurs in these rats is prevented if a high-fat diet is fed from weaning. It was hypothesised that hypopituitarism (as evidence by the decrease in pituitary weight) was caused by the high-fat diet which subsequently prevented the increase in SBP. Although the high-fat-fed animals had a decrease in pituitary weight compared with the chow-fed animals this was present in both the 9% and 18% offspring, so cannot be the sole explanation. As the high-protein diet also has an effect it could therefore be due to the amount of carbohydrate in these diets and in particular the ratio between fat and carbohydrate. Although the protein : carbohydrate ratio may also be important as, if rats are allowed to self-select between diets of differing ratios they maintain a relatively close ratio of between 0.2-0.43 although the range of ratios available was much broader, i.e. 0.07-3.60 (Theall et al 1984). If the amount of carbohydrate is important this may further influence insulin secretion and action.

The idea that dietary carbohydrate promotes hypertension has been a topic of some discussion. The main areas which it could affect are:

(i) Hyperinsulinaemia and/or insulin resistance

(ii) Effect on sympathetic nervous system

(iii) Other actions of glucose or insulin

Fagerberg (1981) has investigated this area and concluded that there were many studies which do indicate a dietary effect on blood pressure mediated via a carbohydrate induced changes in plasma insulin and sympathetic activity. It is entirely possible that a similar effect is operating in this animal model.

The glucose tolerance test in the female offspring indicated the postnatal diets had a greater effect on the plasma insulin concentrations as there were few differences between the 9% and 18% groups. All animals were able to clear the glucose in the 60 minutes but the high-fat fed animals required higher insulin levels in the latter part of the test to return the glucose levels towards baseline. This increase in insulin could be suggestive of insulin resistance, or a change in insulin sensitivity. The increase in insulin levels in this study agree with the insulin increases seen in chapter four when the animals were maintained on a macronutrient self-selection diet, which is similarly high in fat and low in carbohydrate.

The changes in insulin levels may be related to changes in carbohydrate metabolism and could also be reflected by the changes in the proportion of muscle fibre types. However it is not known which is cause or effect as each of these measurements were only taken at age 12 weeks and perhaps need to be repeated at weaning to help clarify this.

To summarise it has been shown that disproportionate growth, insulin action and blood pressure can be 'programmed' *in utero* via exposure to a lower maternal protein dietary intake, but post-natal diet is also critical for the maintenance of the expression of this relative hypertensive state. Furthermore, this reflects for the first time an interaction (in an animal model) between pre and postnatal dietary experience in the pathogenesis, expression and maintenance of the hypertensive state.

Chapter 8.0
GENERAL DISCUSSION

This programme of work shows that there is a programming effect when rats are exposed to a low protein diet *in utero* and these changes which are occur are many and varied. This present study originated from work which is published by McCarthy et al 1994. Dams were fed either a 9% protein diet for two weeks prior to and throughout pregnancy or a control diet according to the model devised by this department to explore the idea that changes in maternal intake during pregnancy may programme the offspring (Levy & Jackson 1993).

In the studies the maternal weight gain and food intake were measured. The results were consistent across all the studies in this program of work in that the 9% dams were able to maintain a similar weight gain prior to pregnancy, however when the extra metabolic demand of pregnancy was added, weight gain fell behind that of the control group. Both groups increased their food intake during pregnancy, although the 18% groups increased their intake significantly more than the 9% groups. In the last few days of pregnancy both groups dropped their dietary intake to below pre-pregnancy intakes, possibly primarily due to the pressure of the offspring on the stomach and intestine. These findings were similar to those of previous studies (Langley & Jackson 1993, Menaker & Navia 1973).

In the pilot study (McCarthy et al 1994), at weaning the offspring from these dams were allowed to self-select for macronutrients and their food and energy intakes were measured. At age 20 weeks the animals were killed and dissected for organ and regional adipose tissue weights. It was found that the female offspring which had been exposed to a 9% protein diet *in utero* had a decrease in carbohydrate intake, an increase in fat and protein intake and significantly heavier regional adipose site weights compared with those female offspring which had been exposed to the control 18% protein diet. In the male offspring, those which had been exposed to the 9% protein diet also had significantly higher intestinal and omental adipose masses, notably the intraabdominal adipose sites. This was the outcome from selecting a lower carbohydrate intake compared to the 18% control animals (McCarthy et al 1994). It was from this background that chapter 4 was undertaken.

The changes in appetite for specific macronutrients and resulting changes in regional adipose sites also had an effect on the plasma insulin and glucose response to an intravenous glucose load. The glucose response was similar between prenatal dietary groups for both sexes which agreed with a similar previous study where the same model was used but where the offspring obtained from the maternal 9% and 18% diets had been

maintained on standard laboratory chow (Langley et al 1994a). The insulin response however had a biphasic response. There was a decrease in initial insulin release followed by a secondary peak after 20 minutes. A similar pattern has been seen in the Zucker rat (Fletcher et al 1986) and was attributed to a change in pancreatic structure. In a similar animal model to the one used in this present study pancreatic changes have been investigated (Snoeck et al 1990). The changes in insulin secretion are suggestive of insulin resistance which is a risk factor for non-insulin dependent diabetes mellitus (NIDDM), one of the non-communicable disease for which this model is being used to investigate.

The increases in regional adipose site masses agree with comparable animal studies where the pregnant dams have had restricted food intake (Anguita et al 1993, Jones et al 1984) and can be correlated with findings in humans after the Dutch Hunger Winter (Ravelli et al 1976). Surprisingly the body composition data did not correlate with these increases in body fat when the fat was calculated by Soxhlet extraction, however inconsistencies were found using this method and therefore the solvent was washed after extraction in subsequent work.

There were only small changes in body composition between prenatal groups although large differences in macronutrient intake were recorded which suggests that the utilisation and metabolism of macronutrient is different in the low-protein exposed offspring, although total energy intake was similar.

During the progress of this study, it was shown that the low protein exposed offspring from this programming model had significantly increased blood pressure compared with the control rats, when maintained on standard laboratory chow (Langley & Jackson 1993). However when the animals are maintained on a macronutrient self-selection diet, the composition of the diet selected is very different to that of chow (McCarthy et al 1994). Most notably is the amount of fat consumed, 3% by weight on the chow diet compared to 30-40% on the macronutrient self-selection diet. Therefore it was investigated to whether this could affect the relative hypertension in this model. It was found that the 9% exposed offspring had fluctuations of their blood pressure such that when fed the macronutrient self-selection diet for two weeks there was a reduction in systolic blood pressure which increased when these animals were fed chow for a similar period of time. This was against the 18% exposed animals which were unaffected by postweaning dietary manipulation. The macronutrient self-selection diet is a high-fat diet compared to standard laboratory chow and the reduction in blood pressure has been seen previously in the spontaneously hypertensive rat (SHR) when fed a high-fat diet (Wexler et al 1981). The mechanisms as to why the systolic blood pressure is reduced in these two groups of rats is not known although hypopituitarism (Wexler 1981), insulin and/or androgens (Chen & Meng 1994) have all been hypothesed as having an effect.

As discussed previously there were a number of similarities between the rat model being used in this program of study and the SHR, therefore the study was performed which investigated whether there were similarities between the 9% exposed animals and the SHR in terms of macronutrient intake, when offered a self-selection diet. It was therefore some what surprising that there were no comparisons to be made between these two groups. However the study did show some interesting results, in that throughout the whole study the SHR group consumed significantly less energy in terms of protein than both the Wistar groups. This may be due to the fact that the SHR offspring may be 'programmed' into handling a certain amount of protein as it has been shown that SHR dams produce less protein in their milk compared to Wistar rats (Rose & McCarty 1994).

Another interesting result was at age 8 weeks there was a stepwise reduction in the amount of carbohydrate consumed across the groups, such that the SHR were consuming the most carbohydrate and the 18% group the least. This is interesting as much of the present work was based on the difference of proportion of carbohydrate to fat consumed leading to differences in carbohydrate metabolism.

The ability to manipulate the blood pressure in the 9% group and our hypothesis that it is the proportion of macronutrients consumed which is critical for the expression of hypertension in this model gave rise to the experiments of feeding different composite diets, particularly rich in one macronutrient as investigated in chapter 7. The important finding in the composite diet study is that once again the postweaning diet is able to alter the systolic blood pressure of the 9% exposed offspring. In this case it also has an effect on the 18% offspring but this is the reverse effect to the 9% offspring. There was an increase in the blood pressure in the 9% group compared to the 18% at weaning which is consistent with an increasing number of studies (Langley & Jackson 1993, Langley et al 1994, Langley-Evans & Jackson 1995 a &b, 1996, Langley-Evans et al 1996). It appears that if the postweaning diet contains a high proportion of carbohydrate such as in standard laboratory chow or in the high-carbohydrate diet the relative hypertension is maintained, however if a decrease in the proportion of carbohydrate occurs, the blood pressure in the 9% animals is also reduced, while the blood pressure in the 18% animals increases. The muscle, regional adipose tissue and organ weight data together with the insulin response to a glucose load for the different postweaning dietary groups may give some explanation for these changes in blood pressure.

The differences in muscle weight for both male and female offspring appeared to be more strongly affected by the post-natal rather than pre-natal diet. The type I, slow twitch, oxidative fibres which are found predominately in the anterior tibularis use carbohydrate, fat and protein as an energy source whereas the type II muscle fibres are fast twitch and rely primarily on carbohydrate for energy. There was a trend to an increase in type II fibres in the high-fat and high-protein-fed female offspring. Both these diets were low in carbohydrate, but it is type II fibres which predominately use

carbohydrate for their substrate. Although this finding was some what unexpected it does correlate with a similar finding in the SHR (Lewis et al 1994) and also these rats had an increased percentage body fat and total carcass energy compared to the chow-fed offspring. There has been shown to be a negative correlation between obesity and the proportion of type I fibres in skeletal muscle (Hickey et al 1995, Lillioja et al 1987).

There were also differences across the regional adipose sites, which may again relate to metabolism. Brown adipose tissue is important in diet-induced thermogenesis and hence energy expenditure and so the changes in weight will affect the function of this tissue. This adipose mass is also related to the interscapular white adipose tissue. As the rat ages brown adipose tissue (BAT) is converted into white adipose tissue (WAT). Increases in this fat mass may therefore reflect either an increase in WAT present from weaning, or an increase in BAT converted to WAT. Increased intraabdominal fat will affect metabolism due to the increase in free fatty acids draining into the portal vein. There is an established relationship that an increase in visceral fat is associated with an increase risk of coronary heart disease (Lapidus et al 1984).

The disproportionate growth of the organs agrees with a previous study in a similar model (Langley-Evans et al 1996a). The changes in weight being related to function, such as heart muscle hypertrophy is an indication of stress on the heart. The increase in adrenal weight may lead to an increase in secretion of hormones although it is not known whether the increase is proportional across the medulla and cortex. Glucorticoids and other adrenal cortex hormones have been implicated in the manifestation of the hypertension seen in this model (Benediktsson et al 1993, Edwards et al 1993, Langley-Evans et al 1996b).

The glucose tolerance test carried out on the female offspring may also be related to changes in carbohydrate metabolism as although all animals were able to clear the glucose load it required higher levels of insulin in the high-fat-fed animals, which was a similar finding to that in chapter four, when the animals were maintained on a macronutrient self-selection diet which was similarly high in fat and relatively low in carbohydrate.

The work so far has concentrated in the energy intake side of the energy balance equation and so the area of energy expenditure and whether there are changes in this aspect could also be investigated in both fed and fasted animals from 9% and 18% prenatal groups. The effect of feeding a choice of a saturated or unsaturated fat diet in the macronutrient regimen may also be considered as the type of fat is important in determining the degree of obesity. It was found that rats fed on a diet with the majority of fat from the polyunsaturated fat corn oil became heavier and fatter than those fed a saturated diet using lard (Hill et al 1992). Whereas fish oils seem to be protective against obesity (Pan et al 1994). The role of fat in the formation of hypertension is also under some debate. In animal models coconut oil has been shown to increase blood pressure

upto 30mmHg above those fed corn oil (Langley-Evans & Jackson 1995), however similarly to human studies when fed a diet high in n-6 or n-3, systolic blood pressure is decreased (Hoffman et al 1986, Iacono & Doughty 1993, Langley-Evans et al 1996).

Finally to summarise, the changes which have been seen in these studies occurred without moving to the extremes of deficiency during pregnancy and could therefore be of great importance in understanding the possibility of the onset of factors which could lead to coronary heart disease and NIDDM in the next generation.

APPENDIX A

Composition of Standard Laboratory Chow

SDS CRM(X)

Nutrients	% weight
Oil	3.4
Protein	18.8
Fibre	12.3
Pectin	1.3
Hemicellulose	6.8
Cellulose	3.3
Lignin	1.0
Starches	44.8
Sugars	4.7

Major Minerals	% weight
Calcium	0.80
Phytate phosphorus	0.21
Available phosphorus	0.43
Sodium	0.30
Choline	0.32
Magnesium	0.22
Potassium	0.64

Trace Minerals	mg/kg
Iron	110.0
Copper	16.0
Manganese	70.0
Zinc	65.0
Fluorine	9

Trace Minerals	µg/kg
Cobalt	280.9
Iodine	603.0
Selenium	217.0

Vitamins	iu/kg
Vitamin A	16843.4
Vitamin D_3	3228.6

Vitamins	mg/kg
α-Tocopherol	93.8
Vitamin E	103.2
Vitamin B_1	16.8
Vitamin B_2	13.6
Vitamin B_6	18.5
Vitamin C	8.0
Vitamin K_3	184.9
Folic acid	4.4
Nicotinic acid	78.7
Pantothenic acid	26.1
Choline	951.0
Inositol	2355.4
ß-Carotene	0.9

Vitamins	µg/kg
Retinol	5104.1
Cholecalciferol	80.7
Vitamin B_{12}	83.5
Biotin	359.1

Composition of Standard Laboratory Chow

SDS CRM(X)

Amino Acids	% weight
Arginine	1.29
Lysine	1.08
Methionine	0.28
Cystine	0.29
Tryptophan	0.22
Histidine	0.49
Threonine	0.72
Isoleucine	0.81
Leucine	1.48
Phenylalanine	0.90
Valine	0.93
Tyrosine	0.69
Glycine	1.61
Aspartic acid	1.11
Glutamic acid	3.71
Proline	1.27
Serine	0.83
Hydroxyproline	0.05
Alanine	0.15

Fatty Acids	% weight
Myristoleic	0.01
Palmitoleic	0.12
Oleic	0.91
Linoleic	0.89
Linolenic	0.11
Arachidonic	0.19
Clupanodonic	0.03
Lauric	0.03
Myristic	0.15
Palmitic	0.33
Stearic	0.05

APPENDIX B

Composition of AIN-76 Vitamin and Mineral Mixes

Vitamin Mix

Substance	% weight
Biotin PMX 2%	0.100
Cal Pant 100	0.160
Folic acid	0.020
Niacin	0.300
Sugar - granulated	98.049
Vitamin A 500	0.080
Vitamin B1 Thiamine	0.060
Vitamin B2 Riboflavine	0.060
Vitamin B6 Pyridoxine	0.070
Vitamin B12 1000mg/kg	0.100
Vitamin E 50% ADS	1.000
Vitamin K3 MSB	0.001

Mineral Mix

Substance	% weight
Chromic potassium sulphate	0.055
DCP Dutch 46%	34.000
Dipotassium hyd phosphate	16.001
Fat premix 80% -K118	0.600
Magnesium oxide	2.400
Potassium sulphate	5.200
Salt PDV	7.400
Sugar - icing	11.963
Potassium citrate C8385	22.000
Cupric carbonate	0.030
Manganese carbonate	0.350

APPENDIX C

Methods of Diet Analysis

C.1 Protein Analysis

The amount of protein in the diet was determined by using the Kjeldahl method. (Osbourne & Voogt 1978) This directly measures nitrogen content and protein was calculated from this by multiplying by 6.25 or specifically for casein by multiplying by 6.38.

There were three stages to the analysis, digestion, distillation and titration.

A: Digestion

The diet first was digested to reduce the organic nitrogen to ammonium sulphate. This was carried out using the Teccator system. Approximately 1g of the diet was accurately weighed onto nitrogen-free paper and added as a whole to the digestion tube. A Kjeldahl catalyst tablet was added, containing K_2SO_4 to raise the boiling point so that oxidation proceeded without the excessive loss of acid.

20ml of concentrated sulphuric acid was added to the tube with a few anti-bumping granules. The solutions were mixed well and 5ml of 25-30% hydrogen peroxide added dropwise to further catalyse the experiment by increasing the concentration of hydrogen ions.

After mixing well the digest was heated for seven hours until it was clear and then for a further 30 minutes to ensure that all the ammonia was converted to ammonium sulphate. The solution was to cooled before transferring to a 100ml volumetric flask which was then made up to volume ready for distillation.

B: Distillation

The sample was distilled using the Markham steam distillation apparatus.(Markham 1942) Before starting the distillation several flasks containing 100ml of boric acid indicator were set up. One of these flasks had 10ml of distilled water added to it and was used as the colour check for the end-point.

At the onset of the distillation the round bottomed flask containing water and anti-bumping granules (the steam generator) was connected up to the steam jacket and run for 10 minutes. Cold water from the reservoir was then allowed to run into the still and was drained by opening the waste clip. The steam generator was then disconnected.

A standard was used to titrate against and was done by adding 2ml of ammonium sulphate containing 1mg N/ml to the still using a Gilson pipette. This was washed in with 5ml of distilled water. The stopper on the top of the still was replaced and 10ml of 40% sodium hydroxide was pipetted into the reservoir and then the stopper lifted slightly to

allow the sodium hydroxide to run gently into the still. Distilled water was then added to the reservoir to form a seal, and the steam generator reconnected to the jacket. One of the conical flasks containing the indicator was held under the condenser so that the tip was in the indicator, this was done in order to trap any gaseous ammonia.

When the first drop of distillate had been collected and the indicator had turned green the distillation was continued for a further 90 seconds, the flask was then removed and put aside for titration.

The heat was removed from the steam generator and once the solution had flushed out of the still, the water was added from the reservoir. The waste clip was then opened to allow the draining of the apparatus. The steam generator was disconnected and the distillation was repeated in triplicate for the standard and then also for each diet sample, but using 5ml sample volume, not 2ml as for the standard.

C: Titration

The distilled standard and samples were titrated against N/14 H_2SO_4 until the colour had changed from green to the pale pink of the flask containing distilled water and indicator. From knowing the amount of acid needed to neutralise a known amount of N/ml, the nitrogen content of the samples could be calculated and hence the protein content of the diets.

C.2 Gross Energy Analysis

The gross energy of the diets were determined by using Ballistic Bomb Calorimetry. (Miller & Payne 1959)

Firstly a blank was fired to record the galvanometer deflection of just the wick combusting. The bomb was turned on and all the parts cleaned and dried, ensuring that the black O-ring seal was in place. 7cm of the wick was cut and one end inserted between the coils of the platinum firing wire, the other end was placed in the crucible. the top of the bomb was replaced, care being taken to screw the collar on to the top and not turning the top of the bomb and the side valve was then closed and the thermocouple was placed in the top of the bomb. The valve on the oxygen cylinder was opened and then the front valve on the bomb to allow oxygen to fill the bomb until 25 bar pressure was reached. The front valve was then closed and the galvanometer set to zero.

The bomb was fired and the maximum deflection recorded. The pressure was released by opening the side valve and the bomb disassembled. It was cooled by immersing the upper section in cold water so that the bomb was at the same starting temperature each time. It was then cleaned and dried and the experiment repeated twice more for the blank.

The galvanometer was then standardised by weighing out 1 benzoic acid tablet and placing it in the crucible, care was taken that the wick was placed under the tablet. The same procedure was then carried out as for the blank. This was repeated in triplicate.

The diets were then analysed by weighing out accurately approximately 1.0g into the crucible and repeating the procedure as before in triplicate for each diet.

C.3 Fat Analysis

The fat content of the diets was determined by the Soxhlet method. (Osbourne & Voogt 1978)

Soxhlet extraction thimbles were dried overnight in an oven at 40°C. These were then allowed to cool in a desiccator with cotton wool for stoppers. Triplicate samples for each diet and duplicate samples for the control were used. Each thimble and stopper was weighed before and after accurately weighing 3g of diet into the thimble. For the control thimbles 1g of lard accurately weighed was used as this contains 1g of fat/ 1g of lard. The thimbles were replaced in the desiccator as they quickly absorb water from the atmosphere.

The extractions were run in batches of five thimbles. Extraction was carried out by boiling the ether for six hours in reflux in a fume cupboard, condensing it through the thimbles. The thimbles were then placed in the oven at 40°C overnight to allow all the ether to evaporate and then they were placed in a desiccator before weighing.

The difference in weight before and after extraction was calculated and hence the percentage fat content in the diet.

APPENDIX D

Staining Solutions for Actomyosin ATPase Activity

1. Acid pre-incubation solution

Calcium chloride (0.18M)	100ml
Glacial acetic acid	3.0ml
Distilled water	850ml

pH adjusted to 4.35 with Potassium hydroxide and final volume brought to 1000ml with distilled water

2. Rinse solution

Tris (MW 121)	12.1g
Calcium chloride	100ml
Distilled water	850ml

pH adjusted to 7.8 with Hydrochloric acid and final volume brought to 1000ml with distilled water

3. Incubation solution

Sigma 221 buffer (1.5M)	13.4ml
Calcium chloride (0.18M)	20ml
Potassium chloride (MW 75)	740mg
ATP, Disodium (MW 551.2)	304mg
Distilled water	150ml

pH adjusted to 9.4 with Hydrochloric acid and final volume brought to 200ml with distilled water

4. Wash solution (1% $CaCl_2$, w/v)

Calcium chloride (MW 147)	10g
Distilled water	1000ml

5. Cobalt chloride solution (2% w/v)

Cobalt chloride (MW 238)	4g
Distilled water	200ml

6. Alkaline washing solution

Sigma 221 buffer (1.5M)	26.8ml
Distilled water	320ml

pH adjusted to 9.4 with Hydrochloric acid and final volume brought to 400ml with distilled water

7. Ammonium sulfide solution (1% w/v)

Ammonium sulfide	2ml
Distilled water	200ml

Solutions 1,2 and 4 are stable and were made up in large quantities and stored in the refrigerator. The other solutions are not stable and were made up immediately before use.

APPENDIX E

Individual Data for Validation

Rat 1

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	10.2	13.3	9.9	164.79	377
2	3.5	8.9	12.9	149.13	379
3	9.2	7.9	9.8	140.94	389
4	11.8	6.4	8.3	175.23	396
5	8.1	7.7	10.6	142.74	404
6	12.2	6.5	9.4	143.46	411
7	10.5	3.3	5.0	90.18	411
8	10.8	4.7	7.8	118.98	410
9	6.2	4.3	8.1	103.41	414
10	5.5	4.1	9.2	109.08	414
11	1.4	1.9	10.0	92.88	416
12	3.7	3.6	10.3	109.71	418
13	3.5	4.1	10.6	65.52	427
14	3.8	3.9	9.9	107.91	426
15	2.7	2.8	8.4	87.84	426
16	1.1	1.8	6.6	63.9	425

Rat 2

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	8.3	11.5	8.4	139.32	350
2	3.6	7.1	9.3	113.85	352
3	7.5	7.5	10.4	138.24	363
4	8.2	6.3	10.5	137.25	369
5	7.9	5.1	10.5	131.85	380
6	5.8	3.4	9.7	111.69	378
7	10.6	4.2	6.9	109.17	385
8	6.6	5.4	8.2	109.62	385
9	3.7	4.4	9.6	106.92	392
10	5.5	3.8	7.4	93.42	393
11	0.1	3.5	9.0	85.86	393
12	3.9	5.4	10.7	120.15	399
13	2.2	4.2	12.7	68.76	405
14	2.7	2.4	9.2	92.88	404
15	3.7	3.6	5.6	71.64	404
16	3.0	6.2	10.5	118.17	413

Rat 3

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	15.6	10.5	4.1	127.17	359
2	19.0	9.6	3.4	130.50	369
3	13.8	11.4	4.8	129.60	374
4	16.4	12.7	3.1	129.87	376
5	16.1	11.4	5.0	139.50	380
6	9.6	10.0	1.8	85.14	374
7	17.5	11.5	1.8	118.98	382
8	25.7	11.6	3.3	161.01	388
9	13.1	13.7	2.3	115.11	393
10	8.9	8.9	3.2	90.00	386
11	7.1	11.4	5.4	110.34	389
12	11.7	14.1	6.5	145.53	399
13	10.7	15.9	5.8	116.64	403
14	9.6	13.0	5.5	125.91	400
15	9.8	11.7	3.0	101.70	401
16	8.6	16.7	5.4	134.82	414

Rat 4

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	14.2	3.2	4.2	96.66	294
2	11.9	6.0	6.1	113.85	301
3	10.9	4.9	5.3	99.81	303
4	10.2	5.6	5.9	104.67	306
5	9.0	5.2	5.0	91.62	311
6	9.8	6.8	7.2	118.08	313
7	10.6	5.6	5.8	105.30	316
8	6.0	7.5	2.7	70.47	314
9	4.0	4.3	6.7	84.15	314
10	1.1	4.7	6.3	71.91	315
11	8.8	6.3	7.1	111.87	321
12	7.1	5.9	7.9	110.79	324
13	3.8	6.5	8.1	66.24	330
14	3.5	5.7	4.5	69.57	324
15	5.4	6.1	8.4	109.44	332
16	5.9	5.9	7.2	100.80	337

Rat 5

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	13.0	7.8	6.5	127.33	330
2	8.6	7.3	4.0	89.64	326
3	11.9	8.2	5.8	119.34	336
4	14.3	8.1	7.1	138.15	344
5	12.0	7.4	6.9	125.73	351
6	11.6	9.1	5.4	118.26	351
7	16.5	5.7	2.6	110.98	351
8	13.6	8.0	4.9	117.45	359
9	12.0	8.3	5.2	115.20	366
10	6.0	7.9	6.9	105.93	370
11	6.4	8.2	7.8	115.74	366
12	8.5	5.5	4.1	83.61	366
13	7.4	7.7	5.0	72.36	372
14	9.2	8.3	6.0	111.60	376
15	11.3	8.3	6.6	124.02	379
16	7.2	7.1	7.9	115.47	382

Rat 6

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	17.1	10.2	5.8	145.26	316
2	13.0	10.1	4.5	119.61	321
3	14.7	9.8	5.6	133.56	329
4	15.6	9.2	5.9	137.07	332
5	12.6	5.4	5.7	110.97	339
6	14.1	6.1	4.9	112.41	340
7	19.0	6.6	4.5	128.61	347
8	16.3	9.9	6.1	143.73	346
9	7.1	5.2	5.1	85.59	347
10	2.8	8.4	5.2	82.44	347
11	8.0	8.7	7.3	119.25	355
12	12.2	9.1	5.8	123.66	356
13	8.1	4.9	6.5	70.20	361
14	5.3	7.9	5.0	88.02	358
15	6.4	7.2	8.0	113.76	363
16	5.5	8.2	8.2	119.34	367

Rat 7

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	13.3	9.7	8.7	153.27	365
2	7.4	9.8	8.0	126.72	370
3	10.4	2.1	5.4	88.74	368
4	9.3	8.1	8.2	129.06	377
5	11.1	6.6	6.2	113.94	382
6	5.4	8.3	8.8	120.60	384
7	12.1	3.7	5.3	99.81	385
8	4.6	4.5	9.0	105.66	388
9	4.3	5.5	8.8	106.56	394
10	3.7	6.3	9.6	113.76	400
11	0.1	7.0	14.3	141.39	401
12	5.1	4.0	9.0	105.66	405
13	3.6	4.0	10.6	65.52	410
14	2.7	4.7	11.0	115.74	413
15	2.0	4.2	12.2	121.14	416
16	2.8	4.4	10.1	107.73	421

Rat 8

Day	Cho (g)	Protein (g)	Fat (g)	Energy (cal)	Body wt (g)
1	11.3	8.5	6.3	122.31	308
2	6.8	6.2	6.0	95.40	306
3	8.7	6.0	4.1	86.13	311
4	14.8	6.2	5.3	118.53	316
5	10.2	4.8	7.4	113.94	325
6	7.7	3.6	4.5	77.13	321
7	15.4	5.0	2.5	93.69	324
8	14.0	7.0	3.1	110.71	327
9	10.3	4.9	5.3	97.65	331
10	6.9	3.9	5.6	84.24	329
11	3.9	4.0	5.6	73.80	330
12	8.9	6.1	4.9	93.69	332
13	14.2	6.0	4.3	88.20	337
14	8.8	5.1	9.7	128.61	342
15	8.6	7.4	5.6	102.96	341
16	5.3	5.1	7.2	95.76	340

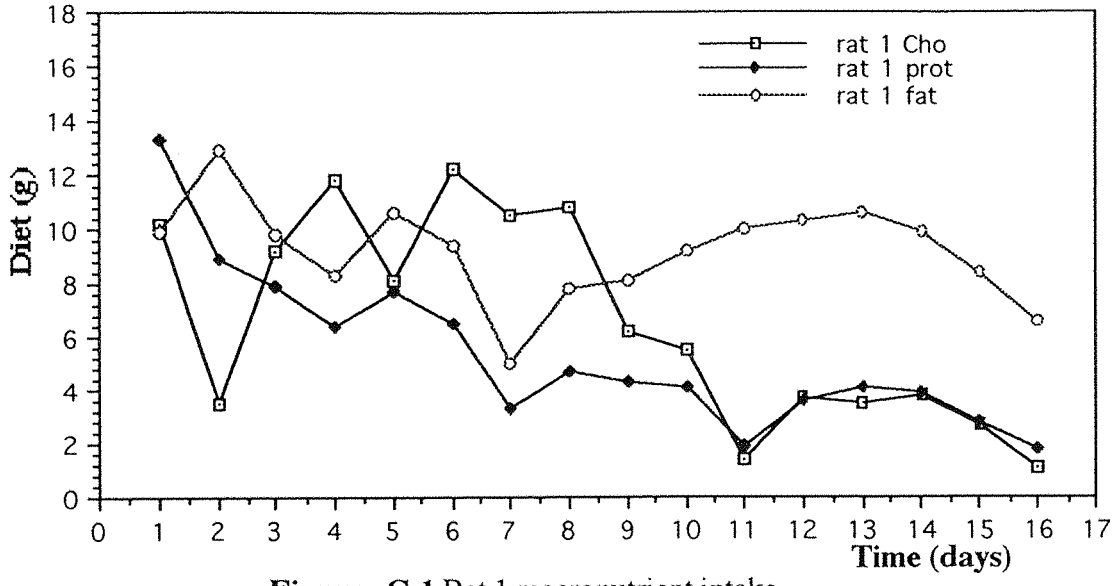

Figure C.1 Rat 1 macronutrient intake

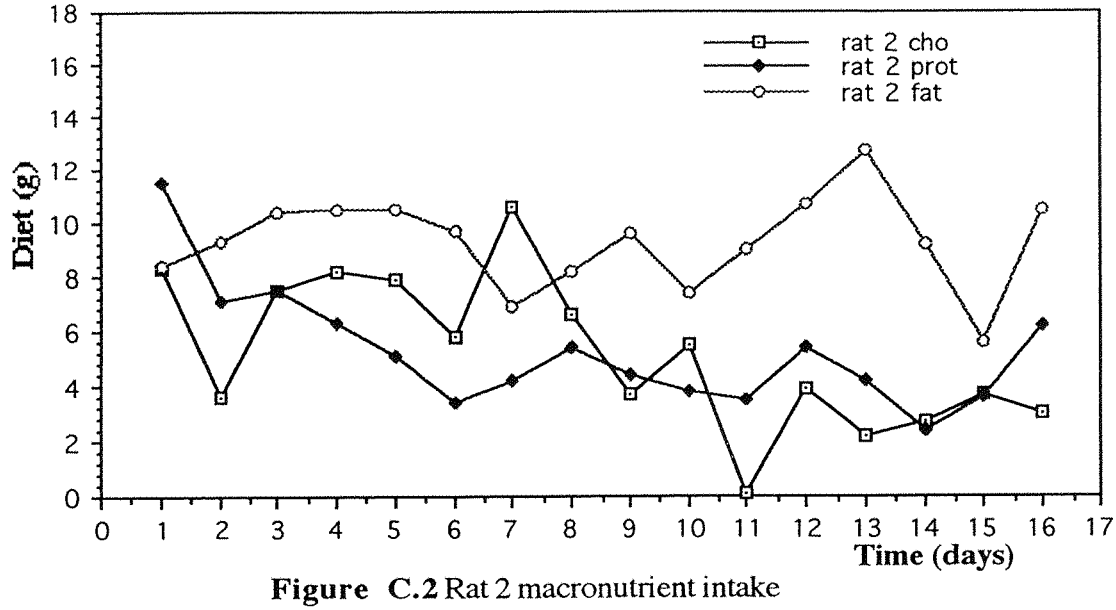

Figure C.2 Rat 2 macronutrient intake

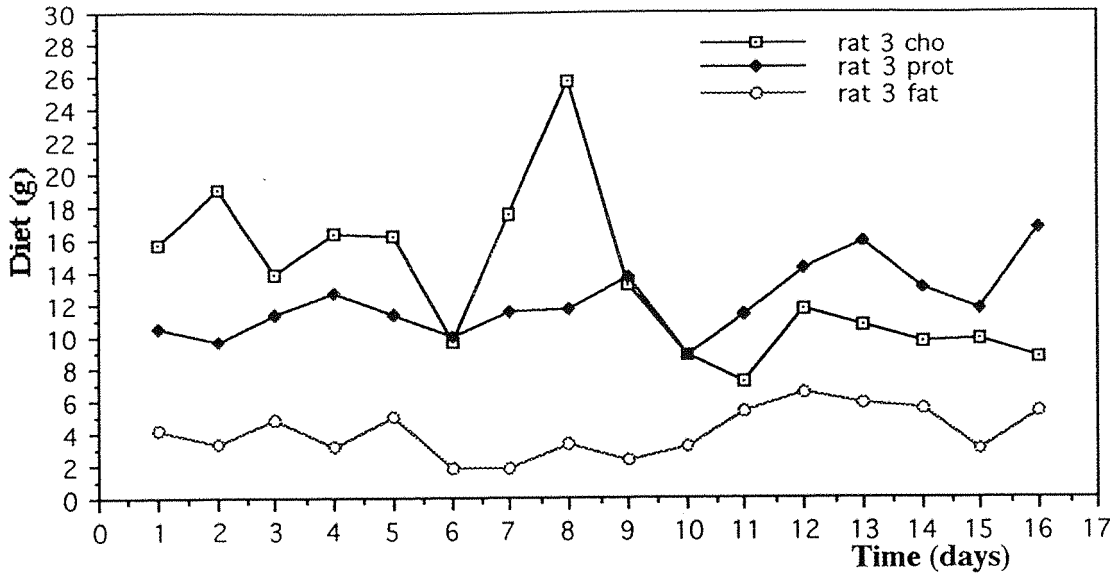

Figure C.3 Rat 3 macronutrient intake

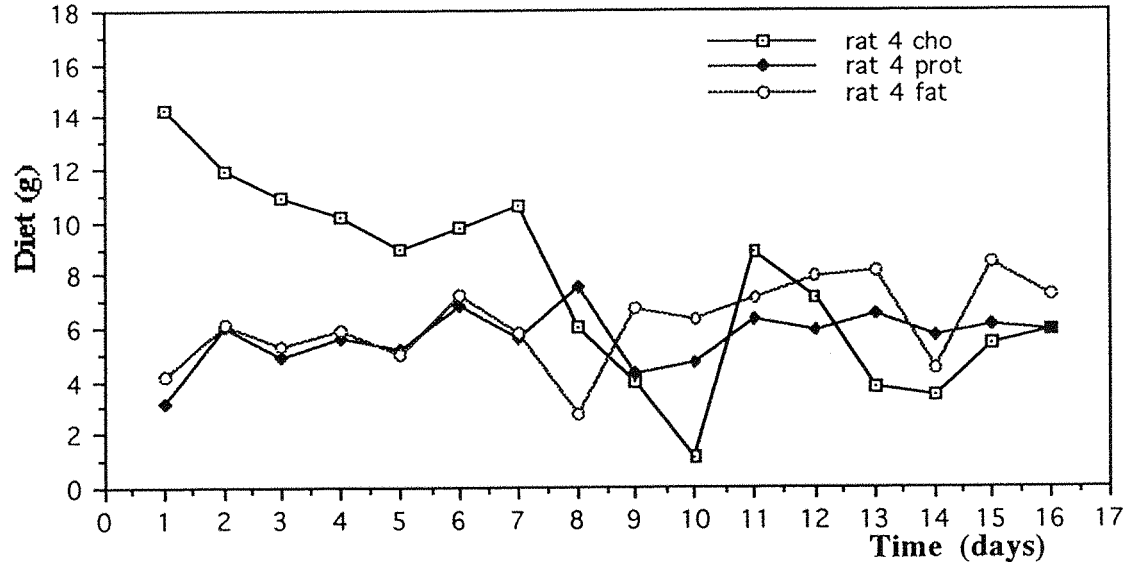

Figure C.4 Rat 4 macronutrient intake

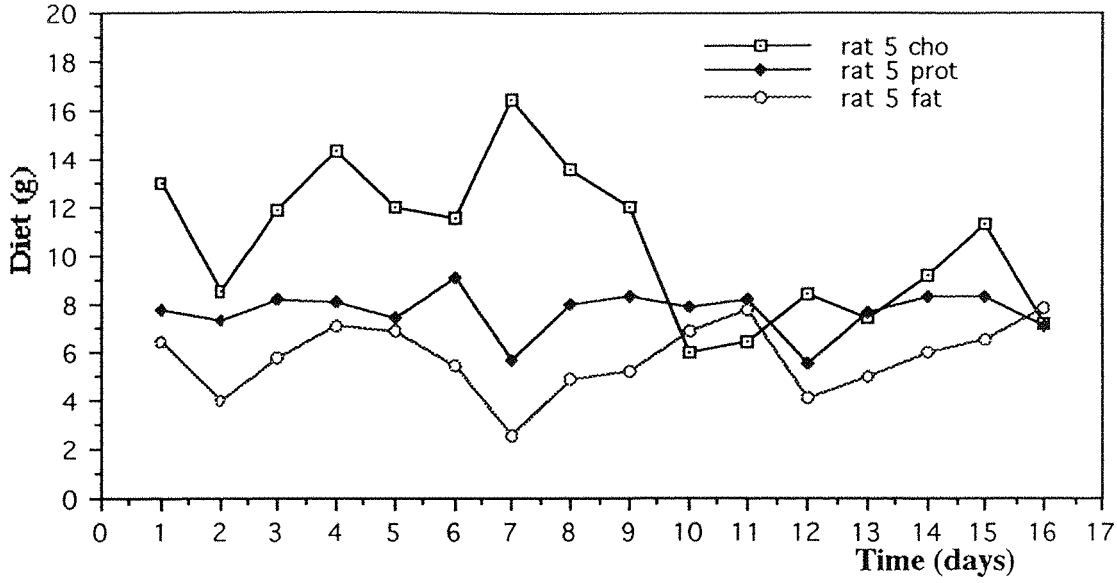

Figure C.5 Rat 5 macronutrient intake

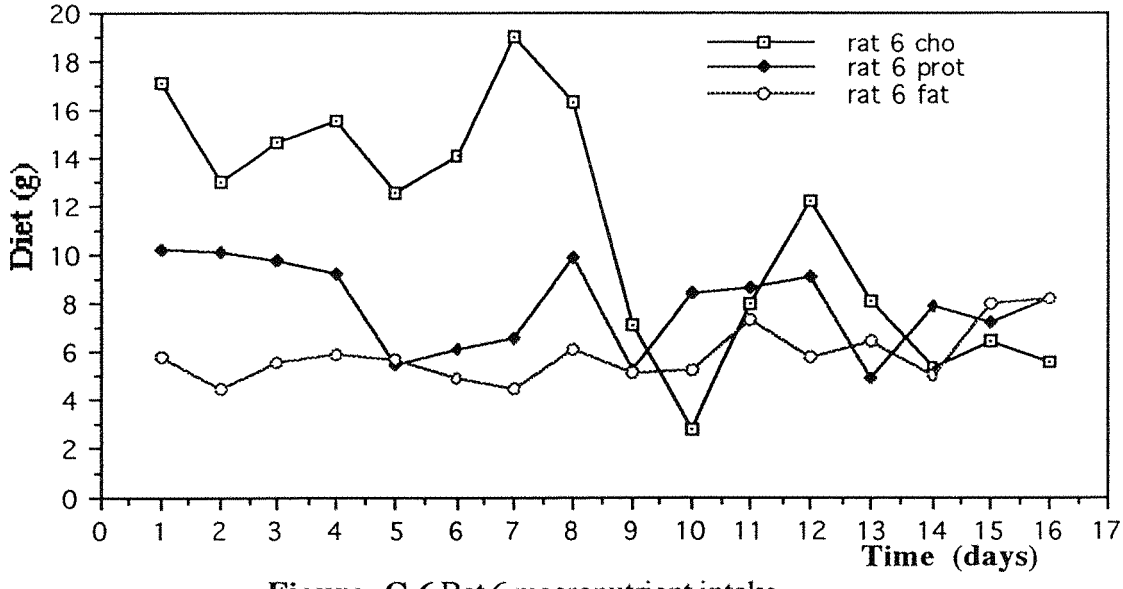

Figure C.6 Rat 6 macronutrient intake

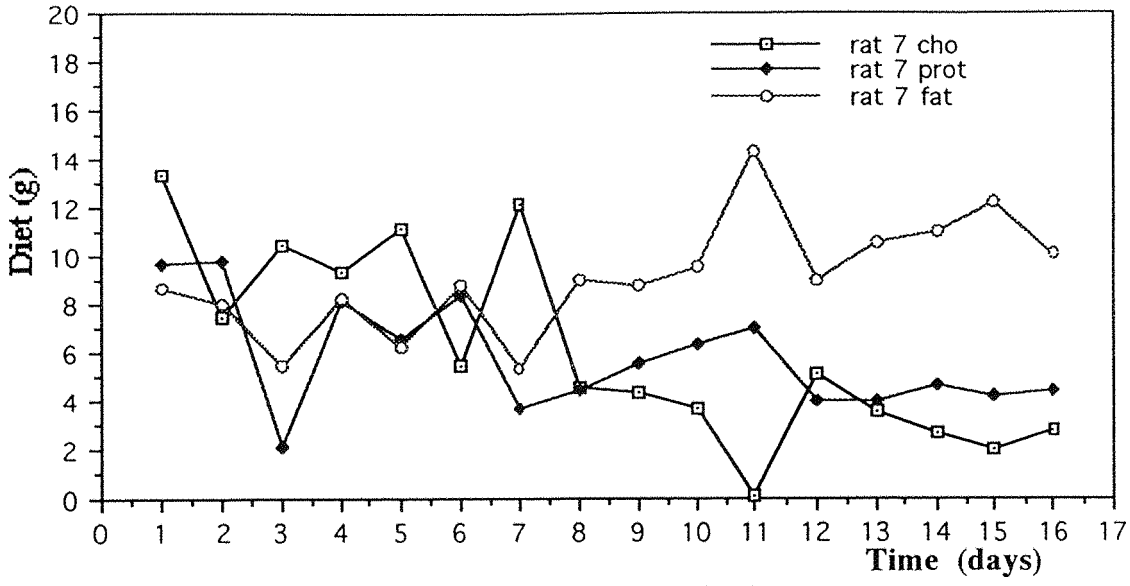

Figure C.7 Rat 7 macronutrient intake

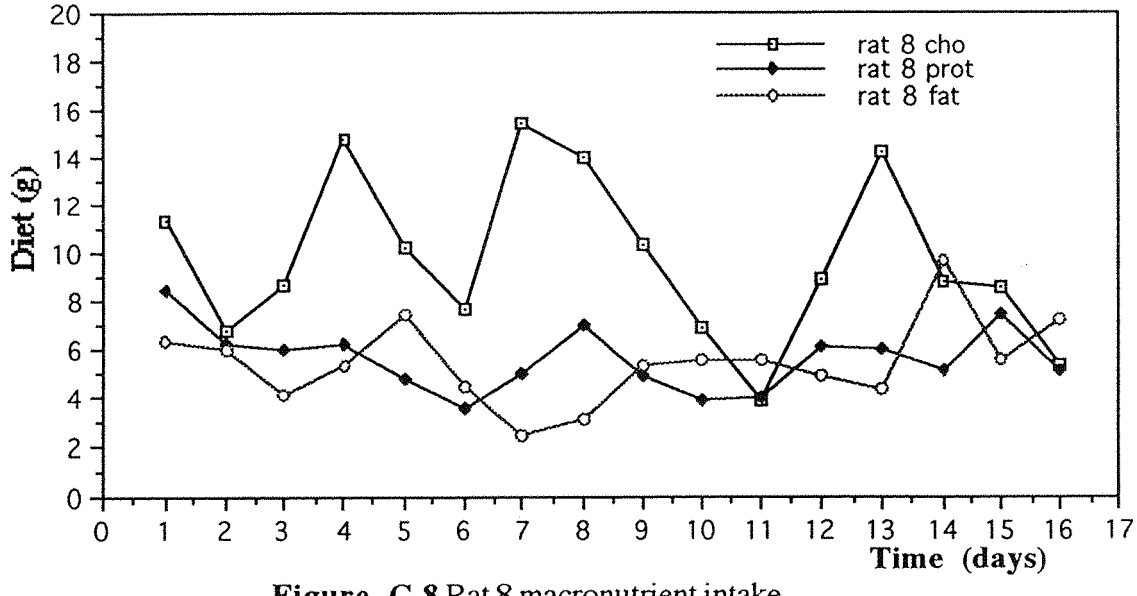

Figure C.8 Rat 8 macronutrient intake

APPENDIX F

Full Analysis of Muscle, Organ and Adipose Data

Prenatal diet	Postnatal diet	Muscle (mg) Anterior tibularis		Extensor digitorum longus		Gastrocnemius		Soleus	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	409.8	16.6	94.9c	3.0	1120	54	165.0bc	22
	h/prot	392bc	9.4	94.0c	3.1	1008bc	40	182.0bc	8.7
	h/cho	439.5	12.8	108.4a	3.0	1144	50	208.4	9.4
	chow	438.6	12.7	102.0	2.5	1152	30	203.1	4.9
18%	h/fat	417.4	9.0	101.8	3.8	1174	105	150.1bc	21.4
	h/prot	412.4	14.8	100.4	2.5	1072	36	165.0bc	14.9
	h/cho	438.1	10.5	100.0	2.3	1060	40	194.4	4.8
	chow	449.4	13.6	103.6	4.2	1152	40	207.6	6.9

Table F.1 : Weight of four muscles for female offspring (Mean ± SE). a : sig diff to 18%, b : sig diff to chow, c : sig diff to high-carbohydrate. P<0.05

Prenatal diet	Postnatal diet	Muscle (mg) Anterior tibularis		Extensor digitorum longus		Gastrocnemius		Soleus	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	533.9bc	26.9	125.7bc	5.1	1408b	80	265.4bc	15.3
	h/prot	523.0bc	7.0	133.1bc	5.4	1562	80	236.8bc	26.7
	h/cho	634	10.0	152.0	3.0	1509	60	309.0	8.0
	chow	631.9a	17.1	151.4	4.4	1656	60	314.5	12.4
18%	h/fat	592.6b	25.5	143.1b	5.8	1549b	70	280.1	27.7
	h/prot	581.9bc	23.4	143.0b	5.2	1455b	70	254.8b	20.9
	h/cho	638.0b	10.0	148.0b	3.6	1573b	50	304.8	11.1
	chow	674.9	10.1	159.6	3.1	1749	30	290.4	44.6

Table F.2 : Weight of four muscles for male offspring (Mean ± SE). a : sig different to 18%, b : sig diff to chow, c : sig diff to high-carbohydrate (P<0.05)

Prenatal diet	Postnatal diet	Regional adipose site weight (g)							
		Inguinal		Popliteal		Interscapular white adipose tissue (I-WAT)		Interscapular brown adipose tissue (I-BAT)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	high-fat	2.400	0.40	0.209bcd	0.02	0.556	0.05	0.557bd	0.05
	high-protein	1.869	0.33	0.148	0.01	0.431	0.06	0.299abc	0.02
	high-cho	1.727	0.09	0.138	0.01	0.466	0.04	0.579ab	0.04
	chow	1.755	0.19	0.152	0.01	0.454	0.03	0.405	0.02
18%	high-fat	3.060bcd	0.36	0.257bcd	0.03	0.847bcd	0.14	0.708bcd	0.08
	high-protein	1.915	0.16	0.170b	0.01	0.538bc	0.03	0.387c	0.03
	high-cho	1.769	0.10	0.150	0.01	0.442	0.02	0.470	0.02
	chow	1.665	0.09	0.149	0.01	0.426	0.02	0.410	0.03

Prenatal diet	Postnatal diet	Regional adipose site weight (g)							
		Parametrial		Intestinal		Omental		Retroperitoneal	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	high-fat	9.117d	1.43	2.956d	0.37	0.378	0.06	6.209	1.39
	high-protein	5.322b	0.43	1.979bc	0.16	0.310b	0.03	4.077abc	0.22
	high-cho	6.463	0.55	2.788	0.13	0.418	0.05	5.488	0.34
	chow	7.435	0.8	2.857	0.20	0.494	0.04	5.456	0.52
18%	high-fat	10.910bcd	1.30	3.539d	0.47	0.506d	0.048	8.507bcd	0.80
	high-protein	6.278	0.30	2.299b	0.19	0.288bc	0.04	4.890	0.33
	high-cho	7.108	0.61	2.789	0.21	0.473	0.03	4.726	0.30
	chow	6.847	0.61	3.193	0.23	0.501	0.04	5.508	0.31

Table F.3 : Weights of fat masses for female offspring. a: sig diff to 18%, b: sig diff to chow, c: sig diff to h/cho, d: sig diff to h/p ($P < 0.05$)

Prenatal diet	Postnatal diet	Regional adipose site weight (g)							
		Inguinal		Popliteal		Interscapular white adipose tissue (I-WAT)		Interscapular brown adipose tissue (I-BAT)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	high-fat	2.600c	0.47	0.251	0.05	0.529ac	0.07	0.666ad	0.05
	high-protein	1.767b	0.22	0.171b	0.01	0.511bc	0.04	0.515bc	0.03
	high-cho	1.548b	0.19	0.158b	0.03	0.379b	0.04	0.651	0.06
	chow	2.917	0.20	0.267	0.01	0.609	0.03	0.731	0.04
18%	high-fat	3.024cd	0.36	0.296cd	0.03	0.752bcd	0.07	0.932bcd	0.05
	high-protein	2.139b	0.20	0.206b	0.02	0.591	0.04	0.567c	0.06
	high-cho	2.114b	0.24	0.193b	0.03	0.499	0.05	0.770	0.07
	chow	3.181	0.14	0.273	0.02	0.523	0.04	0.713	0.09

Prenatal diet	Postnatal diet	Regional adipose site weight (g)							
		Epididymal		Intestinal		Omental		Retroperitoneal	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	high-fat	8.153	2.07	4.165b	0.71	0.687	0.17	9.336	2.24
	high-protein	5.261b	0.55	3.187b	0.27	0.426ab	0.04	6.848b	0.83
	high-cho	5.303b	0.64	3.235b	0.38	0.525b	0.07	7.408b	1.14
	chow	10.090	0.43	5.660a	0.27	0.945	0.05	13.540	0.82
18%	high-fat	11.010cd	1.41	5.480cd	0.60	0.737b	0.13	13.108cd	1.13
	high-protein	6.182b	0.53	3.319b	0.26	0.592b	0.07	7.562b	0.76
	high-cho	6.318b	0.65	3.645b	0.37	0.550b	0.10	7.863b	0.83
	chow	10.660	0.44	6.350	0.20	1.073	0.09	14.140	1.00

Table F.4 : Weights of fat pads for male offspring. a: sig diff to 18%, b: sig diff to chow, c: sig diff to h/cho, d: sig diff to h/p ($P < 0.05$)

Prenatal diet	Postnatal diet	Organ weight: Liver (g)		Kidney (g)		Adrenal (g)		Heart (g)		Brain (g)		Pituitary (mg)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	9.729	0.49	1.830bd	0.08	0.092c	0.01	1.038bc	0.04	1.839a	0.06	12.25	0.85
	h/prot	9.455	0.45	2.216bc	0.05	0.092c	0.01	0.983b	0.07	1.785a	0.08	12.50	0.53
	h/cho	9.350	0.40	1.959b	0.07	0.068ab	0.01	0.924b	0.03	1.895	0.04	13.75	0.40
	chow	8.883	0.33	1.594	0.08	0.080	0.01	0.816	0.02	1.864	0.02	13.88	0.58
18%	h/fat	9.413b	0.25	1.742cd	0.04	0.098	0.01	0.954b	0.04	1.968bc	0.03	13.75	0.60
	h/prot	8.884	0.46	2.116bc	0.06	0.103bc	0.01	0.867	0.04	1.944bc	0.01	12.40	0.64
	h/cho	9.279	0.32	1.836b	0.02	0.081	0.01	0.917b	0.02	1.856	0.03	13.75	0.40
	chow	8.600	0.33	1.621	0.05	0.079	0.01	0.829	0.03	1.846	0.02	13.38	0.70

Table F.5 : Weight of organs for female offspring. a: significantly different to 18%, b: significantly different to chow, c: significantly different to high-carbohydrate, d: significantly different to high-protein ($P < 0.05$)

Prenatal diet	Postnatal diet	Organ weights: Liver (g)		Kidney (g)		Adrenal (g)		Heart (g)		Brain (g)		Pituitary (mg)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	13.130	1.12	2.439d	0.14	0.079c	0.01	1.545b	0.18	2.002	0.03	10.40c	0.46
	h/prot	13.556	0.58	3.069bc	0.13	0.078c	0.01	1.308b	0.05	1.994	0.03	10.10	0.80
	h/cho	12.690	0.44	2.608	0.12	0.055b	0.02	1.240b	0.04	2.046	0.03	11.88	0.55
	chow	14.224	0.80	2.466a	0.09	0.067	0.04	1.125	0.02	1.998	0.03	11.13	0.32
18%	h/fat	15.074	0.88	2.778	0.14	0.089bc	0.01	1.513bcd	0.06	2.107	0.03	11.90d	1.17
	h/prot	13.364b	0.68	3.086bc	0.15	0.092bc	0.01	1.240	0.05	2.048	0.03	8.63bc	0.60
	h/cho	13.204b	0.67	2.634	0.08	0.060	0.04	1.308b	0.04	2.021	0.04	11.75	0.17
	chow	15.071	0.38	2.710	0.05	0.069	0.03	1.219	0.02	1.998	0.03	11.63	0.45

Table F.6 : Weight of organs for male offspring. a: significantly different to 18%, b: significantly different to chow, c: significantly different to high-carbohydrate, d: significantly different to high-protein ($P < 0.05$)

Prenatal diet	Postnatal diet	Total Body Composition											
		Final Body weight (g)		%Water		%Protein		%Fat		Total Energy (kJ)		Energy /g body weight (kJ)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	233.3	12.4	60.5	1.0	18.5	0.7	8.5	0.3	3235	371.7	31.3	1.4
	h/protein	216.1	6.3	63.5	0.3	19.4	0.4	5.7	0.3	2089	102.5	25.4	0.9
	h/cho	243.8	6.2	63.0	0.4	19.9	0.3	6.5	1.0	2407	111.0	25.7	0.3
	chow	245.6	7.3	62.4	0.8	19.9	0.2	6.0	0.7	2676	169.9	28.9	0.7
18%	h/fat	244.6	7.1	56.9	1.0	19.2	0.7	9.8	1.1	3935	375.8	34.3	0.8
	h/protein	223.8	6.5	61.5	0.3	18.5	0.4	6.7	0.3	2173	96.9	24.3	0.9
	h/cho	237.6	2.6	63.0	0.6	19.5	0.5	5.5	0.4	2317	87.1	26.5	0.4
	chow	250.3	7.1	62.5	0.5	19.5	0.4	5.9	0.3	2440	112.4	26.9	1.0

Table F.7 : Total body compostion of female offspring expressed as percentage of wet weight (mean ± SE)

Prenatal diet	Postnatal diet	Total Body Composition											
		Final Body weight (g)		%Water		%Protein		%Fat		Total Energy (kJ)		Energy /g body weight (kJ)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
9%	h/fat	307.7	19.9	62.0	1.2	19.3	0.7	7.0	1.2	4325	655.6	33.0	0.8
	h/protein	291.4	9.6	64.4	0.4	19.7	0.5	4.9	0.3	3175	265.5	26.9	1.0
	h/cho	344.0	10.7	65.1	0.9	20.5	0.4	4.2	0.6	3613	263.5	29.7	0.5
	chow	371.5	13.3	60.7	0.3	19.8	0.5	6.8	0.3	4293	87.1	27.7	0.6
18%	h/fat	349.8	14.1	60.4	1.5	18.3	0.6	7.4	0.8	5116	532.4	32.6	0.8
	h/protein	308.3	9.7	62.9	0.7	19.4	0.2	6.0	0.6	3096	239.7	25.3	0.6
	h/cho	350.5	7.3	63.6	0.8	20.0	0.3	5.1	0.6	3839	227.4	29.6	0.5
	chow	394.5	5.5	60.6	0.5	19.4	0.3	6.7	0.6	4530	170.2	27.5	0.6

Table F.8 : Total body compostion of male offspring expressed as percentage of wet weight (mean ± SE)

REFERENCES

Abadie J, Mathew M, Happel M, Kumar S, Prasad A, Dela Houssaye A, Prasad C 'Regulation of dietary fat preference : Establishing a reproducible profile of dietary fat preference in rats' Life Sci 1993; 53 : 131-139

Ahokas RA, Reynolds SL, Wang Y-F, Anderson GD, Lipshitz J 'The effect of carbohydrate overfeeding on vlood pressure in the pregnant, spontaneously hypertensive rat' Am J Obstet Gynecol 1986; 155 : 1113-1118

Allen LH, Zeman FJ 'Influence of increased postnatal food intake on body composition of progeny of protein-deficient rats' J Nutr 1971; 101 : 1311-1318

Allison DB, Paultre F, Heymsfield SB, Pi-Sunyer FX 'Is the intrauterine period really a critical period for the development of adiposity' Int J Obes 1995; 19 : 397-402

Anderson EA 'Insulin and the sympathetic nervous system' Int J Obes 1993 17 (suppl 3) S86-S90

Anderson GH & Johnston JL 'Nutrient control of brain neurotransmitter synthesis and function' Can J Physiol Pharmacol 1983; 61 : 271-281

Anderson GH & Li ETS 'Protein and amino acids in the regulation of quantitative and qualitative aspects of food intake' Int J Obes 1987; 11(suppl 3) : 97-108

Anguita RM, Sigulem DM, Sawaya AL 'Intrauterine food restriction is associated with obesity in young rats' J Nutr 1993; 123 : 1421-1428

Antonov AN 'Children born during the siege of Leningrad in 1942' J Paediatr 1947; 30 : 250-259

Astrup A 'Dietary compostition, substrate balances and body fat in subjects with a predisposition to obesity' Int J Obes 1993; 17 (suppl 3) : S32-S36

Barker DJP 'Progamming of coronary heart disease' BNF Nutr Bull 1992; 17 (suppl 1) : 31-40

Barker DJP 'Fetal origins of coronary heart disease' BMJ 1995; 311 : 171-174

Barker DJP, Bull AR, Osmond C, Simmonds SJ 'Fetal and placental size and risk of hypertension in adult life' BMJ 1990; 301 : 259-262

Barker DJP, Gluckman PD, Robinson JS 'Conference report : Fetal origins of adult disease - Report of the first interational study group, Sydney, 29-30 October 1994' Placenta 1995; 16 : 317-320

Barker DJP, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO 'Relation of birth weight and childhood respiratory infection to adult lung function and chronic obstuctive airways disease' BMJ 1991; 303 : 671-675

Barker DJP, Godfrey KM, Osmond C, Bull A ' The relation of fetal length, ponderal index and head circumference to blood pressure and the risk of hypertension in adult life' Paediatr Perinat Epidemiol 1992; 6 : 35-44

Barker DJP, Hales CN, Fall CHD, Osmond C, Phipps K, Clark PMS 'Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X) : Relation to reduced fetal growth' Diabetologia 1993a; 36 :62-67

Barker DJP, Martyn CN, Osmond C, Hales CN, Fall CHD 'Growth in utero and serum cholesterol concentrations in adult life' BMJ 1993b; 307 : 1524-1527

Barker DJP & Osmond C 'Infant mortality, childhood nutrition and ischaemic heart disease in England and Wales' Lancet 1986; 1 : 1077-1081

Barker DJP, Osmond C, Simmonds SJ, Wield GA 'The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life BMJ 1993c; 306 : 422-426

Barker DJP, Winter PD, Osmond C, Margetts B, Simmonds SJ 'Weight in infancy and death from ischaemic heart disease' Lancet 1989; 2 : 577-580

Beck B, Burlet A, Nicolas JP, Burlet C 'Hypothalamic neuropeptide Y (NPY) in obese Zucker rats : Implications in feeding and sexual behaviors' Physiol & Behav 1990; 47 : 449-453

Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CRW Glucocorticoid exposure in utero : New model for adult hyertension' Lancet 1993; 341 : 339-341

Bergman RN & Ader M 'Pathogenesis of non-insulin-dependent diabetes mellitus' In Diabetes Nestle Nutr Workshop Series 1995; 35 : 99-117

Bjorntorp P 'Regional fat distribution - implications for type II diabetes' Int J Obes 1992; 16 (suppl 4) : S19-S27

Bjorntorp P 'Fatty acids, hyperinsulinemia, and insulin resistance : Which comes first?' Curr Opinion Lipid 1994; 5 : 166-174

Blake HH & Henning SJ 'Control of protein intake in the young rat' Physiol Behav 1986; 38(5) : 607-611

Bligh ME, DeStefano MB, Kramlik SK, Douglass LW, Dubuc P, Castonguay TW 'Adrenal modulation of the enhanced fat intake subsequent to fasting' Physiol & Behav 1990; 48 : 373-381

Bock BC, Kanarek RB, Aprille JR 'Mineral content of the diet alters sucrose-induced obesity in rats' Physiol & Behav 1995; 57 : 659-668

Bonanome A, Visona A, Lusiani L 'Carbohydrate and lipid metabolism in patients with non-insulin-depedent diabetes mellitus : Effects of a low-fat, high-carbohydrate diet vs a diet high in monounsatured fatty acids' Am J Clin Nutr 1991; 54 : 586-590

Boozer CN, Schoenbach G, Atkinson RL 'Dietary fat and adiposity : A dose-response relationship in adult male rats fed isocalorically' Am J Physiol 1995; 268 ; E546-E550

Bouchard C & Perusse L 'Genetics of Obesity' Ann Rev Nutr 1993; 13 : 337-354

Bowman WC & Rand MJ 'Textbook of pharmacology 2nd ed.' Blackwell Scientific 1990 Chapt 13

Bray GA 'The obese patient' Saunders Philadelphia 1976

Bray GA 'Obesity : A disease of nutrient or energy balance?' Nutr Rev 1987; 45 : 33-43

Brenner BM & Chertow GM 'Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury' Am J Kid Dis 1994; 23 : 171-175

Brenner BM, Garcia DL, Anderson S 'Glomeruli and blood pressure : Less of one, more of the other?' Am J Hypertens 1988; 1: 335-347

Brussaard JH, Van Raaij JMa, Stasse-Wolthuis M, Katan MB, Hautvast JGAJ 'Blood pressure and diet in normotensive volunteers : Abscence of an effect of dietary fiber, protein or fat' Am J Clin Nutr 1981; 34 : 2023-2029

Bursztyn P 'Nutrition and blood pressure' Libby 1987

Campbell DM, Hall MH, Barker DJP, Cross J, Shiell AW, Godfery KM 'Diet in pregnancy and the offspring's blood pressure 40 years later' Brit J Obs Gyn 1996; 103 : 273-280

Campbell LV, Marmot PE, Dyer JA, Borkman M, Storlien LH 'The high-monounsaturated fat diet as a practical alternative for NIDDM' Diabetes Care 1994; 17 : 177-182

Caprio S, Hyman LD, Limb C, McCarthy S, Lange R, Sherwin RS, Shulman G, Tamborlane WV 'Central adiposity and its metabolic correlates in obese adolescent girls' Am J Physiol 1995; 269 : E118-E126

Castonguay TW, Hartman WJ, Fitzpatrick EA, Stern JS 'Dietary self-selection and the Zucker rat' J Nutr 1982; 112 : 796-800

Chang S, Graham B, Yakubu F, Lin D, Peters JC, Hill JO 'Metabolic differences between obesity-prone and obesity-resistant rats' Am J Physiol 1990; 259 : R1103-R1110

Chen Y-F, Meng Q-C ' Sexual dimorphism of blood pressure in spontaneously hypertensive rats is androgen dependent' Life Sci 1991; 48 : 85-96

Chow BF & Lee C-J 'Effect of dietary restriction of pregnant rats on body weight gain of the offspring' J Nutr 1964; 82 : 10-18

Christensen K, Vaupel JW, Holm NV, Yashin AI 'Mortality among twins after age 6 : Fetal origins hypothesis versus twin method' BMJ 1995; 310 : 432-436

Cipres G, Urcelay E, Butta N, Ayuso MS, Parrilla R, Martin-Requero A 'Loss of fatty acid control of gluconeogenesis and PDH complex flux in adrenalectomized rats' Am J Physiol 1994; 267 : E528-E536

Coffigny H, Dupouy J-P 'The fetal adrenals of the rat : Correlations between growth, cytology, and hormonal activity, with and without ACTH deficiency Gen Compar Endocrin 1978; 34 : 312-322

Dahri S, Reusens B, Remacle C, Hoet JJ 'Nutritional influences on pancreatic development and potential links with non-insulin dependent diabetes' Proc Nutr Soc 1995; 54 : 345-356

Daly PA & Landsberg L 'Hypertension in obesity and NIDDM : Role of insulin and sympathetic nervous system'Diabetes Care 1991; 14(3) : 240-248

Department of Health 'The health of the nation : A strategy for health in England' HMSO 1992

Desai M, Crowther NJ, Ozanne SE, Lucas A, Hales CN 'Adult glucose and lipid metabolism may be programmed during fetal life' Biochem Soc Transact 1995; 23 : 331-335

Despres J-P 'Lipoprotein metabolism in visceral obesity' Int J Obes 1991; 15 : 45-52

Devenport L, Knehans A, Sundstrom A, Thomas T 'Corticosterone's dual metabolic actions' Life Sci 1989; 45 : 1389-1396

Dietz WH 'Critical periods in childhood for the development of obesity' Am J Clin Nutr 1994; 59 : 955-959

Dornfeld LP, Maxwell MH, Waks AU, Schroth P, Tuck ML 'Obesity and hypertension: longterm effects of weight reduction on blood pressure' Int J Obes 1985; 9 : 381-389

Drewnowski A 'Energy intake and sensory properties of food' Am J Clin Nutr 1995; 62(suppl) : 1081S-1085S

Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR 'Dysfunction of placental glucocorticoid barrier : Link between fetal environment and adult hypertension?' Lancet 1993; 341 : 355-357

Enns MP & Wilson MW 'Prenatal food restriction and subsequent weight gain in male rats' Science 1983; 219 : 1093-1094

Fagerberg B 'Do carbohydrates promote hypertension?' Int J Obes 1981; 5(suppl 1) : 151-155

Fagerberg B, Andersson O, Nilsson U, Hedner T, Isaksson B, Bjorntorp P 'Weight-reducing diets : Role of carbohydrates on sympathetic nervous activity and hypotensive response' Int J Obes 1984; 8 : 237-243

Fall CHD, Barker DJP, Osmond C, Winter PD, Clark PMS, Hales CN 'Relation of infant feeding to adult serun cholesterol concentration and death from ischaemic heart disease' BMJ 1992; 304 : 801-805

Fall CHD, Osmond C, Barker DJP, Clark PMS, Hales CN, Stirling Y, Meade TW 'Fetal and infant growth and cardiovascular risk factors in women' BMJ 1995; 310 : 428-432

Felber J-P, Ferrannini E, Golay A, Meyer HU, Theibaud D, Curchod B, Maeder E, Jequier E, DeFronzo RA 'Role of lipid oxidation in pathogenesis of insulin resistance of obesity and type II diabetes' Diabetes 1987; 36 : 1341-1350

Ferrannini E, Buzzigoli G, Bonadonna R, Giorico MA, Oleggini M, Graziadei L, Pedrinelli R, Brandi L, Bevilacqua S 'Insulin resistance in essential hypertension' N Engl J Med 1987; 317 : 350-357

Flatt JP 'The difference in the storage capacities for carbohydrate and for fat, and its implications in the regulation of body weight' Ann NY Acad Sci 1988 ; : 104-123

Flatt JP 'Body composition, respiratory quotient, and weight maintenance' Am J Clin Nutr 1995; 62 (suppl) : 1107S-1117S

Fletcher JM, Haggarty P, Wahle KWJ, Reeds PJ 'Hormonal studies of young lean and obese Zucker rats' Horm Metabol Res 1986; 18 : 290-295

Frayn KN & Coppack SW 'Insulin resistance, adipose tissue and coronary heart disease' Clin Sci 1992; 82 : 1-8

Friedmann MI 'Control of energy intake by energy metabolism' Am J Clin Nutr 1995; 62 (suppl) : 1096S-1100S

Fuller JH 'The epidemiology of non-insulin-dependent diabetes mellitus' In Diabetes Nestle Nutr Workshop Series 1995; 35 : 91-98

Galef BG 'A contrarian view of the wisdom of the body as it relates to dietary self-selection' Psychol Rev 1991; 92(2) : 218-223

Gambardella P, Greco AM, Sticchi R, D'Aponte D 'Circadian rhythm variations in the adult rat induced by low and high protein diets administered at various stages of development' Chronobiol Int 1990; 7 : 43-49

Ganong WF 'Review of medical physiology 15th ed.' Appleton & Lange 1991 Chapt 19

Garlick PJ, Millward DJ, James WPT, Waterlow JC 'The effect of protein deprivation and starvation on the rate of protein synthesis in tissues of the rat' Biochem Biophys Acta 1975; 414 : 71-84

Garrow DS, James WPT 'Human nutrition and dietetics 9th ed.' Churchill Livingstone 1993 Chapts. 25,32,35,41

Gennser G, Rymark P, Isberg PE 'Low birth weight and risk of high blood pressure in adult hood' BMJ 1988; 296 : 1498-1500

Glore SR & Layman DK 'Loss of tissues in female rats subjected to food restriction during lactation or during both gestation and lactation' J Nutr 1985; 115 : 233-242

Godfrey KM 'Maternal nutrition, fetal growth and adult degenerative disease' BNF Nutr Bull 1992; 17(suppl 1) : 31-40

Godfrey KM, Forrester T, Barker DJP, Jackson AA, Landman JP, Hall JStE, Cox V, Osmond C 'The relation of maternal nutritional status during pregnancy to blood pressure in childhood' Brit J Obs Gyn 1994; 101(5) : 398-403

Godfey KM, Redman CWG, Barker DJP, Osmond C 'The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight' Brit J Obs Gyn 1991; 98 : 886-891

Godfey KM, Robinsion S, Barker DJP, Osmond C, Cox V 'Maternal nutrition in early and late pregnancy in relation to placental and fetal growth' BMJ 1996; 312 : 410-414

Goldberg GR & Prentice AM 'Maternal and fetal determinants of adult diseases' Nutr Rev 1994; 52(6) :191-200

Goldberg RJ, Ellison RC, Hosmer DW Jr, Capper AL, Puleo E 'Effects of alterations in fatty acid intake on the blood pressure of adolescents : The Exeter-Andover Project' Am J Clin Nutr 1992; 56 : 71-76

Goodman MN, Dluz SM, McElaney MA, Belur E, Ruderman NB 'Glucose uptake and insulin sensitivity in rat muscle : Changes during 3-96 weeks of age' Am J Physiol 1983; 244 : E93-E100

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM 'Weight-reducing effects of the plasma protein encoded by the obese gene' Sci 1995; 269 : 543-546

Hales CN, Barker DJP, Clark PMS, Cox LJ, Osmond C, Winter PD 'Fetal and infant growth and impaired glucose tolerance at age 64' BMJ 1991; 303 : 1019-1022

Harris RBS, Martin RJ, Bruch RC 'Dissociation between food intake, diet composition and metabolism in parabiotic partners of obese rats' Am J Physiol 1995; 268 : R874-R883

Hastings-Roberts MM & Zeman FJ 'Effects of protein deficiency, pair-feeding, or diet supplementation on maternal, fetal and placental growth in rats' J Nutr 1977; 107 :973-982

Herrera E, Knopp RH, Freinkel N 'Carbohydrate metabolism in pregnancy : VI Plasma fuels, insulin, liver composition, gluconeogenesis, and nitrogen metabolism during late gestation in the fed and fasted rat' J Clin Invest 1969; 48 : 2260-2272

Hickey MS, Carey JO, Azevedo JL, Houmard JA, Pories WJ, Israel RG, Dohm GL 'Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans' Am J Physiol 1995; 268 : E453-E457

Hill JO, Lin D, Yakubu F, Peters JC 'Development of dietary obesity in rats : Influence of amount and composition of dietary fat' Int J Obes 1992; 16 : 321-333

Hoffmann P, Block HU, Beitz J, Taube C, Forster W 'Comparative study of the blood pressure effects of four different vegetable fats on young spontaneously hypertensive rats' Lipids 1986; 21 : 733-737

Hsueh AM, Agustin CE, Chow BF 'Growth of young rats after differential manipulation of matneral diet' J Nutr 1967; 91 : 195-200

Hulman S, Falkner B, Freyvogel N 'Insulin resistance in the conscious spontaneously hypertensive rat : Euglyemic hyperinsulinemic clamp study' Metabolism 1993; 42 : 14-18

Iacono JM & Dougherty RM 'Effects of polyunsaturated fats on blood pressure' Ann Rev Nutr 1993; 13 : 243-260

Iwase M, Kodama T, Himeno H, Yoshinari M, Tsutsu N, Sadoshima S, Fujishima M 'Effect of aging on glucose tolerance in spontaneously hypertensive rats' Clin Exptl Hypertension 1994; 16 (1) : 647-76

Jackson AA 'How can early diet influence later disease?' BNF Nutr Bull 1992; 17(suppl 1) :23-30

Jeanrenaud B 'An hypothesis on the aetiology of obesity: Dysfunction of the central nervous system as a primary cause' Diabetologia 1985; 28 : 502-513

Jelliffe DB 'The assessment of the nutritional status of the community' WHO Monogr Ser 1966; 33 : 3-271

Johnston BM 'Fetal growth retardation and increased placental weight in the spontaneously hypertensive rat' Reprod Fertil Dev 1995; 7 :

Jones AP & Friedman MI 'Obesity and adipocyte abnormalities in offspring of rats undernourished during pregnancy' Science 1982; 215: 1518-1519

Jones AP, Pothos EN, Rada P, Olster DH, Hoebel BG 'Maternal hormonal manipulations in rats cause obesity and increase madial hypothalamic norepinephrine release in male offspring' Dev Brain Res 1995; 88 : 127-131

Jones AP, Simson EL, Friedman MI 'Gestational undernutrition and the development of obesity in rats' J Nutr 1984; 114 : 1484-1492

Jones CT 'Fetal metabolism and fetal growth' J Reprod Fert 1976; 47 : 189-201

Kahn HS & Williamson DF 'Abdominal obesity and mortality risk among men in nineteenth-century North America' Int J Obes 1994; 18 : 686-691

Kaiyala KJ, Woods SC, Schwartz MW 'New model for the regulation of energy balance and adiposity by the central nervous system' Am J Clin Nutr 1995; 62 (suppl) : 1123S-1134S

Kanarek RB 'Determinants of self-selection in experimental animals' Am J Clin Nutr 1985; 42 : 940-950

Kanarek RB, Aprille JR, Hirsch E, Gualtieri L, Brown C 'Dietary-induced obesity : Relation between diet composition, obesity and brown adipose tissue' Am J Physiol 1987; 253 : R158-R166

Kanarek RB & Marks-Kaufmann R 'Developmental aspects of sucrose -induced obesity in rats' Physiol Behav 1979; 23 : 881-885

Kanarek RB & Orthen-Gambill N 'Differential effects of sucrose, glucose and fructose on the development of carbohydrate-induced obesity in rats' J Nutr 1982; 112 : 1546-1554

Katayama S, Abe M, Kashiwabara H, Kosegawa I, Ishii J 'Evidence against a role of insulin in hypertension in spontaneously hypertensive rats : CS-045 does not lower blood presssure despite improvement of insulin resistance' Hypertension 1994; 23 : 1071-1074

Kim S-H, Mauron J, Gleason R, Wurtman R 'Selection of carbohydrate to protein ratio and correlations with weight gain and body fat in rats allowed three dietary choices' Int J Vit Nutr Res 1991; 61 : 166-179

Kissebah AH 'Insulin resistance in visceral obesity' Int J Obes 1991; 15 : 109-115

Kleinman JC, Donahue RP, Harris MI, Finucane FF, Madano JH, BrockDB 'Mortality among diabetics in national sample' Am J Epidemiol 1988; 128 : 389-401

Koda JE, Fineman M, Rink TJ, Dailey GE, Muchmore DB, Linerall LG 'Amylin concentrations and glucose control' Lancet 1992; 339 : 1179-1180

Kon SK 'LVII The self-selection of food constituents by the rat' Biochem J 1931; 25 : 473-481

Kortner G, Petrova O, Vogt S, Schmidt I 'Sympathetically and nonsympathetically mediated onset of excess fat deposition in Zucker rats' Am J Physiol 1994; 267 : E947-E953

Krieger DR, Landsberg L 'Mechanisms in obesity related hypertension : Role of insulin and catecholamines' Am J Hypertension 1988; 1 : 84-90

Lachall M & Yung CY 'Insulin resistance and hypertension' Mol Cell Biochem 1992; 109 : 119-125

Landman JP & Hall JStE 'Dietary patterns and nutrition in pregnancy in Jamaica' J Trop Paediatr 1989; 35 : 185-190

Landsberg L 'Diet, obesity and hypertension : An hypothesis involving insulin sympathetic nervous system and adaptive thermogenesis' Quart J Med New Series 1986; 61(236) : 1081-1090

Landsburg L 'The sympathoadrenal system, obesity and hypertension : An overview' J Neurosci Methods 1990a; 34 : 179-186

Landsburg L 'Insulin resistance, energy balance and sympathetic nervous system activity' Clin Exptl Hypertension 1990b; 12(5) : 817-830

Landsberg L 'Hyperinsulinemia : Possible role in obesity-induced hypertension' Hypertension 1992a; 19 : I61-I66

Landsberg L 'Obesity and hypertension : Experimental data' J Hypertension 1992b; 10(suppl 7) : S195-S201

Landsberg L 'Diabetes, obesity and hypertension : Role of insulin and the sympathetic nervous system' Cardio Risk Factors 1993; 3(3) : 153-158

Landsberg L 'Pathophysiology of obesity-related hypertension : Role of insulin and the sympathetic nervous system' J Cardio Pharm 1994; 23(suppl1) : S1-S8

Landsburg L & Krieger DR 'Obesity, metabolism and the sympathetic nervous system' Am J Hypertension 1989; 2 : 125S-132S

Landsberg L, Troisi R, Parker D, Young JB, Weiss ST 'Obesity, blood pressure and the sympathetic nervous system' Ann Epidemiol 1991 1(4) : 295-303

Langley SC, Browne RF, Jackson AA 'Altered glucose tolerance in rats exposed to maternal low protein diets in utero' Comp Biochem Physiol 1994a; 109A : 223-229

Langley SC & Jackson AA 'Increased systolic blood pressure in adult rats, induced by fetal exposure to maternal low protein diet' Clin Sci 1993; 86 :217-222

Langley SC, Phillips GJ, Jackson AA 'In utero exposure to maternal low protein diets induces hypertension in weanling rats independently of maternal blood pressure changes' Clin Nutr 1994b; 13 : 239-242

Langley SC, Seakins M, Grimble RF, Jackson AA 'The acute phase response of adult rats is altered by in utero exposure to maternal low protein diets' J Nutr 1994; 124 : 1588-1596

Langley-Evans SC 'Early life origins of hypertension : A rat model' BNF Bull 1994; 19 (suppl) : 69-77

Langley-Evans SC, Clamp AG, Grimble RF, Jackson AA 'Influence of dietary fats upon systolic blood pressure in the rat' Int J Food Sci Nutr 1996; 47 :

Langley-Evans SC, Gardner DS, Jackson AA 'Association of disproportionate growth of fetal rats in late gestation with raised blood pressure in later life' J Reprod Fert 1996a; 106 : 307-312

Langley-Evans SC & Jackson AA 'Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets' Comp Biochem Physiol 1995a; 110A : 223-228

Langley-Evans SC & Jackson AA 'The blood pressure response of normotensive rats and rats with maternal-diet-induced hypertension to diets of differing fatty acid composition' Proc Nutr Soc 1995b; 54 : 138A

Langley-Evans SC, Phillips GJ, Gardner DS, Jackson AA 'Role of glucocorticoids in programming of maternal diet-induced hypertension in the rat' J Nutr Biochem 1996; 7 : 173-178

Lapidus L, Bengtsson C, Larsson B, Pennert K, Rybo E, Sjostrom L 'Distribution of adipose tissue and risk of cardiovascular disease and death : A 12 year follow-up of participants in the population study of women in Gothenburg, Sweden.' BMJ 1984; 289 : 1257-1261

Larsson B 'Obesity, fat distribution and cardiovascular disease' Int J Obes 1991; 15 : 53-57

Larue-Achagiotis C, Martin C, Verger P, Louis-Sylvestre J 'Dietary self-selection vs. complete diet : Body weight gain and meal pattern in rats' Physiol Behav 1992; 51 : 995-999

Laurerio TJ, Bond JP, Ulman EA 'Development and characterization of a purified diet to identify obesity-susceptible and resistant rat populations' J Nutr 1994; 124 : 2172-2178

Law CM, Barker DJP, Osmond C, Fall CHD, Simmonds SJ 'Early growth and abdominal fatness in adult life' J Epidemol Community Health 1992; 46 : 184-186

Law CM, De Swiet M, Osmond C, Fayers PM, Barker DJP, Cruddas AM 'Initiation of hypertension in utero and its amplification throughout life' BMJ 1993; 306 : 24-27

Lee C-J & Chow BF 'Protein metabolismin the offspring of underfed mother rats' J Nutr 1965; 87 : 439-443

Lee C-J & Chow BF 'Metabolism of proteins by progeny of underfed mother rats' J Nutr 1968; 94 : 20-26

Leibowitz SF 'Neurochemical-neuroendocrine systems in the brain controlling macronutrient intake and metabolism' TINS 1992; 15(12) : 491-497

Leibowitz SF, Lucas DJ, Leibowitz KL, Jhanwar YS 'Developmental patterns of macronutrient intake in female and male rats from weaning to maturity' Physiol Behav 1991; 50(6) : 1167-1174

Leon DA, Koupilova I, Lithell HO, Berlund L, Mohsen R, VageroD, Lithell U-B, McKeigue PM 'Failure to realise growth potential in utero and adult obesity in relation to blood pressure in 50 year old Swedish men' BMJ 1996; 312 : 401-406

Leprohon CE & Anderson GH 'Maternal diet affects feeding behaviour of self-selecting weanling rats' Physiol Behav 1980; 24(3) : 553-559

Leprohon CE & Anderson GH 'The effect of maternal diet on weanling serotonin metabolism and protein feeding behaviour of offspring' J Nutr 1992;112(1) : 29-38

Levin BE 'Reduced norepinephrine turnover in organs and brains of obesity-prone rats' Am J Physiol 1995; 268 : R3849-R394

Levin BE & Sullivan AC 'Glucose-induced norepinephrine levels and obesity resistance' Am J Physiol 1987; 253 : R475-R481

Levin BE & Sullivan AC 'Glucose-induced sympathetic activation in obesity-prone and resistant rats' Int J Obes 1989; 13 : 235-246

Levin BE, Triscari J, Hogan S, Sullivan AC 'Resistance to diet-induced obesity : Food intake, pancreatic sympathetic tone and insulin' Am J Physiol 1987; 252 : R471-R478

Levine RS, Hennekens CH, Jesse MJ 'Blood pressure in prospective population based cohort of newborn and infant twins' BMJ 1994; 308 : 298-302

Levy L & Jackson AA 'Modest restriction of dietary protein during pregnancy in the rat : Fetal and placental growth' J Dev Physiol 1993;19(3) : 113-118

Lew EA, Garfinkel L 'Variations in mortality by weight among 750,000 men and women' J Chronic Diseases 1979; 32 : 563-576

Lewis DM, Levi AJ, Brooksby P, Jones JV 'A faster twitch contraction of soleus in the spontaneously hypertensive rat is partly due to changed fibre type composition' Exptl Physiol 1994; 79 : 377-386

Li ETS & Anderson GH 'Meal composition influences subsequent food selection in the young rat' Physiol Behav 1982; 29(5) : 779-783

Lilloja S, Young AA, Culter CL, Ivy JL, Abbott WGH, Zawadzki JK, Yki-Jarvinen H, Christin L, Secomb TW, Bogardus C 'Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man' J Clin Invest 1987; 80 : 415-424

Lissner L, Heitmann BL 'Dietary fat and obesity : Evidence from epidemiology' Euro J Clin Nutr 1995; 49 : 79-90

Lithell HO, McKeigue PM, Berlund L, Mohsen R, Lithell U-B, Leon DA 'Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50-60 years

Lucas A 'Programming by nutrition in man' BNF Nutr Bull 1991; 16 (suppl 1) : 24-28

Lumey CH, Stan GA, Ravelli AC, Stein ZA 'Birth weight, birth cohort and adult weight among women born during the Dutch famine of 1944-45' Am J Epidemiol 1992; 136 : 951

Mackenzie HS & Brenner BM 'Fewer nephrons at birth : A missing link in the etiology of essential hypertension' Am J Kid Dis 1995; 26 : 91-98

Margetts BM, Beilin LJ, Armstrong BK, Rouse IL, Vandongen R 'Blood pressure and dietary polyunsaturated and saturated fats : A controlled trial' Clin Sci 1985; 69 : 165-175

Margetts BM, Rowland MGM, Foord FA, Cruddas AM, Cole TJ, Barker DJP 'The relation of maternal weight to the blood pressures of Gambian children' Int J Epidemiol 1991; 20 : 938-943

Markham R 'A steam distillation apparatus suitable for Micro-Kjeldahl analysis' Biochem J 1942; 36 : 790-791

Mayel-Afshar S & Grimble RF 'Changes in protein turnover during gestation in the foetuses, placentas, liver, muscle and whole body of rats given a low-protein diet' Biochem Biophys Acta 1983; 756 : 182-190

Mayer J 'Causative factors in overnutrition' In II Occurance, causes and prevention of overnutrition : Symposia of the Swedish Nutrition Foundation 1963

McCarthy HD, Pickard C, Speed J, Jackson AA 'Sexual dimorphism of macronutrient selection and regional adipose tissue accumulation following in utero exposure to maternal low protein diet' Proc Nutr Soc 1994; 53(3) : 172A

McDonald H, Stamford S, McCarthy HD 'Acute effect of peripheral interleukin-1K administration on macronutrient selection in the rat' Proc Nutr Soc 1993; 53 : 358A

McLeod KI, Goldrick RB, Whyte HM 'The effect of maternal malnutrition on the progeny in the rat : Studies on nitrogen balance' AJEBAK 1972; 50 : 731-738

Mercer LP, Gustafson JM, Higbee PT, Geno CE, Schweisthal MR, Cole TB 'Control of physiological response in the rat by dietary nutrient concentration' J Nutr 1984; 114 : 144-152

Mercer LP, Watson DF, Ramlet JS 'Control of food intake on the rat by dietary protein concentration' J Nutr 1981; 111 : 1117-1123

Miller DS, Payne PR 'A ballistic bomb calorimeter' Brit J Nutr 1959; 13 : 501-508

Menaker L & Navia JM 'Appetite regulation in the rat under various physiological conditions : The role of dietary protein and calories' J Nutr 1973;103 : 347-352

Modan M, Halkin H, Almog S, Lusky A, Eshkol A, Shitrit MA, Fuch Z, 'Hyperinsulinaemia : A link between hypertension, obesity and glucose tolerance' J Clin Invest 1985; 75 : 809-817

Mondon CE & Reaven GM 'Evidence of abnormalities of insulin metabolism in rats with spontaneous hypertension' Metaoblism 1988; 37 : 303-305

Morley JE, Levine AS, Gosnell BA, Kneip J, Grace M 'Effect of neuropeptide Y on ingestive behaviours in the rat' Am J Physiol 1987; 252 : R599-R609

Morris P & Anderson GH 'The effects of early dietary experience on subsequent protein selection in the rat' Physiol Behav 1986; 36 : 271-276

Morriss FH, Boyd RDH, Mahendran D 'Placental transport' Phys Reprod 2nd ed. 1994; Chapt 51 :813-851

Muaku SM, Underwood LE, Selvais PL, Ketelslegers JM, Maiter D 'Maternal protein restriction early or late in rat pregnancy has differential effects on fetal growth, plasma insulin-like growth factor-I (IGF-I) and liver IGF-I gene expression' Growth Regulation 1995; 5 : 125-132

Mullen BJ, Martin RJ 'Macronutrient selection in rats : Effect of fat type and level' J Nutr 1990; 120 : 1418-1425

Musten B, Peace D, Anderson GH 'Food intake regulation in the weanling rat : Self-selection of protein and energy' J Nutr 1974; 104 : 563-572

Narimiya M, Azhar S, Dolkas CB, Mondon CE, Sims C, Wright DW, Reaven GM 'Insulin resistance in older rats' Am J Physiol 1984; 246 : E397-E404

O'Hare JA, Minaker KL, Meneilly GS, Rowe JW, Pallotta JA, Young JB 'Effect of insulin on plasma norepinephrine and 3,4-dihydroxyphenylalanine in obese men' Metabolism 1989; 38 : 322-329

Osbourne DR, Voogt P 'The analysis of nutrients in foods' Academic Press 1978 Chapts. 3,5

Oscai L, Brown MM, Miller WC 'Effect of dietary fat on food intake, growth and body composition in rats' Growth 1984; 48 : 415-424

Pagliassotti MJ, Knobel SM, Shahrokhi KA, Manzo AM, Hill JO 'Time course of adaptation to a high-fat diet in obesity-resistant and obesity-prone rats' Am J Physiol 1994; 264 : R659-R664

Pan DA, Hulbert AJ, Storlien LH 'Dietary fats, membrane phospholipids and obesity' J Nutr 1994; 124 : 1555-1565

Paneth N, Susser M 'Early origin of coronary heart disease (the 'Barker hypothesis')' BMJ 1995; 310 : 411-412

Parillo M, Rivellese AA, Ciardullo AV 'A high-monounsaturated-fat/low carbohydrate diet improves peripheral insulin sensitivity in non-insulin-dependent diabetes mellitus

Peach H, Heller RF 'Epidemiology of common dieases' Heinmann Medical Books London 1984

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F 'Effects of the obese gene product on body weight regulation in ob/ob mice' Sci 1995; 269 : 540-543

Phillips DIW, Hirst S, Clark PMS, Hales CN, Osmond C 'Fetal growth and insulin secretion in adult life' Diabetologia 1994; 37 : 592-596

Phillips GJ, Langley-Evans SC, Benediktsson R, Seckl JR 'The role of dietary protein restriction during pregnancy on the activity of placental 11ß-hydroxysteroid dehydrogenase' Proc Nutr Soc 1994; 53(3) : 170A

Phipps K, Barker DJP, Hales CN, Fall CHD, Osmond C, Clark PMS 'Fetal growth and impaired glucose tolerance in men and women' Diabetologia 1993; 36 : 225-228

Plato PA, Mondon CE, Reaven GM 'Nutritional status modifies insulin-mediated glucose uptake in spontaneously hypertensive rats' Blood Pressure 1994; 3 : 336-339

Poehlman ET, Gardner AW, Arciero PJ, Goran MI, Calles-Escandon J 'Effects of endurance training on total fat oxidation in elderly persons' J App Physiol 1994; 76 : 2281-2287

Pond WG, Schoknecht PA, Fiorotto ML, Walton L, Tran V, Davis TA, Mersmann HJ 'Differential nutrient partitioning in pregnant rat dams restricted amount of a balanced diet versus restricted nonprotein calories' Nutr Res 1994; 14 : 1067-1076

Pond WG, Strachan DN, Sinha YN, Walker EF jr, Dunn JA, Barnes RH 'Effect of protein deprivation of swine during all or part of gestation on birth weight, postnatal growth rate and nucleic acid content of brain and muscle of progeny' J Nutr 1969; 99 : 61-67

Powley T 'The ventromedial hypothalmic syndrome : Satiety and a cephalic phase hypothesis' Physiol Rev 1977; 84 : 89-126

Prentice AM & Jebb SA 'Obesity in Britain : Gluttony or sloth?' BMJ 1995; 311 : 437-439

Ramirez I 'Diet texture, moisture and strach type in dietary obesity' Physiol Behav 1987; 41 : 149-154

Ravelli G-P, Stein ZA, Susser MW 'Obesity in young men after famine exposure in utero and early infancy' New Engl J Med 1976; 295(7) :349-354

Reaven E, Wright D, Mondon CE, Solomon R, Ho H, Reaven GM 'Effect of age and diet on insulin secretion and insulin action in the rat' Diabetes 1993; 32 : 175-180

Reaven GM 'Role of insulin resistance in human disease' Diabetes 1988; 37: 1595-1607

Rolls BJ & Hammer VA 'Fat, carbohydrate and the regulation of energy intake' Am J Clin Nutr 1995; 62(suppl) : 1086S-1095S

Rona RJ, Gulliford MC, Chinn S 'Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood' BMJ 1993; 306 : 817-820

Rose JL, McCarty R 'Maternal influences on milk intake in SHR and WKY pups' Pysiol Behav 1994; 56 : 901-906

Rose WC ' The nutritive significance of amino acids' Phys Rev 1938; 38 : 109-136

Rosso P 'Nutrition and maternal-fetal exchange' Am J Clin Nutr 1981; 34 :744-755

Saltzmann E & Roberts SB 'The role of energy expenditure in energy regulation : Findings from a decade of research' Nutr Rev 1995; 53 : 209-220

Schoknecht PA, Pond WG, Mersmann HJ, Maurer RR 'Protein restriction during pregnancy affects postnatal growth in swine progeny' J Nutr 1993; 123 : 1818-1825

Schutz Y 'The adjustment of energy expenditure and oxidation to energy intake : The role of carbohydrate and fat balance' Int J Obes 1993; 17(suppl 3) : S23-S27

Sharma NB & Gelsema AJM 'Central nucleus of the amygdala and the development of hypertension in spontaneously hypertensive rats' Am J Pysiol 1995; 268 : R1171-R1177

Sheard NF 'The diabetic diet : Evidence for a new approach' Nutr Rev 1995; 53 : 16-19

Shetty PS, Jung RT, James WPT 'Postprandial thermogenesis in obesity' Clin Sci 1981; 60 : 519-525

Shor-Prosnor G, Brennan G, Ian C, Jasaitis R, Madhu K, Leibowitz SF 'Meal patterns of macronutrient intake in rats with particular dietary preferences' Am J Physiol 1994; 226 : R1395-R1402

Shrader RE, Zeman FJ 'Effect of maternal protein deprivation on morphological and enzymatic development of neonatal rat tissue' J Nutr 1969; 99 : 401-421

Smith SR, Edgar PJ, Pozefsky T, Chetri MK, Prout TE 'Insulin secretion and glucose tolerance in adults with protein-calorie malnutrition' Metab 1975; 24 : 1073-1084

Snoeck A, Remacle C, Reusens B, Hoet JJ 'Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas' Biol Neonate 1990; 57 : 107-118

Sobotka TJ, Cook MP, Brodie RE 'Neonatal malnutrition : Neurochemical, hormonal and behavioral manifestations' Brain Res 1974; 65 : 443-457

Stamler R, Stamler J, Riedlinger WF, Algera G, Roberts RH 'Weight and blood presure : Findings inhypertension sceening of 1 million Americans' Am J Med Assoc 1978; 240 : 1607-1610

Stephan JK, Chow B, Frohman LA, Chow BF 'Relationship of growth hormone to the growth retardation associated with maternal dietary restriction' J Nutr 1971; 101 : 1453-1458

Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW 'Influnce of dietary fat composition on development of insulin resistance in rats : Relationship to muscle triglyceride and n-3 fatty acids in muscle phospholipid' Diabetes 1991; 40 : 280-289

Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG,Pascoe WS, 'Fish oil prevents insulin resistance induced by high-fat feeding in rats' Sci 1987; 237 : 885-888

Susser M, Stein Z Timing in prenatal nutrition : A reprise of the Dutch famine study' Nutr Rev 1994; 52(3) : 84-94

Swislocki A & Tsuzuki A 'Insulin resistance and hypertension : Glucose intolerance, hyperinsulinemia, and elevated free fatty acids in the lean spontaneously hypertensive rat' Am J Med Sci 1993; 306 : 282-286

Takezawa H, Hayasti H, Sano H, Saito H, Ebihara S 'Circadian and estrous cycle-dependent variations in blood pressure and heart rate in female rats' Am J Physiol 1994; 267 : R1250-R1256

Theall CL, Wurtman JJ, Wurtman RJ 'Self-selection and regulation of protein:carbohydrate ration in foods adult rats eat. J Nutr 1984; 114 : 711-718

Tremblay A, Nadeau A, Despres J-P, Bouchard C 'Hyperinsulinemia and regualtion of energy balance' am J Clin Nutr 1995; 61 : 827-830

Tse EO, Gregoire FM, Magrum LJ, Johnson PR, Stern JS 'A low protein diet lowers insulin secretion but does not alter hyperinsulinemia in obese Zucker (fa/fa) rats' J Nutr 1995; 125 : 1923-1929

Vaccarino FJ 'Growth hormone-releasing factor and feeding : Behavioural evidence for direct central actions' Ann NY Acad Sci 1990; 579 :227-232

Vagero D & Leon D 'Ischaemic heart disease and low birth weight : A test of the fetal-origins hypothesis from the Swedish Twin Registry' Lancet 1994; 343 : 260-263

Vander Tuig JG & Romsos DR 'Effects of dietary carbohydrate, fat and protein on norepinephrine turnover in rats' Metabolism 1984; 33 : 26-33

Van Dijk JP, Challis JRG 'Control and ontogeny of hypothalmic-pituitary-adrenal function in the fetal rat' J Dev Physiol 1989; 12 : 1-9

Van Marthens E & Shimomaye SY 'In-utero fetal and placental development following maternal protein repletion in rats' J Nutr 1978; 108 : 959-966

Vettor R, Cusin I, Ganten D, Rohner-Jeanrenaud F, Ferrannini E, Jeanrenaud B 'Insulin resistance and hypertension : Studies in transgenic hypertensive TGR(mREN-2)27 rats' Am J Physiol 1994; 267 : R1503-R1509

Wade GN & Gray JM ' Gonadal effects on food intake and adiposity : A metabolic hypothesis' Physiol & Behav 1979; 22 : 583-593

Weigle DS 'Appetite and the regulation of body composition' FASEB J 1994; 8 : 302-310

Welborn TA, Breckenbridge A, Rubinsten AH, Dollery CT, Fraser TR 'Serum-insulin in essential hypertension and in peripheral vascular disease' Lancet I 1966; 1336-1337

Welle S 'Sympathetic nervous system response to intake' Am J Clin Nutr 1995; 62 (suppl) : 1118S-1122S

Wexler BC 'Inhibition of the pathogenesis of spontaneous hypertension in spontaneously hypertensive rats by feeding a high fat diet' Endocrinology 1981; 108(3) : 981-989

White BD, He B, Dean RG, Martin RJ 'Low protein diets increase neuropeptide Y gene expression in the basomedial hypothalamus of rats' J Nutr 1994; 124 : 1152-1160

Widdowson EM 'Intrauterine growth retardation in the pig : I.Organ size and cellular development at birth and after growth to maturity' Biol Neonate 1971; 19 : 329-340

Widdowson EM & McCance RA 'The effect of finite periods of undernutrition at different ages on the composition and subsequent development of the rat' Proc Roy Soc B 1963; 158 : 329-342

Wilding JHP, Gilbey SG, Bailey CJ 'Increased neuropeptide Y messenger ribonucleic acid (mRNA) and decreased neurotensin mRNA in the hypothalamus of the obese (ob/ob) mouse' Endocrin 1993; 132 : 1939-1944

Williams PT, Fortmann SP, Terry RB, Garay SC, Vranizan KM, Ellsworth N, Wood PD 'Associations of dietary fat, regional adiposity, and blood pressure in men' JAMA 1987; 257 : 3251-3256

Woodger TL, Sirek A, Anderson GH 'Diabetes, dietary tryptophan, and protein intake regulation in weanling rats' Am J Phys 1979; 236(5) : R307-R311

Wright GL, McMurtry JP, Wexler BC 'Food restriction reduces the blood pressure of the spontaneously hypertensive rat' Life Sci 1981; 28 : 1253-1259

Young CM & Rasmussen KM 'Effects of varying degrees of chronic dietary restriction in rat dams on reproductive and lactational performance and body composition in dams and their pups' Am J Clin Nutr 1985; 41 : 979-987

Young JB, Daly PA, Uemura K, Chaouloff F 'Effects of chronic lard feeding on sympathetic nervous system activity in the rat' Am J Physiol 1994; 267 : R1320-R1328

Young JB & Landsberg L 'Effect of oral sucrose on blood pressure in the spontaneously hypertensive rat' Metabolism 1981; 30 : 421-424

Young JB, Mullen D, Landsberg L 'Caloric restriction lowers blood pressure in the spontaneously hypertensive rat' Metabolism 1978; 27 : 1711-1714

Young VR & Marchini JS 'Mechanisms and nutrition siginificance of metabolic responses to altered intake of protein and amino acids, with reference to nutritional adaptation in humans' Am J Clin Nutr 1990; 51 : 270-289

York DA 'Neural activity in hypothalmic and genetic obesity' Proc Nutr Soc 1987; 46 : 105-117

Zamboni M, Armellini F, Cominacini L, Turcato E, Todesco T, Bissoli L, Micciolo R, Bergamo-Andreis IA, Bosello O 'Obesity and regional body-fat distribution in men : Separate and joint relationships to glucose tolerance and plasma lipoproteins' Am J Clin Nutr 1994; 60 : 682-687

Zeman FJ 'Effect of protein deficiency during gestation on postnatal cellular development in the young rat' J Nutr 1969; 100 : 530-538

Zeman FJ & Stanbrough EC 'Effect of maternal protein deficiency on cellular development in the fetal rat' J Nutr 1969; 99 : 274

Zhang Y, Proenka R, Massei M, Barone M, Leopold L, Friedmann JM 'Postional cloning of the mouse obese gene iand its human homologue' Nature 1994; 372 : 425-432

Additional References

Department of Health 'Our Healthier Nation' HMSO 1996

Eriksson JG, Forsen T, Tuomilehto J, Winter PD, Osmond C, Barker DJP 'Catch-up growth in childhood and death from coronary heart disease : longitudinal study' BMJ 1999 ; 318 : 427-431

Langley-Evans SC, Sherman RC, Welham SJM, Nwagwu MO, Gardner DS, Jackson AA 'Intrauterine programming of hypertension : the role of the renin-angiotensin system' Biochem Soc Trans 1999 ; 27 : 88-93

Leon DA 'Fetal growth and adult disease' Euro J Clin Nutr 1998; 52 (suppl 1) : S72-S82

Ravelli ACJ, Van der Meulen JHP, Osmond C, Barker DJP, Bleker OP 'Obesity at the age of 50y in men and women exposed to famine prenatally' Am J Clin Nutr 1999 ; 70 : 811-816

Rees WD, Hay SM, Buchan V, Antipatis C, Palmer RM 'The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply' Br J Nutr 1999 ; 81 : 243-250

Seckl JR, Nyirenda MJ, Walker BR, Chapman KE 'Glucocorticoids and fetal programming' Biochem Soc Trans 1999 ; 27 : 97-103

Symonds ME & Stephenson T 'Maternal nutrition and endocrine programming of fetal adipose tissue development' Biochem Soc Trans 1999 ; 27 : 97-103