Nutritional programming of behaviour in the rat

1.0 Introduction

1.1.1 Obesity – definitions, diagnosis, prevalence, future projections, co-morbidities, causes and treatment.

1.1.2 Definitions and diagnosis of obesity

The World Health Organisation (WHO) has defined obesity as 'abnormal or excessive fat accumulation that may impair health' (WHO, 2011, p1). A recent report from the NHS Information Centre for Health and Social Care stated: 'overweight and obesity are terms that refer to an excess of fat and they usually relate to increased weight for height' (NHS, 2011, p9). Currently in the UK, under the advice of the National Institute for Clinical Excellence, the Body Mass Index (BMI) is the easiest and most commonly used method of determining if a patient is overweight or obese. BMI is calculated by dividing the weight of an individual in kilograms by the square of height in meters (kg/m^2). A BMI over 25 is classified as overweight, over 30 obese, over 35 morbidly obese and over 40 super-morbidly obesity (NHS, 2011). It is worth noting that these cut-offs may be inappropriate for some ethnic groups and are generally not used for children (Flower *et al.,* 2007).

Despite the fact that BMI is effective without the need for expensive specialist equipment, it does not take into account factors such as the abnormal distribution of visceral adipose tissue that may be undetectable by the kg/m^2 calculation (NHS, 2011). It is also worth noting that the BMI calculation cannot distinguish between fat mass and high muscle mass (Frankenfield *et al.,* 2001). Waist circumference has been proven to be useful in measuring visceral

adiposity associated with cardiometabolic health problems associated with obesity (Brown, 2009; Burton, 2010). Patients displaying a waist circumference in excess of 103cm in males and 88cm in females are regarded as having a raised waist circumference and are at increased risk of the multitude of pathologies associated with obesity (Brown, 2009). BMI and waist circumference can be used effectively in conjunction to determine the risk of a patient to serious health problems associated with obesity (Burton, 2010).

1.1.3 UK prevalence of obesity and associated illness

Epidemiological evidence detailing the prevalence of obesity in the UK shows a largely uniform increase over the past two decades with some degree of stabilization in recent years. Recent statistics report that in the adult population (age 16+) of England, 22% of males and 24% of females had a BMI of over 30 in 2009 (NHS, 2011). This was an increase from 15% in 1993, but was virtually the same as 2006 where the prevalence of obesity in both sexes was 24% (NHS, 2008, 2011). Additionally 44% of males and 32% of females were classed as overweight in England in 2009 with 32% of males and 44% of females exhibiting an increased waist circumference, representing a marked increase from 23% since 1993 (NHS, 2011).

The number of people admitted to hospital with a primary diagnosis of obesity in England increased rapidly from 979 in 2000 to 7,988 in 2009 and 10,571 in 2010 (NHS, 2011). A greater number of hospital admissions with a primary clinical diagnosis of obesity were in the 35-44 and 45-54 age groups

(NHS, 2011). The number of people seeking pharmacological therapy for obesity in England is also rising exponentially with 1.45 million prescriptions distributed in 2009 up from 127, 000 in 1993 (NHS, 2011).

Obesity has been demonstrated to be of critical importance in determining risk of serious health problems, along with other lifestyle factors such as smoking, alcohol consumption and lack of exercise. When BMI and waist circumference measurements were both used to determine susceptibility to serious illness in England in 2009, statistics revealed 19% of males were at an enhanced risk, 14% at a high and 20% at a very high risk of developing health problems (NHS, 2011). In females, 14% had an enhanced risk, 18% percent a significant risk and 23% at a very high risk of developing health problems associated with being overweight and obese (NHS, 2011). These statistics were virtually identical to those recorded in 2006 (NHS, 2008). Risks commonly include cardiovascular disease, cancer and a range of metabolic abnormalities such as type-2-diabetes.

Childhood obesity is also a significant issue in the UK and has also been demonstrated to be on the increase. The prevalence of being overweight in young to teenage children (ages 2-15) was 31% in males and 28% in females, with 16% of males and 15% of females being recorded as obese (NHS, 2011). The prevalence of obesity in children aged 2-15 had increased significantly from previous records more than a decade earlier, whereas the prevalence of being overweight remained similar (NHS, 2011). In reception class children of 4-5 years of age, 13.3% were classed as overweight and 9% obese, with

20% of children being overweight and 14.6% being obese in year 6 at 10-11 years of age (NHS, 2011). In 2009 in Scotland, just fewer than 30% of children had a BMI outside the healthy range with 31% in boys and 28% in girls (Scottish Health Survey, 2009).

1.1.4 Projected increases in the UK prevalence of obesity

Projected increases in the prevalence of obesity in the UK in years to come present an increasingly grim picture for the future, predicting that by 2035 47% of males and 36% of females in England will be obese (Foresight, 2007). According to the same group, by 2050 obesity has been predicted to rise to as high as 60% in males 50% in females. Even more short term projections to adult obesity present a considerable increase with 36% in males and 28% in females being classed as obese by 2015 (Foresight, 2007). If such projections are correct, by today's prices the cost of obesity for the NHS could reach around £9.7 billion by 2050 with an extra cost of around £50 billion to account for the wider expense to society.

1.1.5 Global trends in obesity

The high and ever increasing prevalence of obesity is not just a problem localised to the UK. Sharp increases in prevalence of obesity in the UK in recent years are consistent with trends observed in other industrialised Western countries such as European nations and the US. In the US levels of obesity in the adult population stood at 35% in females and 32% in males in 2008 and are projected to rise to over 40% by 2015 (Wang & Beydoun, 2007; Yanovski & Yanovski, 2011). European countries such as Germany, Poland

and the Czech Republic also have similar rates of obesity compared to the UK (European Commission, 2010). The prevalence of obesity is also increasing in countries with fast growing economies such as China (Wang *et al*., 2007; Shen *et al.,* 2012), India (Garg *et al.,* 2010; Goyal *et al.,* 2010) and Brazil (Oliveira *et al.,* 1996; Seki *et al.,* 2009) where in parallel to development and the creation of wealth, nutritional habits are changing with chronic overnutrition becoming more common.

1.1.6 Clinical obesity and associated co-morbidities

Obesity has been demonstrated to be an important risk factor for a substantial number of disorders and diseases, across numerous fields of medicine including cardiology, oncology, endocrinology, reproductive medicine, neurology, psychiatry and rheumatology (WHO, 1999). Of particular importance is the role of obesity in the development of serious life threatening illnesses including diabetes (Greenway, 1999; Eid, 2011), cardiovascular disease (Mathew *et al.,* 2008; Berg & Bonner, 2005), renal disease (Rutkowski *et al.,* 2006; Kopple & Feroze, 2011) and cancer (Fair & Montgomery, 2009; Harvey *et al.,* 2011).

Commonly associated with obesity is a group of disorders referred to collectively as the metabolic syndrome, the presence of which can significantly increase the likelihood of the development of cardiovascular disorders and type-2-diabetes (Kassi *et al.,* 2011; Barth, 2011). According to the diagnostic criteria outlined by the WHO, a patient presenting with one of either type of diabetes, impaired glucose tolerance or either impaired fasting

glucose and insulin resistance, in combination with two of either hypertension, dyslipidaemia, central visceral adiposity and microalbuminuria meets the criteria for diagnosis of the metabolic syndrome (WHO, 1999). According to the International Diabetes Federation (IDF) the presence of an increased waist circumference in combination with two of either elevated serum triglycerides, attenuated high density lipoprotein cholesterol, hypertension or enhanced plasma glucose, meets the criterion for diagnosis of the metabolic syndrome (IDF, 2006). Type-2 insulin dependent diabetes mellitus is a disease synonymous with obesity and chronic overnutrition which consists of enhanced plasma serum concentrations of glucose in the presence of insulin resistance and insulin deficiency. Of the numerous serious diseases associated with obesity, type-2 diabetes has the strongest association (Diabetes UK, 2010).

According to the WHO, the diagnostic criterion for type-2 diabetes is either significantly elevated fasting plasma serum glucose concentrations, or significantly elevated plasma serum glucose concentrations 2-hours after the administration of oral glucose via a glucose tolerance test (WHO, 2006). Measures of fasting glucose levels and glucose tolerance indicative of an impairment are often observed in conjunction with symptoms such as increased hunger (polyphagia), thirst (polydipsia), unrination (polyuria), chronic fatigue and sustained weight loss (Cooke & Plotnick, 2008). The prevalence of diabetes in England is currently 5.1% and is projected to almost double by 2025 (Diabetes UK, 2010).

1.1.7 Genes vs. environment

Although research has shown that obesity has a strong genetic component, the contribution of the environment cannot be ignored when considering the universal availability and affordability of energy rich food in modern industrialised societies. In such circumstances an obesogenic diet combined with sedentary lifestyle can be crucial in driving the increase in the prevalence of obesity (Du & Feskens, 2010; Chaput *et al*., 2011). Obesity is a characteristic feature of several genetic diseases such as Prader-Willi syndrome, Cohen syndrome, MOMO syndrome and Bardet-Biedl syndrome, although such conditions are rare and account for only a tiny percentage of obesity in the general population (Goldstone & Beales, 2008; Jin *et al.,* 2011; Herrera *et al*., 2011; Rooryck & Lacombe, 2008).

Numerous polymorphisms to genes responsible for appetite related physiology and metabolism are known to constitutionally predispose an individual to weight gain, which may lead to obesity in conditions where food supply is more than adequate (Jafar-Mohammadi & McCarthy, 2008). For example, single nucleotide polymorphisms in the region of the FTO gene (fat mass and obesity associated gene) have been associated with adiposity, elevated BMI and metabolic abnormalities (Fawcett & Barroso, 2010; Larder *et al.,* 2011). In societies where food stocks are plentiful and energy dense and food items are ubiquitous and affordable, genes which may have functioned in the past to prevent starvation in nutritionally austere times are ill-equipped to cope with intake and thus lead to overweight and obesity

(Champion *et al.,* 2010). An extreme example of such an instance has been seen in Pima Indians, a tribe of Native Americans who conventionally farmed the semi-arid desserts of South East Arizona. When exposed to the obesogenic dietary conditions of the modern day US, levels of obesity, type-2 diabetes, cardiovascular disease and renal failure increased rapidly in Pima Indians compared to other ethnic groups (Ravussin, 1993; Goran, 2000; Lemley, 2008; Jimenez-Corona *et al.,* 2006). Despite obesity and its causes being of major scientific interest, the magnitude of the current obesity epidemic suggests much is still to be elucidated.

1.1.8 Treatment of obesity

The range of treatments available for obesity is vast and span varying dietary regimens, to pharmacological treatments and in extreme circumstances surgical interventions, all with varying degrees of effectiveness. Dietary interventions have been demonstrated to lead to some short-term success in inducing weight-loss but require self-discipline and commitment and do not always work long-term (Wooley & Garner, 1991; Clifton, 2008; Clifton *et al.,* 2009). Pharmacological interventions acting on the CNS have had some limited success but rarely induce sustained weight-loss. Drug treatments are often associated with a myriad of side effects that can be unpleasant for the user (Halford *et al.,* 2010). Drugs such fenfluramine and sibutramine have been withdrawn from the market in many countries due to the combination of the potential to cause adverse cardiovascular complications and lack of effectiveness (Blanck *et al.,* 2004; Williams, 2010; Garrow, 2010; Cheung,

2011). In addition to fenfluramine and sibutramine, the cannabinoid receptor antagonist rimonabant has also recently been withdrawn due to the occurance adverse side effects including negative affect and enhanced suicidality (Derosa & Maffioli, 2012; Kang & Park, 2012).

Surgical interventions such as bariatric surgery, usually consisting of gastric bypass surgery or gastric banding, can be effective at inducing sustained weight-loss but can be prone to serious complications and of all the treatment options are the most expensive (Mitchell *et al.,* 2009; Hong *et al.,* 2011). Psychological treatments such as cognitive behavioural therapy have also been demonstrated to be effective but are also expensive (Melchionda *et al.,* 2003; Teufel *et al.,* 2011). Systematic reviews have shown that treatment of obesity early on in childhood can be critical in reversing long-lasting complications associated with obesity (Parsons *et al.,* 1999; Lloyd *et al.,* 2010; 2012). Despite the extensive range of treatments available, the fact that the prevalence of obesity continues to rise and the effect of treatment overall appears to be limited presents a dire picture for the future. Despite decades of research investigating the science behind obesity and its consequences many aspects of the effects of obesity and hyperenergetic diet remain to be elucidated in great detail.

1.2 Obesity and Behaviour

1.2.1 Diet, obesity and behaviour in humans

As discussed above, obesity is synonymous with the development of metabolic abnormalities and disease that can be severely detrimental to the

quality of life of the affected individual. Although the harmful effects of obesity upon a wide range of physiological systems are well documented, the effects of chronic overnutrition and obesity on behaviour are still far from clear. There are many ambiguities regarding the contribution of nutritional status toward behaviour. Research examining the effects of obesity and/ or hyperenergetic diet upon behaviour in humans does appear to show some notable effects, however studies are often biased and flawed making the overall picture ambiguous. The majority of studies undertaken have involved children, adolescents, the elderly and groups vulnerable to behavioural disorders (Schmidt *et al.,* 1997; Stevenson *et al.,* 2006; Lamport *et al.,* 2009; Paile-Hyvarinen*et al.,* 2009; Hassenstab *et al.,* 2010; Bruehl *et al.,* 2010).

The 'Avon Longitudinal Study of Parents and Children' (ALSPAC) conducted in Avon in the UK has provided some interesting, if somewhat inconclusive findings regarding the effects of high fat and energy dense food items upon behaviour in children. One study examining data from 4000 children from the ALSPAC cohort explored whether consumption of junk food in children aged 4 was related to numerous measures of behaviour aged 7, scored by parents (Wiles *et al.,* 2009). Consumption of high fat energy dense food items at 4 years of age was related to increases in measures of hyperactivity at 7 years of age, with no effect upon measures of conduct and peer problems, emotional problems and pro-social behaviour. This association remained after adjusting for confounding variables such as IQ. A study drawing 12,942 children from the same cohort examined associations between a measure of consumption of high fat energy dense food items at age 6 and behaviour 16 months later.

Results demonstrated associations between intake and measures indicative of dysfunction in measures of pro-social behaviour and total difficulties (Peacock *et al.,* 2011).

The question of whether hyperenergetic diet can influence behaviour has never been a more salient issue than when considering the question of the role of diet in the exacerbation of behavioural disorders in children, adolescents and other vulnerable groups. Such disorders include attention deficit hyperactivity disorder (ADHD), conduct disorder, autism and Asperger's syndrome (Schmidt *et al.,* 1997; Stevenson *et al.,* 2006). Children and adolescents whose dietary pattern consisted of more food items high in fat and sugar were more likely to receive a diagnosis of ADHD than subjects who adhered to a dietary pattern which was deemed to be 'healthy' (Howard *et al.,* 2011). Multiple studies have demonstrated associations between diet and ADHD in children and adults (Blunden *et al.,* 2011; Kim & Chang, 2011). Looking at a community based cohort of 1,663 individuals in Germany aged between eighteen and sixty-four using self-report measures, de Zwaan *et al.,* (2011) reported that a higher percentage of obese individuals scored highly on psychometric measures of ADHD compared to overweight individuals and people of ideal weight. The associations remained after adjusting data for scores on measures of anxiety and depression. Similarly Kim *et al.,* (2011) reported that children of both sexes with ADHD had an increased risk of obesity if un-medicated and were less likely to engage in organised sports and physical activity. There has been speculation that additives, refined sugars or food allergy/sensitivity may exacerbate the expression of challenging

behaviours associated with ADHD, raising the question of whether the elimination of foods containing products such as artificial food colouring would be beneficial to ADHD sufferers (Schnoll *et al.,* 2003; Stevens *et al.,* 2011).

According to Lamport *et al.,* (2009), who conducted a comprehensive review of studies investigating the relationship between glucose tolerance and cognitive function, studies investigating the administration of glucose prior to testing on performance upon cognitive measures found both young and older participants with poor glucose tolerance exhibited deficits on measures of memory using immediate and delayed recall memory tests (Allen *et al.,* 1996; Kaplan *et al.,* 2000; Messier *et al.,* 1997).

Research has revealed that sufferers of type-2 diabetes often perform worse on psychometric measures of memory, learning and attention compared to age matched controls (Paile-Hyvarinen *et al.,* 2009; Hassenstab *et al.,* 2010; Bruehl *et al.,* 2010) and that diabetes and Alzheimer's disease may share some common features and pathologies (Haan, 2006; Liu *et al.,* 2011; Park, 2011). A number of studies have demonstrated cognitive impairments in sufferers of type-2-diabetes and individuals with metabolic abnormalities, suggesting impairments may occur in middle age in parallel to the onset of the metabolic syndrome even prior to the onset of diabetes. Hassenstab *et al.,* (2010) reported that of five measures of the metabolic syndrome, only insulin resistance predicted lower performance on measures of recall, learning and

executive function in non-diabetic middle aged sufferers of the metabolic syndrome.

Bruehl *et al.,* (2010) demonstrated that in non-diabetic, middle aged sufferers of the metabolic syndrome, participants with higher insulin resistance performed worse than participants with lower insulin resistance upon psychometric measures of executive function and declarative memory. Paile-Hyvarinen *et al.,* (2009) demonstrated deficits in performance on measures of working and episodic memory, as well as visual attention in suffers of type-2 diabetes compared to individuals with normal glucose tolerance. Multiple studies have been published demonstrating associations between type-2 diabetes and the development of Alzheimer's disease, reporting that diabetes may be a key risk factor for the development of dementia. The two disorders may have related pathologies and effective management of diabetes may be advantageous in delaying the onset of dementia (Craft, 2007; Neumann *et al.,* 2008; Salacz & Csibri, 2011; Akter *et al.,* 2011; Bosco *et al.,* 2011). It is clear from this raft of evidence from clinical and epidemiological research, that cognitive impairments are commonly associated with obesity related pathologies.

Obesity and the consumption of energy dense food items has often been associated with negative emotional states such as feelings of depression, anxiety and lack of control (Luppino *et al.,* 2010; Vogelzangs *et al.,* 2010; Faith *et al.,* 2011; Pervanidou & Chrousos, 2011). Despite these associations there are obvious ambiguities regarding the exact nature of the relationship

between obesity and mood. Studies have reported associations between obesity, binge eating disorder and feelings such as lack of control, shame, anger, guilt and low self-esteem in both males and females (Fandino *et al.,* 2010; Munsch & Herpertz, 2011; Zeeck *et al.,* 2010; Albohn-Kuhne & Rief, 2011). Elevated scores on measures more indicative of psychopathology, including assays of severe depression, paranoia, psychoticism, obsessive-compulsivity and interpersonal sensitivity, have been detected in obese women also suffering from binge eating disorder (Fandino *et al.,* 2010). One prime example of when mood is often associated with diet is the notion of 'comfort food', referring to the consumption of hyper-calorific food items which may temporarily enhance comfort, pleasure and well-being, but consumption of which, in combination with sedentary lifestyles characterized by little exercise, can be harmful and lead to obesity (Chaput *et al*., 2011).

Interestingly, it is worth noting that in many instances glucose has been demonstrated to enhance performance on many psychometric measures of learning and memory in humans. This effect has been shown to be tied into factors such as age, the ability to respond to glucose and the type of psychometric assessment used (McNay & Gold, 2002; Gold, 2005; Morris *et al.,* 2010). Glucose has been demonstrated to significantly enhance performance on psychometric measures in elderly subjects and sufferers of Alzheimer's disease and Down syndrome, with it originally being assumed that this was not the case with younger participants (Hoyer, 1991; Gold, 1995; Manning *et al.,* 1998; Korol & Gold, 1998; Gold, 2005).

Despite this assumption, it has been shown that glucose administration can enhance performance on psychometric measures in young participants (late teens early 20s), however the task utilized had to be of a certain level of difficulty. Glucose administration increased performance in young participants upon more complex versions of measures previously used such as immediate and delayed recall tasks of narrative prose, verbal working memory tests, measures of attention, word and face recognition tasks (Benton & Sargent 1992; Benton & Owens, 1993; Korol & Gold, 1998; Gold, 2005). Also demonstrated to be of importance is the individual's ability to respond to glucose. Participants who took longer to clear glucose from circulation have been demonstrated to benefit the most from glucose administration, measured by performance on verbal measures of declarative memory such as remembering passages of narrative prose (Craft *et al.,* 1994; Curwin *et al.,* 1995, Korol & Gold, 1998). Despite this effect seen in both humans and animals, studies demonstrating the effects of chronic global overnutrition in rodents show generally detrimental effects upon cognitive processes involved in the mediation of a wide range of behaviours.

1.2.2 Diet, obesity and behaviour in rats

Due to the lack of information regarding the relationship between obesity and hyperenergetic diet in determining behaviour in humans, research involving rodents has been useful in providing insights into the precise nature of the relationship between overnutrition and behaviour. Several studies have been published demonstrating the consequences of chronic overnutrition and

obesity upon behaviours related to emotional state, ingestive behaviour and behaviours relating to learning and memory (Souza *et al.,* 2007; Abildgaard *et al.,* 2011; La Fleur *et al.,* 2007; Wincour *et al.,* 2005).

1.2.2.1 Emotional Behaviour

Several studies have been published demonstrating associations between obesity and/or exposure to hyperenergetic diet and emotional behaviour in rodents. Souza *et al.,* (2007) reported that when tested in a light-dark exploration task, Wistar rats which had been exposed to a highly palatable hyperenergetic diet enriched with sucrose for 4 months, spent significantly less time in a lightened chamber compared to offspring fed a nutritionally balanced control chow. Since rats are naturally nocturnal and dislike bright light spending less time in the lightened chamber has been purported to represent greater anxiety. Animals exposed to the obesogenic diet also had increased fat mass, alterations to glucose and insulin metabolism and enhanced levels of protein degradation in the frontal cortex, but not in the hippocampus compared to controls. Using operant conditioning procedures combined with a behavioural measure of anxiety, Alsiö, *et al.,* (2009b) demonstrated in Wistar rats that an enhanced motivation to press a lever for 95% sucrose pellets was related to more time spent upon the aversive open arms of the elevated plus maze, which was indicative of reduced anxiety. It was also demonstrated that a preference for a high fat diet was also associated with an anxiolytic behavioural profile on the elevated plus maze in male rats (Alsiö *et al.,* 2009a).

In Sprague-Dawley rats, behavioural profiles indicative of anxiety and depression in adulthood induced by early maternal separation were ameliorated by exposure to an obesogenic cafeteria diet (Maniam & Morris, 2010). Hilakivi-Clarke *et al.,* (1996) reported that exposure of Sprague-Dawley rats to a diet high in polyunsaturated fats increased the duration of time, and reduced the latency of time to engage in aggressive behaviour in response to the introduction of an intruder to the home cage, compared to rats sustained upon a low fat diet.

As well as behaviours associated with anxiety and aggression, one study reported that a high fat diet could increase the occurrence of behaviours sensitive to amelioration with anti-depressant drugs in animals prone to depression. Using a genetic rodent model of depression known as the Flinders Sensitive Line, when rats sensitive to depression were compared to depression resistant animals, Abildgaard *et al.,* (2011) demonstrated that exposure to a high fat diet increased the propensity of sensitive animals to engage in behaviours known to be ameliorated by anti-depressants upon a forced swim test. The forced swim test is often used as a behavioural measure to investigate the behavioural effects of anti-depressant drugs in rodents.

The forced swim test conventionally consists of a rat being placed within a water filled container for a short period of time to be given the chance to swim (Porsolt *et al.,* 1977; 1978; 2001). Immobility (when the rat stops swimming) has been reported to be sensitive to the administration of anti-

depressant drugs (Porsolt, 1977; 1978; Shimazoe *et al.,* 1987; Porsolt *et al.,* 2001). Drugs which reduce the frequency and duration of immobility in the forced swim test have been purported to have anti-depressant properties (Porsolt *et al.,* 1977; 1978; Nomura *et al.,* 1982). High fat feeding was also associated with deficits upon measures of object recognition memory. It is worth noting that in this instance behavioural changes were not accompanied by significant increases in weight gain.

1.2.2.2 Ingestive behaviour

Destruction of the ventromedial nucleus of the hypothalamus (VMH) in rats has been demonstrated to cause hyperphagia (overeating) and severe obesity (Smith, 1927; Hetherington, 1941; Hetherington & Ranson, 1942; Brobeck et *al.,* 1943; Albert *et al.,* 1971; Anand & Brobeck, 1951). Originally this led researchers to assume the VMH was the key 'satiety centre' of the brain. Further research ascertained that in early studies rudimentary lesioning techniques may have destroyed tracts passing through the VMH (Gold *et al.,* 1972; King, 2006). Sprague-Dawley rats subjected to lesions of the VMH have been demonstrated to show lower locomotor activity levels and possess several abnormalities to appetite related behaviour (Jen, 1980). Food consumption in rats subjected to VMH lesions is highly sensitive to adulteration with quinine and animals are less motivated to respond for food on operant measures of food self-administration and also have the propensity to exhibit an enhanced reaction to mild electric shock when responding for food (Levine & Soliday, 1960; Ferguson & Kessey; Grossman, 1966; 1972;

Porter & Allen, 1977 cited in Jen, 1980). Sclafani and Springer (1976) reported that in a similar manner to VMH lesioned animals, female CFE rats made obese by exposure to a range of highly palatable human food items showed no desire to consume food made bitter by the adulterant quinine and showed no desire to respond for food upon operant measures of food self-administration. Also similarly to VMH lesioned rats, they displayed no increase in activity when food deprived and after periods of food deprivation gained lost weight at a slower rate.

Conversely Jen (1980) reported that obese male Sprague-Dawley rats exposed to a high fat diet did not exhibit any of the behavioural characteristics of rats rendered obese through ablation of the VMH. A later study confirmed obese female rats exhibited abnormalities in operant responding for food similar to VMH animals (Jen *et al.* 1981). La Fleur *et al.,* (2007) reported that Wistar rats subjected to chronic exposure to an obesogenic diet showed an enhanced motivation to consume sucrose rich pellets in operant studies of self-administration.

1.2.2.3 Learning and memory

Evidence from human studies reporting associations between obesity, overnutrition and cognition and behaviour are compelling but do little to elucidate the exact nature of the relationship (Lamport *et al.,* 2009). Studies using rodent models of learning and memory have been useful in shedding light upon the effects of chronic overnutrition on multiple types of memory function in rats. The Morris water maze originally pioneered by Richard Morris

in 1982 is a rodent memory test in which a rat is placed within a tank of water and must remember the location of a hidden escape platform located under the waterline. This test has been widely used as a measure of the effects of genetic, physiological or pharmacological manipulations upon spatial memory (Morris *et al.,* 1982). Molteni *et al.,* (2002) demonstrated that Sprague-Dawley rats fed a diet high in fat and sugar took significantly longer to find the escape platform when tested on a water maze, compared to control animals. This deficit in spatial memory was observed in conjunction with attenuated levels of proteins known to mediate memory function such a hippocampal expression of brain derived neurotrophic factor (BDNF) and mRNA levels of signalling pathways associated with BDNF.

Similarly Goldbart *et al.,* (2006) found that feeding Sprague-Dawley rats a high fat refined carbohydrate diet exacerbated deficits in performance upon the Morris water maze induced by intermittent hypoxia. Stranahan *et al.,* (2008) demonstrated that rats fed a high fat/glucose diet in conjunction with fructose rich corn syrup for eight months, took significantly longer to find the hidden escape platform in the Morris water maze. This was noted in conjunction with numerous metabolic abnormalities indicative of diabetes. Additionally Jurdak *et al.,* (2008) reported that Long-Evans rats subjected to conditions of sucrose induced obesity, exhibiting metabolic impairments, had impaired performance on the Morris water maze, compared to controls and rats fed a high fat diet. Such changes were observed in combination with alterations to neurochemistry and the structural morphology of the hippocampus.

Other measures of spatial memory in rats include the radial the arm maze in which the rat must learn to remember the location of sweet food pellets hidden within a multi-armed maze (Olton & Samuelson, 1976) and similarly the radial arm water maze where the rat must learn to remember the location of the escape platform hidden within a multi-armed maze (Buresova *et al.,* 1985). Granholm *et al*., (2008) reported that when tested upon a water radial arm maze, Fischer 334 rats that had been fed a high fat carbohydrate rich diet were more likely to make errors when trying to locate the escape platform. Errors included repeatedly entering an arm of the maze where the escape platform was either not present or had previously been removed earlier in the session. Deficits in performance were indicative of severe impairments to spatial memory. Kanoski and Davidson (2010) reported that in Sprague-Dawley rats trained to find sucrose pellets in an eight arm radial maze, animals that had been exposed to a high fat diet for 72-hours made significantly more errors, including entering non-baited arms or entering arms which had already been cleared of bait during that trial.

Alternatively the novel object discrimination procedure has been used to measure recognition memory in rodents. In this procedure a rat is given the opportunity to learn to detect the difference between familiar and novel objects (Ennaceur & Delacour, 1988). Jurdak and Kanarek (2009) demonstrated, using a novel object discrimination procedure to measure recognition memory, that male Long-Evans rats exposed to conditions of sucrose induced obesity exhibited a deficit in object recognition after a 1-hour interval, compared to controls.

Overnutrition has also been demonstrated to impair performance upon operant measures of learning and memory such as the variable interval delay alternation task. The variable interval delayed alternation (VIDA) task is an operant conditioning procedure used to measure rule-learning memory in rats (Winocur, 1985; 1986; Winocur & Greenwood, 1990). A rat is placed within a box which contains a pellet dispenser operated by a lever than can be automatically retracted by the experimenter. The settings of the box can be automatically set so a lever press dispenses a pellet, dispenses no pellet or the lever is retracted entirely. After a shaping period in which the animal learns that a press of the lever will result in a reward, a twenty second trial in which each press of the lever is rewarded by a sweet food pellet is followed by a twenty second trial in which a lever press gives no reward. During testing after two reward trials followed two no-reward trials a variable time interval (varying from zero, five, ten, twenty, forty or eighty seconds) occurs when the response lever is withdrawn from the box. After the interval the lever is lowered to signify the beginning of a reward trial. A measure of memory performance can be calculated by dividing the average latency of first response during the reward trial by the average latency of first response during the no reward trial. Rats will conventionally learn the alternation rule and learn that the reward trial precedes the no reward trial and that no response is required.

Greenwood and Winocur (1990) reported that after two and half months exposure to a lard based diet rich in fat saturated fat, lard fed Long-Evans rats exhibited impaired performance upon a VIDA task, compared to controls.

According to Winocur and Greenwood (1999), Long-Evans rats fed high fat diets consisting of beef tallow and soybean oil, exhibited impairments in a VIDA task compared to rats fed standard laboratory chow. This effect was unaltered by the level of home-cage enrichment (enriched vs. impoverished) received by each animal. Also Winocur *et al.,* (2005) reported that obese Zucker rats performed less effectively in the VIDA task compared to lean Zucker rats. Deficits in learning and memory were accompanied by alterations to the function of insulin sensitive glucose transporters in the hippocampus in obese animals compared to controls.

The conclusion of the majority of studies investigating the effects hyperenergetic diet upon behavioural measures of learning and memory in rodents is that exposure to overnutrition leads to sustained impairments to performance upon numerous measures of learning and memory. Such deficits in performance are often accompanied in conjunction with the effects of obesity and metabolic abnormalities indicative of the metabolic syndrome and type-2-diabetes.

1.3. Fetal programming and the developmental origins of adult disease

1.3.1 Introduction

Research has shown that the gestation and neonatal periods may act as critical windows when the long-term development of the fetus, neonate or young infant can be permanently altered by adverse environmental conditions. This may potentially 'programme' physiological function and risk of disease in adulthood. Programming has been defined by McMillen and

Robinson (2005) as, “the induction, deletion, or impaired development of a permanent somatic structure, or the ‘setting’ of a physiological system by an early stimulus or insult operating at a ‘sensitive’ period, resulting in long-term consequences for function”. The concept of programming was originally theorised by the neuroendocrinologist Gunter Dörner who speculated that perinatal or neonatal nutrition could contribute to the development of obesity, diabetes, arteriosclerosis and neuroendocrine abnormalities (Dorner & Mohnike, 1973; Dorner, 1973a; 1973b; Dorner *et al.,* 1973). Programming is an essential component of the ‘developmental origins of adult disease’ hypothesis which was proposed by David Barker at the University of Southampton in the late 1980’s.

Epidemiological literature examining historical records, documented an association between the number of ‘early life risk factors’ recorded from hospital birth records and the prevalence of non-communicable diseases in the same cohort many years later in adulthood (Barker & Bagby, 2005; Calkins & Devaskar, 2011). Associations have been documented between low birth-weight and cardiovascular disease, facets of the metabolic syndrome, type-2-diabetes and chronic respiratory infection (Barker *et al.,*1989; Hales, *et al.,* 1991; Barker, 1991a; 1991b; 1991c; Barker & Martyn, 1992).

Programming is often associated with effects of less than optimal quality of maternal environment during pregnancy upon fetal development, but also can be associated with maternal and direct health and wellbeing during the early neonatal period (Meaney, 2001; Plagemann & Harder, 2005). Research

from animal models has demonstrated that perinatal programming insults can include nutritional factors (maternal obesity, maternal exposure to hypo- or hypercaloric diet, maternal diet deficient in essential micro or macronutrients) and other maternal factors such as smoking, psychological stress, and obstetric complications resulting in conditions such as placental insufficiency or hypoxia (Langley-Evans & McMullen, 2010). Particular attention has been afforded to the relationship between maternal nutritional constraints during pregnancy and the development of obesity and the metabolic syndrome, cardiovascular disease, renal and liver disorders (Langley-Evans, 2006). Interestingly, reviews of studies examining new born babies have demonstrated that breastfeeding may have a protective effect against childhood obesity compared to infants fed formula milk, highlighting the fact that the early postnatal period is also a period of sensitivity to programming insults (Arenz *et al.,* 2004; Ryan, 2007; Cope & Allison, 2008).

It has been proposed that early critical periods of development act as 'windows' of susceptibility during which the ontogenesis of a physiological system can be permanently altered, resulting in lasting implications that potentially predispose to disease in later life. During such periods organ systems may be susceptible to developmental plasticity in the form of molecular and cellular changes resulting in degradation of structural integrity and permanently compromised functional capacity of that particular system (McMillen & Robinson, 2005). Researchers have speculated as to reasons underlying such changes in response. In the event of maternal exposure to a nutritionally sub-optimal environment, fetal cellular and molecular

adaptations may represent an attempt to optimise the success of the pregnancy at the expense of the longitudinal structural or functional integrity of an organ system (McMillen & Robinson, 2005; Armitage *et al.,* 2004). Alterations in later physiological function may occur when a discrepancy or a 'mismatch' exists between the responses set during pregnancy in an attempt to reduce the likelihood of miscarriage and the requirements of the post-natal environment (Langley-Evans & McMullen, 2010). Also even if the event of miscarriage is unlikely, perturbations to maternal nutrient supply may trigger an adaptive response set during pregnancy which attempts to predict postnatal nutrient supply. This may be at odds with the prevailing environment actually encountered in reality, resulting in maladaptation (McMillan & Robinson, 2005).

1.3.2 Epidemiological evidence for programming

The foundation of the developmental origins of adult disease hypothesis lies within human epidemiological literature documenting instances in which, through war or catastrophe, women have been subjected to severe nutritional constraints during pregnancy. Circumstances in recent history in which populations accustomed to adequate nutrition have been subjected famine and severe malnourishment have included war time occurrences such as the Dutch 'hunger winter', the siege of Leningrad and other instances of catastrophe such as the great Chinese famine (Roseboom *et al.,* 2011; Stanner & Yudkin, 2001; Li *et al. ,*2011). Socio-demographic research undertaken upon babies whose gestation period fell within the Dutch winter famine during the

2nd world war has provided researchers with a rare insight into the effects of severe maternal undernutrition upon offspring in a western population conventionally accustomed to adequate nutrition (Hart, 1993; Roseboom *et al.,* 2011). During the winter of 1944-45 in the Nazi occupied Netherlands, a complete blockade of food and fuel combined with an extraordinarily severe winter saw the civilian population approach the brink of starvation. As the situation worsened vulnerable groups including pregnant mothers received supplemented rations. However, by the end of winter 1945 severe hunger was universal with daily calorie consumption reaching as low as 400 calories a day in the worst affected areas (Hart, 1993).

Studies documenting metabolic abnormalities and disease in offspring of women subjected to severe undernutrition during the Dutch famine have provided valuable insights into the consequences of maternal dietary perturbations during critical periods of development, experienced many years later in adulthood (Roseboom *et al.,* 2011). Offspring exposed to severe maternal undernutrition during the Dutch winter famine possessed a significantly greater chance of developing obesity (Roseboom *et al.,* 2006), facets of the metabolic syndrome (De Rooij *et al.,* 2007), type-2-diabetes (De Rooij *et al.,* 2006a; 2006b; Roseboom *et al.,* 2006), coronary heart disease (Roseboom *et al.,* 2000; 2001) and renal disease (Painter *et al.,* 2005a; Roseboom *et al.,* 2006) in adulthood. The majority of alterations were attributable to undernutrition during early and mid gestation. Additionally offspring subjected to maternal undernutrition throughout early gestation during the Dutch winter famine possessed a greater susceptibility to develop

psychiatric illness including schizophrenia (Susser *et al.,* 1996; Hoek *et al.,* 1998) and depression (Brown *et al.,* 2000; Stein, *et al.,* 2009), as well as to engage in addictive behaviours (Franzek *et al.* ,2008). Interestingly, Lussana *et al.,* (2008) demonstrated that adults subjected to famine during early gestation had an enhanced propensity to consume a diet high in fat and were less likely to engage in exercise.

A key feature of the developmental origins of adult disease hypothesis, as demonstrated by epidemiological literature from the Dutch feminine, is the importance of the timing of exposure. Early gestational exposure to famine was associated with cardiovascular disease (Roseboom *et al.,* 2000; 2001), schizophrenia in males (Roseboom *et al.,* 2011) and breast cancer in females (Roseboom *et al.,* 2006), whereas blood and respiratory disorders were associated with exposure during mid gestation (Roseboom *et al.,* 2006). Coronary heart disease and stress induced hypertension were also associated with undernutrition during early gestation (Painter *et al.,* 2006b; 2006c). Impairments to glucose tolerance have been associated with undernutrition throughout the entire gestation period (Roseboom *et al.,* 2006).

Numerous studies examining populations of people born in various regions of England in the early-to-mid 20th century have reported associations between low birth weight and the prevalence of metabolic abnormalities and disease later in adulthood (Barker & Bagby, 2005). Data from a cohort of over 5000 men born in Hertfordshire in the UK from 1911-1930 revealed that babies with the lowest birth weight and lower bodyweights during early infancy were

more likely to succumb to cardiovascular disease many years later (Barker, 1991b; Barker & Martyn, 1992). In men from the same cohort lower birth weight and lower weight during early infancy was associated with fatal chronic obstructive airways disease during adulthood (Baker, 1991b). A similar relationship was observed between low birth weights and fatal ischemic heart disease (Barker *et al.,* 1989).

Despite offering compelling evidence in support of the developmental origins of adult disease hypothesis, human epidemiological literature detailing associations between early life events and disease in adulthood has received criticism for a number of reasons. A major problem has been cited to be the large number of confounding variables which may also influence the development of disease in adulthood in the cohorts of human participants studied. Issues include inadequate control for confounding variables, problems with the replication of findings and cohort selection (Langley-Evans, 2006). In an attempt to explore the relationship between early life events and disease and abnormality in adulthood in greater detail, scientists have used numerous animal models to elucidate the potential mechanism. Despite maternal undernutrition initially being of primary interest to researchers looking to add flesh to the bones of the fetal origins of adult disease hypothesis, the opposite trend has also emerged as an equally salient issue due to the fact that obesity in women of child bearing age is becoming ever more common.

1.3.3 Prevalence of obesity in women of child bearing age in the UK

Epidemiological research has demonstrated that, as with levels of obesity in the general population, the prevalence of obesity in women of childbearing age (16-44) is also rising sharply, increasing from 14.4% in 1997 to 20.2% in 2008 in England (HSE, 2008). Age has also been reported to be an important factor when considering the likelihood of maternal obesity, with older women being more likely to be obese when pregnant compared to younger women (HSE, 2008). Due to the fact that levels of obesity in women of child-bearing age are rising, combined with sharp increases in the number of women who reach obesity at any point during pregnancy, important questions arise as to the consequences of such a phenomenon, not only for the mother but the long-term development of the infant.

1.3.4 Maternal dietary perturbations and programming in rodents

Epidemiological evidence described above reporting the high and ever increasing prevalence of obesity in women of child-bearing age presents an alarming picture for the future and underlines the importance of the fetal origins of adult disease hypothesis. Despite its compelling nature, evidence detailing associations between maternal nutritional perturbations and adult disease in humans, such as that from the Dutch winter famine or the Hertfordshire cohort, do little to provide comprehensive insights into the mechanisms underlying such programming effects. In order to address this issue animal models have been used by researchers to investigate potential mechanisms underlying fetal programming effects including a variety of

murine, ovine, porcine and recently non-human primate species (McMillan & Robinson, 2005; Sullivan *et al.,* 2010; 2011).

Several studies have demonstrated that rats exposed to maternal undernutrition during pregnancy and the early neonatal period may go on to develop obesity, hypertension, the metabolic syndrome, cardiovascular disease and type-2 diabetes as adults (Langley-Evans *et al.,* 1996a; 1996b; 1998; Gardner *et al.,* 1998; Ozanne & Hales *et al.,* 1998). Abnormalities include increased bodyweight and adipose tissue mass, as well as raised systolic blood pressure, hyperinsulinaemia and hyperleptinaemia (Vickers *et al.,* 2001; 2005; 2007; 2008). Maternal exposure to low protein diet has also been show to alter or impair glucose tolerance and insulin resistance in adult offspring (Langley *et al.,* 1994; Petry *et al.,* 2000). Offspring exposed to maternal undernourishment exhibited a greater sensitivity to the obesity inducing effects of high caloric diet as adults despite being smaller at birth (Vickers *et al.,* 2000). As well as hypertension, diabetes and the metabolic syndrome, maternal undernutrition has also been demonstrated to induce lasting alterations to aspects of cardiovascular (Elmes *et al.,* 2007; 2008) and renal function (Langley-Evans *et al.,* 1999a; 1999b; 2003; McMullen *et al.,* 2004; Cornock *et al.,* 2010).

Similarly, Desai *et al.,* (1997a) reported that male offspring subjected to maternal protein restriction were hypersensitive to the effects of a high caloric diet in adulthood, exhibiting enhanced serum concentrations of insulin. Wilson and Hughes (1997) demonstrated that adult offspring subjected to

maternal undernutrition during pregnancy and lactation exhibited impairments to glucose tolerance and pancreatic islet function compared to controls. However, such programming effects were dependent upon the presence or absence of direct postnatal nutritional manipulations, such as exposure to a high fat diet or a sucrose supplement in conjunction to maternal undernutrition. Vickers (2001) reported underlying hypertension, hyperinsulinaemia, hyperleptinaemia and obesity in offspring subjected to maternal undernutrition and direct exposure in adulthood to high caloric diet were results of multiple impairments to the adipoinsular axis.

Interestingly Erhuma *et al.,* (2007) reported that in rats maternally exposed to a low protein diet during early, middle and late pregnancy exhibited signs of insulin resistance and hypertriglyceridemia at eighteen months of age but showed no difference to controls at one or nine months of age. Such observations suggest age may be important in bringing out the insulin resistant phenotype and other metabolic abnormalities in rats subjected maternal undernutrition. Such findings appear to validate the assertions made by epidemiological studies in humans demonstrating associations between maternal nutrient restriction during the Dutch famine and diabetes and the metabolic syndrome in adulthood, as well as Barker's associations between low birth-weight and adult disease.

Due to the high and ever increasing prevalence of obesity in women of child-bearing age in Western cultures, the animal literature has begun to focus upon manipulations that permit examination of the consequences of maternal

obesity and overnutrition during early critical periods of development for adult offspring (Langley-Evans, 2006; Langley-Evans & McMullen, 2010). Interestingly the effects of maternal overnutrition during pregnancy and lactation are remarkably similar to the consequences of maternal undernutrition during the same periods. Although there is still speculation regarding the exact nature of the mechanisms underlying such effects, It appears metabolic abnormalities, type-2 diabetes and other disorders can also be programmed by maternal overnutrition in a similar manner to programming induced by maternal undernutrition.

For example, using mice Samuelsson *et al.,* (2008) reported that offspring of mothers exposed to an obesogenic diet prior to conception and during pregnancy and lactation exhibited enhanced adiposity, showed signs of insulin resistance, hypertension and were hyperphagic at numerous points during adulthood. Maternal overnutrition increased triglyceride, cholesterol, glucose, insulin and leptin in offspring compared to offspring of mothers exposed to a nutritionally balanced control chow.

White *et al.,* (2009) either made female rats obese by exposure to a high fat diet prior to conception, fed dams a nutritionally balanced control chow, or pair-fed dams the same number of calories as the control diet using the high fat diet. Exposure to all diets was continued throughout pregnancy and lactation. Post-lactation offspring were weaned onto the chow diet and then at eight weeks of age animals were either transferred to the high fat diet or continued on chow. Offspring of obese dams weighed significantly more than

offspring of dams fed the control chow, or the pair fed dams at eighteen months of age, with offspring fed the high fat diet directly weighing significantly greater that those fed the control chow. Interestingly, offspring of obese dams who were directly fed the low fat chow from eight weeks of age weighed virtually the same as offspring of dams fed the control chow but directly fed the high fat diet from eight weeks of age. Such findings suggested that maternal obesity has an independent but similar effect upon offspring adiposity to that of direct exposure to the obesogenic diet and that the effect upon offspring was attributable to maternal obesity and not the consumption of dietary fat (as represented by the pair fed group).

In a similar study Shankar *et al.,* (2010) induced maternal obesity in rat dams prior to pregnancy by exposure to a hypercaloric liquid diet infused via intragastrically implanted canuli. At birth offspring of obese dams were cross-fostered to dams fed a nutritionally balanced control chow that had given birth on the same day. Offspring subjected to maternal obesity were insulin resistant and had elevated plasma serum concentrations of insulin, leptin and triglycerides. Such findings also suggest that maternal obesity during pregnancy, induced prior to conception is associated with programming of insulin resistance and metabolic abnormalities in adult offspring. Studies examining the consequences of maternal high-fat diet prior to conception and during pregnancy and lactation demonstrated alterations to the expression of multiple genes related to hepatic adipose metabolism, fetal development and the insulin axis (Zhang *et al.,* 2004; Zhang *et al.,* 2009).

Bayol *et al.,* (2008) exposed dams to a hyperenergetic cafeteria diet during pregnancy and lactation and then weaned offspring onto a nutritionally optimal control chow diet until early adulthood. Adult offspring subjected to maternal overnutrition during pregnancy and lactation were obese and exhibited greater adipose tissue mass and plasma serum concentrations of triglycerides, cholesterol, leptin and insulin compared to offspring fed chow during gestation and lactation but exposed to the experimental diet post-lactation. Offspring fed the hyperenergetic diet during gestation and lactation exhibited greater expression of genes related to regulation of the insulin axis, vasculogenesis, angiogenesis, lipoprotein regulation, glucose transportation, glucose metabolism and fatty acid storage, compared to control offspring. Changes were more pronounced in female offspring.

Benkalfat *et al.,* (2011) exposed dams to either a control chow or a cafeteria diet prior to conception, as well as during pregnancy and lactation and then either fed offspring the experimental diet or the control chow post-lactation. Similarly to the findings described above, weanling and adult offspring subjected to maternal overnutrition exhibited enhanced plasma serum concentrations of insulin, leptin, glucose and adiponectin as well as being obese. Post-lactational exposure to cafeteria diet exacerbated these deficits. Akyol *et al.,* (2011) fed dams on an obesogenic cafeteria diet prior to mating, during pregnancy and lactation, as well as providing the cafeteria diet directly to offspring during the post-weaning period, to examine obesity related parameters and glucose tolerance in offspring at 13 weeks of age. Hyperenergetic diet during all three periods of development impaired glucose

tolerance in the offspring, although any impairment was dependent upon post-lactational cafeteria diet feeding. In contrast to previous studies, diet during the three maternal feeding periods had no effect upon bodyweight, adipose tissue mass and plasma lipid concentrations in the offspring.

1.3.5 Possible mechanisms underlying programming

Several mechanisms have been reported to underlie nutritional programming effects, most notably materno-fetal endocrine exchanges, epigenetic alterations and changes to fetal gene expression (Langley-Evans, 2006). Tissue remodelling may occur when perturbations to maternal nutrition or other developmental insults may alter tissue morphology or the number or type of functional units of a physiological system such as cardiomyocytes in the cardiovascular system, β-cells of the pancreas and nephrons in the renal system (Langley-Evans, 2007; McMillan & Robinson, 2005). Recent studies have reported changes to the structural morphology of the hypothalamic neural circuitry known to mediate appetite in offspring induced by programming insults (Plagemann *et al.,* 1999a; 1999b; 2000a; 2000b; Davidowa & Plagemann, 2003). The prospect that maternal dietary perturbations may alter the structural morphology of the developing hypothalamus and that such changes may contribute to the programming of metabolic abnormalities could be of critical importance understanding the mechanisms underlying programming of appetite, behaviour and body composition.

The placental exchange of hormones between the mother and the fetus during pregnancy has been reported to potentially contribute to the programming of developmental abnormalities in offspring (Langley-Evans, 2007). In particular, maternal exposure to glucocorticoids has received interest from researchers. Glucocorticoids are steroid hormones secreted by the adrenal gland in response to the activation of the hypothalamic-pituitary-adrenal axis (HPA axis) and are known to cross the placental barrier and promote the premature maturation of tissues (Langley-Evans *et al.,* 1996c; 1996d; Langley-Evans, 1997a). Maternal overexposure of the developing fetus to glucocorticoids has been postulated to mediate programming effects of many physiological systems (Langley-Evans *et al.,* 1996c; 1996d; Langley-Evans, 1997a).

To preserve the integrity of the HPA axis of the developing fetus, a placental gradient of glucocorticoid concentration is maintained to protect the fetus from the potentially harmful effects of the elevated maternal glucocorticoid circulation. The gatekeeper enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD2) maintains this gradient between the mother and fetus by inactivating glucocorticoids when passing through the placenta (Langley-Evans *et al.,* 1996c; 1996d). Not only has maternal undernutrition been reported to down-regulate 11β-HSD2 (Langley-Evans *et al.* 1996a), maternal protein restriction has been reported to impair growth (Langley-Evans & Nwagwu, 1998) programme hypertension (Gardner *et al.,* 1997; Langley-Evans, 1997b; McMullen & Langley-Evans, 2005a), programme alterations to renal function (McMullen & Langley-Evans, 2005b) and programme alterations to hepatic

function (Erhuma *et al.,* 2009) in offspring, through glucocorticoid-related processes.

Epigenetic alterations to gene expression have also been postulated to underlie metabolic changes induced by nutritional programming manipulations. Histone acetylation or methylation and DNA methylation have been demonstrated to regulate gene expression and have been shown to be reliant on the number of methyl donors present in diet such as S-adenosyl-methionine (SAM, Burdge *et al.,* 2007; Langley-Evans, 2006; Lillycrop, 2011). DNA methylation is dependent upon the availability of SAM derived from the methionine-homocysteine pathway. Maternal undernutrition via protein restriction has been suggested to provide the developing fetus with excess quantities of methionine and potentially perturb this methionine-homocysteine pathway, resulting in hypomethylation of DNA and over-expression of key genes (Langley-Evans, 2006; Lillycrop & Burdge, 2010). However, little evidence of gross perturbation of these pathways was noted by Langley-Evans *et al*., (2006) after low protein diet. Maternal environmental effects upon the epigenome have mostly been reported to occur during fetal development, but there are also demonstrations of effects during the neonatal period (Weaver *et al.,* 2004a; 2004b; Caldji *et al.,* 2011). An extensive body of literature has been accumulated demonstrating changes to intracellular signalling pathways consistent with DNA methylation in response to environmental factors in early life, such as the quality of maternal care during the suckling period (Meaney & Szyf, 2005; Zhang & Meaney, 2010a; 2010b; Caldji *et al.,* 2011).

One other mechanism which has been postulated to underlie the programming effects of maternal dietary perturbations is direct changes to gene expression. Nutritional manipulations have been reported to alter gene expression in a number of systems, for example maternal undernutrition has been demonstrated to alter the expression of genes related to kidney function which may in turn contribute to hypertension (McMullen & Langley-Evans, 2005a; McMullen & Langley-Evans, 2005b; Cornock *et al.,* 2010). However, there is ambiguity regarding the nature of the relationship between alterations to gene expression and metabolic abnormalities, as it is unclear whether changes to gene expression are a cause or an effect of metabolic changes themselves, tissue remodelling or epigenetic changes (Langley-Evans, 2006).

1.4. Programming brain neurochemistry in rodents

The prospect that maternal nutritional manipulations could programme appetite related neural circuitry in the developing hypothalamus of the fetus or neonate, through whatever mechanism, could account for the metabolic abnormalities seen in offspring as a consequence of such changes. Potentially such changes could involve all, or combinations, of the mechanisms mentioned above. A number of studies have been published demonstrating that brain neurochemistry in weanling and adult offspring is susceptible to programming manipulations through perturbations to maternal diet or neonatal feeding conditions (Plagemann *et al.,* 2000a; 2000b; Davidowa & Plagemann, 2000; Davidowa *et al.,* 2002).

The hypothalamic neural circuitry involved in energy homeostasis involves a wide range of neurotransmitters, neuropeptides, gut-brain peptides and hormones, many of which are potential targets for programming manipulations (Plagemann *et al.,* 2000a; 2000b; Davidowa *et al.,* 2002; Langley-Evans, 2006). It has been proposed that such changes may be the consequences of morphological cytoarchitectural alterations to neurons in hypothalamic substructures, such as alterations to the numerical density of neurons, the numerical density of dendritic spines and the expression of numerous receptors (Plagemann *et al.,* 2000b; Davidowa & Plagemann, 2003; Gorski *et al.,* 2007).

Studies documenting the consequences of perturbations to maternal diet upon hypothalamic neurochemistry in weanling and adult offspring have demonstrated numerous alterations in the function of neurotransmitter, neuropeptide and hormone systems involved in the mediation of energy homeostasis. Maternal exposure to a low protein diet during pregnancy and lactation increased concentrations of the orexigenic neuropeptide neuropeptide-Y (NPY) in the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA), but not the ventromedial nucleus (VMH) in weanling offspring compared to controls (Plagemann *et al.,* 2000a). Low protein-exposed offspring were both underweight and hyperleptinaemic compared to controls. Weanling offspring of dams subjected to maternal undernutrition during pregnancy and lactation exhibited distinct malformations to the VMH and PVN of the hypothalamus, with no changes observed in the dorsomedial hypothalamus (DMH), the arcuate nucleus (ARC) and LHA compared to

controls (Plagemann *et al.,* 2000b). The numerical density of neurons within the VMH and the PVN was enhanced in low protein offspring which also had lower numerical density of neurons responsive to NPY in the ARC.

The programming of brain neurochemistry in offspring, induced by maternal nutritional manipulations during early critical periods of development, has not been demonstrated exclusively by models of maternal undernutrition. Neonatal overnutrition induced by reducing litter size and increasing neonatal access to milk has also been demonstrated to programme brain neurochemistry in weaning and adult offspring (Plagemann, *et al.,* 1999a; 1999b; Davidowa & Plagemann, 2000; Davidowa *et al.,* 2002). Conventionally, animals raised in small litters during the lactation period exhibit hyperphagia, obesity and metabolic abnormalities for the rest of their lives, compared to offspring raised in litters of normal size (Plagemann, *et al.,* 1999a; 1999b; Davidowa & Plagemann, 2000; Zippel *et al.,* 2001; Davidowa *et al.,* 2002). Adult offspring subjected to gestational diabetes displayed an increased numerical density of NPY neurons in the arcuate nucleus (ARC) of the hypothalamus in conjunction to being obese, hyperphagic, hyperinsulinaemic, overweight and having impaired glucose tolerance (Plagemann *et al.,* 1999a).

Changes to the activity of neurons extracted from brain slices of the VMH on exposure to the orexigenic endogenous melanocortin antagonist agouti-related peptide (AgRP) and the biogenic amine neurotransmitter dopamine, were detected in rats subjected to neonatal overnutrition compared to controls (Davidowa *et al.,* 2002; Li *et al.,* 2002). Neurons from brain slices

extracted from the PVN of adult offspring subjected to neonatal overnutrition and obesity exhibited an inhibited response to orexigenic neuropeptides AgRP, NPY and melanin-concentrating hormone (MCH), compared to neurons from control offspring (Davidowa & Plagemann, 2003). Inhibition was also present after exposure to anorectic neuropeptides such as cocaine and amphetamine regulated transcript (CART) and α-melanocyte-stimulating hormone (α-MSH), although to a much lesser degree. Such observations suggest that obesity and metabolic impairments induced by neonatal overnutrition may be, in part, underpinned by alterations to both orexigenic and anorectic systems within multiple hypothalamic sub-regions.

It has been suggested by Plagemann and colleagues that neonatal overnutrition via reductions in litter size may programme impairments to the sensitivity of hypothalamic nuclei to peripheral factors implicated in mediating energy homeostasis, such as the hormone and tonic satiety factor leptin and the anorectic brain gut peptide cholecystokinin (CCK). For example, neonatal overnutrition induced by reducing litter size attenuated leptin induced inhibition of the firing rate of neurons in slices of the ARC in juvenile rats (Davidowa & Plagemann, 2000). Zippel *et al.,* (2003) observed that within the LHA, the number of neurons inhibited by exposure to CCK, in isolation or combination with dopamine, as well as overall responsiveness was greater in rats subjected to neonatal overnutrition compared to rats raised in large litters. A lower firing rate and greater change of basal firing rate was observed in response to dopamine exposure in neurons extracted from the LHA in offspring subjected to neonatal overnutrition, compared to rats from

large litters. Zippel *et al.*, (2001) reported that weanling rats subjected to both neonatal over- and undernutrition exhibited altered neuronal responsiveness in the LHA to the administration of CCK compared to controls. Offspring subjected to neonatal overnutrition displayed an excitatory response to CCK exposure, with the opposite response noted in offspring subjected to neonatal undernutrition. Such findings suggest perturbations to neonatal nutrition via changes to litter size altered the sensitivity of hypothalamic nuclei to peripheral signals of overnutrition.

A number of authors have proposed that 'metabolic imprinting' may occur, which may programme appetite related neural circuitry in individuals genetically predisposed to obesity during pregnancy, or the neonatal period leading to metabolic abnormalities and disease in later life (Levin, 2000a; 2000b; 2006; Muhlhausler *et al.,* 2006; Muhlhausler & Ong, 2011). Research has also shown that the effects of maternal nutritional programming manipulations upon offspring brain neurochemistry can be tied in to constitutional susceptibility to the obesogenic properties of hypercaloric experimental diets in both mothers and offspring. Levin and Govek (1998) compared offspring of rat dams sensitive to the obesogenic effects of hypercaloric diet to animals that were resistant to such effects and added a further two groups by feeding a subset of both groups a hyper-obesogenic liquid diet. Offspring of dams sensitive to the obesogenic effects of the diet exhibited greater adiposity and plasma serum leptin concentrations, with offspring of obesity-sensitive dams additionally fed the liquid diet showing the greatest increase compared to offspring of dams resistant to the effects of

hypercaloric diet. Offspring of mothers subjected to conditions of maternal obesity during pregnancy and lactation and then direct diet-induced obesity in adulthood exhibited enlargements of both the VMH and DMH and exhibited reduced binding of the noradrenaline (NA) transporter in the PVN, VMH, the ARC and the amygdala (Levin & Dunn-Meynell, 2002). Offspring of mothers who were originally obesity resistant but rendered obese by a hypercaloric liquid diet, had increased 5-HT transporter binding in the VMH, the DMH, the PVN, the ARC and the LHA, as well as enhanced PVN and reduced VMH NA transporter binding as adults (Levin & Dunn-Meynell, 2002).

Unlike control offspring, adult offspring subjected to maternal protein restriction during the early neonatal period were not susceptible to the anorectic properties of the selective 5-hydroxytryptamine (5-HT) reuptake inhibitor citalopram (Barreto Medeiros *et al.,* 2002). Data suggests that maternal protein restriction can programme numerous alterations to the hypothalamic neural circuitry implicated in the mediation of energy homeostasis. Using a similar feeding protocol Gorski *et al.,* (2007) reported offspring of dams subjected to dietary induced obesity exhibited enhanced expression of leptin receptor mRNA in the ARC of the hypothalamus.

In summary, it would appear that neonatal overnutrition may impair the sensitivity of both anorectic and orexigenic neural systems involved in the mediation of energy homeostasis. This may, in part, underlie the long-term obesogenic and metabolic effects seen in offspring. There may also be alterations to actions of peripheral hormones or brain gut peptides known to

affect energy homeostasis within the hypothalamus, in offspring subjected to alterations in neonatal nutrition. Many of the systems that have been observed to be responsive to the early nutritional environment are known to mediate behaviours associated with energy homeostasis and appetite. It is likely that if neurochemistry is altered in such a manner, maternal nutritional manipulations during early critical periods of development may also programme behaviour associated with such functions.

Although the effects of nutritional programming manipulations have focused primarily upon changes to hypothalamic systems known to mediate appetite regulation, it is worth noting that such systems mediate a wide a range of other behaviours. For example the monoamine 5-HT is also known to mediate emotional behaviours including aggression (Olivier *et al.,* 1995; Popova, 2008), anxiety (Handley & McBlane, 1993; Graeff *et al.,* 1996) and depression (Graeff *et al.,* 1996; Luki, 1998), as well as learning and memory (McEntee & Crook, 1991; Palmer & DeKosky, 1993, Buhot *et al.,* 2000) and neuroendocrine function across a vast array of brain regions (Lucki*,* 1998). The monoamine DA is also known to mediate reward, reinforcement (Di Chiara, 2002; Ikemoto, 2007; Wise, 2008), learning and memory (Palmer & DeKosky, 1993; Dalley & Everitt, 2009), and executive function (Barnes *et al.,* 2011). When considering the wide range of brain systems altered by nutritional programming manipulations in rodents, the range of behaviours potentially altered by such perturbations could be great in magnitude.

1.5 Programming induced by changes to maternal behaviour

Environmental manipulations during the early neonatal period, such as maternal separation and handling of the mothers, has been shown to induce changes to mother-pup interactions and in turn alter the function of the brain stress systems in the offspring (Smythe, *et al.,* 1996; Francis & Meaney, 1999). It has been reported that in lactating rodents, separating the mother from the pups reduced the propensity of the mothers to engage in behaviours such as licking and grooming of the pups and arched back nursing (LG-ABN) (Francis & Meaney, 1999; Champagne & Meaney 2006). Conversely, repeated handling of the mothers had the opposite effect (Francis & Meaney, 1999; Champagne & Meaney 2006). Arched back nursing allows pups greater access to suckle for maternal milk and, as with maternal licking and grooming, has been shown to reduce the responsiveness of brain stress systems (Francis & Meaney, 1999), and monoamine neurotransmitter systems (Vicentic *et al.,*2006; Arborelius & Eklund, 2007).

It has been reported that during lactation, decreased levels of LG-ABN induced by maternal separation was associated with neurochemical signs of stress in adult offspring, such as enhanced expression of corticotrophin releasing factor (CRF) mRNA within the PVN and the central nucleus of the amygdala, as well as increased expression of markers of CRF related neuronal activity within the locus coeruleus (LC) (Meaney, *et al.,* 1996; Frances & Meaney, 1999). This was observed in conjunction to increased CRF receptor binding in the LC and raphe nuclei, reduced glucocorticoid receptor mRNA expression within the hippocampus and the PVN, reduced $GABA^{\alpha}$ receptor activity in all the regions examined and reduced glucocorticoid mediated

inhibition of CRF (Meaney, *et al.,* 1996; Frances & Meaney, 1999). The exact opposite appears to be true for postnatal handling, with the exception of changes to glucocorticoid receptor mRNA within the PVN (Frances & Meaney, 1999). Changes to brain neurochemistry and the responsiveness of brain stress systems has been shown to be accompanied by alterations to behaviours relating to stress, fearfulness and self-defence in adult offspring (Menard *et al.,* 2004; Cameron *et al.,* 2005; Zhang *et al.* 2004; 2005; 2006). In addition to programmed changes to adiposity, metabolism, and brain neurochemistry, a small number of studies have been published demonstrating that maternal dietary perturbations (primarily through undernutrition) can programme behaviour in offspring.

1.6 Nutritional programming of behaviour in the rat

When considering reports of programmed changes to brain neurochemistry, the idea that programming may extend to offspring behaviour is compelling and is an obvious area for future investigation. Few studies to date have been published examining whether the programming effects of maternal overnutrition during early critical periods of development extend to offspring behaviour. Mirroring human epidemiological literature, most experimental manipulations employed in animal studies examining the possibility of behavioural programming have consisted of models of maternal undernutrition (Almeida *et al.,* 1993; 1996b; Fakuda *et al.,* 2002; Cordoba *et al.,* 1994; Levay *et al.,* 2008; Orozco-Solis *et al.,* 2009). Along with studies demonstrating associations between direct nutritional perturbations and

changes to behaviour in rodents, the effects of maternal dietary perturbations upon behaviour in adult offspring in rodents are displayed in Table 1.1.

1.6.1 Anxiety and Exploratory behaviour

A small number of studies have been published examining the consequences of maternal dietary manipulations upon anxiety and exploratory behaviour in adult offspring, with mixed results. Maternal protein restriction during pregnancy or lactation enhanced exploration and reduced anxiety in offspring at 8 weeks of age compared to controls (Almeida *et al.,* 1991; 1993; 1996b). Such changes were postulated by the authors to represent increased impulsivity in offspring exposed to maternal malnutrition. Conversely, one other study reported that maternal undernutrition increased behavioural signs of anxiety upon multiple measures of rodent anxiety at three months of age (Jaiswal *et al.,* 1996). Such differences may have been attributable to the procedure employed in the latter study in which restricted offspring spent 12 hours a day with non-lactating maternalised females. The anxiogenic effect could have been induced by alterations to mother pup interaction or other stress related changes to maternal behaviour, rather than by undernutrition.

Spencer and Tilbrook (2009) demonstrated that neonatal overnutrition induced by reducing litter size, attenuated anxiety and increased exploratory behaviour in adult female offspring but not in males. Female offspring exposed to neonatal overnutrition exhibited an enhanced response to restraint stress measured by increased Fos-immunoreactivity in the PVN and thalamus compared to controls. Both adult male and female offspring

subjected to neonatal overnutrition weighed significantly more and consumed more food than offspring raised in large litters.

Levay *et al.,* (2008) demonstrated that maternal protein restriction prior to conception, as well as during gestation and lactation, induced alterations to anxiety and exploratory behaviour in adult offspring. Using an emergence test to measure exploration and anxiety related behaviour in Hooded Wistar rats, it was observed that offspring of dams exposed to maternal undernutrition prior to conception showed significantly more signs of anxiety, being less likely to venture out and spend time outside of the safety of a hide box located within an open field. The findings from our own research into maternal exposure to hyperenergetic diet upon anxiety and exploratory behaviour published in Wright *et al.,* (2011b) will be described in the next chapter.

1.6.2 Learning and memory

Several studies have been published demonstrating behavioural alterations indicative of deficits in learning and memory in offspring, programmed by maternal undernutrition during pregnancy and lactation. An early study by Jordan *et al.,* (1981) demonstrated that infant rats maternally subjected to undernourishment during pregnancy and lactation exhibited impairments to spatial memory in adulthood, as measured by performance upon the 16-arm radial maze. Castro *et al.,* (1989) found that infant rats maternally subjected to undernutrition throughout lactation (through daily mother-pup separation) exhibited numerous impairments to spatial memory in adulthood as

measured by performance upon a conditioned spatial discrimination task, although this difference could have been an effect of maternal separation per se.

Fukuda *et al.,* (2002) demonstrated deficits in spatial memory in offspring of Wistar rat dams fed a protein deficient diet during lactation when tested on the Morris water maze. Offspring exposed to lactational protein deprivation took significantly longer to locate the escape platform and travelled significantly further than offspring of dams fed a control chow.

Bedi (1992) demonstrated that infant rats subjected to malnutrition through maternal food restriction followed by direct food deprivation in the first 30 days of life, exhibited deficits in spatial memory when tested immediately after the period of undernutrition. Interestingly despite this effect, deficits were ameliorated by time, with rats being tested in later life showing no deficit. This suggests a recovery of spatial memory function induced by a period of nutritional rehabilitation. Conversely, Cordoba *et al.,* (1994) ascertained that rats exposed to maternal malnutrition during pregnancy exhibited severe deficits in spatial memory in the Morris water maze, when tested as adults. This suggested that even after a period of nutritional rehabilitation a deficit remained. Such findings demonstrate the importance of maternal nutrition during early critical periods of development upon the integrity of learning and memory processes in adult offspring. Despite such findings, to the author's knowledge, no studies have been published

examining the effect of maternal overnutrition during early critical periods of development upon learning and memory in adult offspring.

1.6.3 Ingestive behaviour

Ingestive behaviour in rodents has also been demonstrated to be susceptible to programming. Studies analysing macronutrient selection have ascertained that dietary preferences for palatable or high fat food items in offspring can be manipulated by alterations to maternal diet during critical periods of development. Bayol *et al.,* (2007) showed that offspring exposed to a maternal hyperenergetic diet during pregnancy and lactation exhibited an enhanced propensity to consume high fat, energy dense food as adults, and develop obesity compared to control offspring. Also Bellinger *et al.,* (2004) observed that male and female offspring subjected to maternal protein restriction during pregnancy exhibited an enhanced propensity to consume high fat food items at 12 weeks of age but not at 30 weeks. Bellinger and Langley-Evans (2005) demonstrated that maternal exposure to a low protein diet during early, mid and late pregnancy individually, altered macronutrient selection by reducing fat consumption in female but not male offspring. Such findings suggest maternal dietary perturbations consisting of both over- and undernutrition can alter macronutrient selection in adult offspring.

Measures of macronutrient selection have provided a broad indication that appetite related behaviour may be altered or impaired by maternal dietary manipulations and this may exacerbate the metabolic abnormalities detected in such animals. However, more detailed assays of behaviour examining the

structural integrity of behaviours related to energy intake in rodents could potentially provide a greater insight into alterations to ingestive behaviour that may be induced by maternal dietary manipulations. Orozco-Solis *et al.,* (2009) fed dams a low protein diet during pregnancy and examined appetite related behaviour in offspring at 35 days old and again at 180 days using the behavioural satiety sequence (BSS).

The BSS was originally described by Antin *et al.,* (1975) as a sequence of behaviours expressed by rodents accompanying food intake consisiting of a temporal progression from feeding behaviour, followed by bouts of exploratory behaviour (locomotor and rearing behaviour) and grooming, to finally resting behaviour. The BSS was originally utilized to differentiate the effect of manipulations on 'natural' satiety related mechanisms from non-specific behavioural effects associated with reduced food intake such as enhanced locomotor behaviour, sedation or gustatory aversion.

For example, although *d*-amphetamine has been demonstrated to be an effective appetite suppressant, the temporal structural transition of behaviour associated with feeding and satiety can be eliminated entirely as a result of significant increases in locomotor behaviour (Halford *et al.,* 1998). Drugs such as *d*-fenfluramine and sibutramine have been demonstrated to be equally effective as appetite suppressants compared to amphetamine but in contrast preserve the structural integrity of behaviours associated with natural satiety (Blundell, 1986; Tellett *et al.,* 2009). Conversely, drugs with sedative properties, such as the 5-HT2 receptor agonist MK-212 have been

demonstrated in high doses to eliminate the conventional sequence of satiety by increasing the proclivity to rest at the expense of other behaviours (Halford *et al.,* 1997).

The BSS has been widely utilized in behavioural science to investigate the impact of pharmacological, genetic or physiological manipulations on satiety mechanisms in rodents (Halford *et al.,* 1998). Recently, the analysis of the BSS has focused specifically on the predictable temporal transition from eating through to grooming, to resting behaviour and is often performed under conditions in which the animal is exposed to a palatable test meal to accentuate the behavioural effects over a 1-hour observation session.

Similarly to controls, offspring tested at 35 days of age who were exposed to maternal protein restriction exhibited a conventional pattern of behaviour, with the exception of a slight delay in the onset of satiety measured by the transition from eating to resting. Offspring of low protein fed dams engaged in eating behaviour sooner and consumed more food than control offspring. At 180 days of age no differences in behaviour were observed, with the exception that offspring of dams fed the low protein diet ate less. Overall results suggested that despite changes in the amount of food consumed, the structural integrity of feeding behaviour was preserved in offspring of dams fed the low protein diet. The findings above considered in conjunction with observations that maternal dietary perturbations can programme alterations to neural circuitry in individuals genetically predisposed to obesity, paints an interesting picture. The findings from our own research into maternal

exposure to hyperenergetic diet upon ingestive behaviour using the BSS published in Wright *et al.,* (2011a) will be described in detail in *Chapter 3.*

1.6.4 Conclusion

In summary, to date little research has been published examining the consequences of maternal obesity and/ or maternal hyperenergetic diet during early critical periods of development upon behaviour in adult offspring. If there are such programming effects it is unclear what the contribution of maternal obesity prior to conception may be relative to maternal overnutrition during gestation and/ or lactation. It is also unclear which types of behaviour in offspring may be affected by maternal overnutrition.

Table 1.1 – Studies demonstrating the effects of direct and maternal dietary perturbations upon offspring behaviour in rats.

Authors	**Strain (gender)**	**Diet**	**Domain**	**Test**	**Effect of obesogenic diet**
Souza *et al.,* (2007)	Wistar rats (males)	Sucrose enriched	Anxiety	Light-dark exploration task	Increased signs of anxiety
Sahakian *et al.,* (1982)	Lister Hooded rats (females)	Cafeteria diet	Hyperactivity	Locomotor activity	Increased locomotor activity in isolation reared rats, reduced locomotor activity in group housed rats
Hilakivi-Clarke *et al.,* (1996)	Sprague-Dawley rats (males)	High fat	Aggression	Resident intruder test	Increased signs of aggression
Abildgaard *et al.,* (2011)	Flinders Sensitive Line rats (males)	High fat	Depression	Forced swim test	Increased signs of depression
Sclafani & Springer (1976)	CFE rats (females)	Cafeteria diet	Appetite	Operant (Fixed ratio schedule), Quinine adulteration	Enhanced sensitivity to quinine alteration. Less desire to work and respond for food reward. No increase in activity after food reward.
Jen (1980)	Sprague-Dawley rats (males)	High fat	Appetite	Operant (Fixed ratio schedule), Quinine adulteration, Shuttle Box	No effect
Jen *et al.,* (1981)	Sprague-Dawley rats (males & females)	High fat	Appetite	Operant (Fixed ratio schedule), Quinine adulteration	Enhanced sensitivity to quinine alteration. Less desire to work respond for food reward. No increase in activity after food reward in females only.

La Fleur *et al.,* (2007)	Wistar rats (males)	High fat/high sugar	Appetite	Operant (progressive ratio)	Enhanced motivation to respond for sucrose pellets
Molteni *et al.,* (2002)	Sprague-Dawley rats (females)	High fat/high sugar	Spatial memory	Morris water maze	Reduction in performance
Goldbart *et al.,* (2006)	Sprague-Dawley rats (males)	High fat/ refined carbohydrate	Spatial memory	Morris water maze	Exacerbation of deficits induced by intermittent hypoxia
Stranahan *et al.*, (2008)	Rat (strain or gender not disclosed)	High fat/high glucose supplemented with high fructose corn syrup	Spatial memory	Morris water maze	Reduction in performance
Jurdak *et al.,* (2008)	Long-Evans rats (males)	High fat/ sucrose solution	Spatial memory	Morris water maze	Reduction in performance
Granholm *et al*., (2008)	Fischer 334 (males)	Saturated fat/ high cholesterol	Spatial memory	Water rardial arm maze	Reduction in performance
Kanoski & Davidson (2010)	Sprague-Dawley rats (males)	High fat	Spatial memory	Radial arm maze	Reduction in performance
Jurdak & Kanarek (2009)	Long-Evans rats (males)	Sucrose solution	Recognition memory	Novel object discrimination	Reduction in performance
Greenwood & Winocur (1990)	Long-Evans rats (males)	High fat	Rule learning memory	Variable Interval delayed alteration task	Reduction in performance
Winocur & Greenwood (1999)	Long-Evans rats (males)	High fat	Rule learning memory	Variable Interval delayed alteration task	Reduction in performance
McNeilly *et al.* (2011)	Wistar (males)	High fat	Rule learning memory	Delayed matching to position task	Reduction in performance

Almeida *et al.,* (1991)	Wistar rats (males)	Low protein (Lactation + early post-lactation)	Anxiety	Elevated plus maze	Reduced anxiety
Almeida *et al.,* (1993)	Wistar rats (males)	Low protein (Lactation)	Anxiety	Elevated plus maze	Reduced anxiety
Almeida *et al.,* (1996a)	Sprague-Dawley rats (males & females)	Low protein (Pregnancy)	Anxiety	Elevated plus maze	Reduced anxiety
Almeida *et al.,* (1996b)	Sprague-Dawley rats (males & females)	Low protein (Pregnancy)	Anxiety	Elevated T maze	Reduced anxiety
Jaiswal *et al.,* (1996)	Charles Foster rats (males)	Low protein (Pregnancy + lactation)	Anxiety/exploration	Elevated plus maze Open field	Increased anxiety
Fracolin-Silva *et al.,* (2006)	Wistar rats (males)	Low protein (Lactation)	Anxiety	Elevated plus maze	Reduced anxiety
Levay *et al.,* (2008)	Hooded Wistar rats (males & females)	Low protein (pre-gestation, pregnancy and lactation)	Anxiety/ exploration	Emergence test Open field Elevated plus maze	Pre-gestation offspring enhanced anxiety on emergence test and open field
Spencer & Tilbrook (2009)	Wistar rats (males & females)	Overnutrition via litter size reduction (lactation)	Anxiety/ exploration	Elevated plus maze Open field	Reduced anxiety in females
Jordan *et al.,* (1981)	Lister Hooded rats (males & females)	Undernutrition via food restriction (pregnancy and lactation)	Spatial memory	8-Arm radial maze 16-Arm radial maze	Reduction in performance

Casrto *et al.,* (1989)	Lister Hooded rats (males)	Undernutrition though maternal separation (lactation)	Spatial memory	Conditioned spatial discrimination	Reduction in performance
Bedi (1992)	Hooded Long-Evans rats (males)	Undernutrition	Spatial memory	Morris water maze	Reduction in performance
Cordoba *et al.,* (1994)	Wistar rats (males & females)	Low protein (pregnancy, lactation and early post-lactation)	Spatial memory	Morris water maze	Reduction in performance
Fukuda *et al.,* (2002)	Wistar rats (males)	Low protein (lactation)	Spatial memory	Morris water maze	Reduction in performance
Bellinger *et al.,* (2004)	Wistar rats (males & females)	Low protein (pregnancy and lactation)	Ingestive behaviour	Macronutrient selection	Enhanced propensity to consume high fat food items at 12 weeks of age but not at 30 weeks.
Bellinger and Langley-Evans (2005)	Wistar rats (males & females)	Low protein (pregnancy)	Ingestive behaviour	Macronutrient selection	Reduced fat consumption in female but not male offspring
Bayol *et al.,* (2007)	Wistar rats (males & females)	Overnutrition through cafeteria diet (pregnancy and lactation)	Ingestive behaviour	Macronutrient selection	Enhanced propensity to consume high fat, energy dense food items as adults
Orozco-Solis *et al.,*	Sprague-Dawley rats	Low protein and	Ingestive behaviour	Behavioural satiety	Slight delay in the onset of satiety, shorter

(2009)	(males)	lactation (pregnancy)	sequence	latency to eat, greater amount of food consumed

1.7 ***Aims and Hypothesis***

The aim of this present thesis is to examine the effects of maternal exposure to a hyperenergetic cafeteria diet during early critical periods of development, upon behaviour, metabolism and aspects of brain neurochemistry in adult offspring. The cafeteria diet feeding regimen, originally pioneered in the 1970s by Nancy Rothwell and Michael Stock at the University of Manchester and Anthony Sclafani at Brooklyn College New York, involves daily exposure of rats to multiple human food items known to be high in fat and sugar and, under conditions of chronic exposure, is obesogenic (Sclafani & Springer 1976; Rothwell & Stock, 1979a; 1979b: Rothwell *et al.,* 1982; 1983). As multiple food items are alternated daily, this particular regimen is advantageous compared to other hyperenergetic diets, as the pattern of food intake mirrors the choice and variety of human food consumption in obesogenic dietary conditions (Rothwell & Stock, 1988). Sampey *et al.,* (2011) reported that cafeteria diet feeding was more effective than a lard based high fat diet used to model human obesity and the metabolic syndrome in rats. Wistar rats subjected to chronic exposure to the cafeteria diet exhibited greater levels of obesity, hyperleptinaemia, hyperinsulinaemia, hyperglycaemia, glucose intolerance and insulin resistance than animals fed the high fat diet (Sampey *et al.,* 2010).

The observation that maternal dietary perturbation throughout pregnancy and the neonatal period can 'imprint,' or induce permanent changes to neural circuitry in the brain responsible for the mediation of a wide range of behaviours is compelling (Levin, 2000; 2006; Muhlhausler *et al.,* 2006;

Muhlhausler & Ong, 2011). To date little detailed research has been undertaken investigating the effects of maternal overnutrition using the cafeteria diet feeding model upon behaviour in adult offspring. Based on observations described in *Section 1.6.1* citing that dietary manipulations during pregnancy and during the neonatal period can induce an anxiolytic behavioural profile in adult offspring, in *Chapter 2* it was hypothesized that maternal obesity induced by cafeteria diet feeding prior to mating and/or cafeteria diet feeding during pregnancy and lactation would reduce anxiety related behaviour in adult offspring.

To this end, using a complex experimental design we fed female rat dams either a cafeteria diet or a nutritionally balanced control chow for six weeks prior to mating. Cafeteria diet feeding prior to mating was to model the effects of maternal obesity upon offspring. After mating dams were either fed the cafeteria diet or the control chow during pregnancy and after birth groups were split again being fed either the experimental diet or the control chow during lactation. Emotional behaviour was examined in adult offspring of both sexes. This design would not only determine the relative importance of each individual maternal feeding period in programming offspring behaviour (pregestation vs. gestation vs. lactation), it would also allow a comprehensive examination of the relationships between each feeding period. The effects of maternal cafeteria diet feeding upon obesity related parameters in adult offspring were recorded including; bodyweight, chow consumption, fat mass and fat content. After determining the relative contribution of each period after the initial battery of testing, the maternal feeding period perceived to

have the greatest importance for behaviour was selected for further investigation.

Based on observations described in *Section 1.6.3*, in *Chapter3* in order to investigate the effect of maternal overnutrition on appetite related behaviour in adult offspring, it was hypothesized that offspring of dams exposed to hyperenergetic diet throughout the lactation period would lead to subtle alterations to the BSS in adult offspring potentially resulting in a delay in the onset of satiety compared to controls. Additionally, based on observations described in *Section 1.6.2*, in order to investigate the effect of maternal overnutrition on behaviours relating to learning and memory processes in adult offspring, it was hypothesized that offspring of dams exposed to hyperenergetic diet throughout the lactation period would lead to impairments in performance on measures of habituation memory and novel object discrimination compared to controls.

To this end, lactating dams were either fed the cafeteria diet or the control diet with all offspring being weaned onto a control chow until behavioural testing took place during adulthood. Behavioural measures included assays of ingestive behaviour and measures of learning and memory. It was also hypothesized that offspring of dams exposed to hyperenergetic diet throughout the lactation period would show alterations to monoamine neurochemistry as adults. To this end rats maternally exposed to either the cafeteria diet or the control chow during lactation were weaned onto the control chow and were culled as adults. Concentrations and turnover of 5-HT

and DA and their respective metabolites were measured in the hypothalamus, hippocampus and frontal cortex.

Chapter 2 - The effect of maternal cafeteria diet feeding during critical periods of development upon metabolism and behaviour

2.0 Introduction

As obesity becomes more prevalent in modern society, increasing understanding of how this might impact upon health and behavioural trends is increasingly important. As research has demonstrated that a hyperenergetic diet can programme multiple aspects of the central nervous system (Levin, 2000; 2006; Muhlhausler *et al.,* 2006), the behavioural implications of maternal obesity and overnutrition are becoming critical to comprehensively understanding the full effects of chronic overnutrition. A robust body of rodent literature exists, documenting that maternal dietary perturbations during gestation and the early neonatal period play a key role in determining the risk to metabolic abnormalities and disease experienced in adult offspring (McMillan & Robinson, 2005; Langley-Evans, 2006; 2007).

To date little research has been undertaken investigating the effects of obesity and maternal overnutrition during early development on emotional behaviour in later life. Several studies have investigated the effects of maternal undernutrition during pregnancy and lactation upon emotional behaviour in adult offspring with variable effects (Almeida *et al.,* 1993; 1996b; Jaiswal *et al.,* 1996; Levay *et al.,* 2008). Interestingly, the obesogenic and metabolic effects of maternal under- and overnutrition in offspring have been reported to be similar (Samuelsson *et al.,* 2008), but it is yet to be ascertained if such a finding applies to behaviour.

The aim of the present chapter was to investigate the consequences of maternal obesity prior to conception, as well as maternal overnutrition during pregnancy and lactation, for obesity related parameters, as well as anxiety and exploratory behaviour in adult offspring. A key aim was to identify which maternal feeding period was of primary importance in altering behaviour in adult offspring of both sexes (pre-gestation vs. gestation vs. lactation). This would enable further research to be directed to focus on that particular period. The present experiment had a complex design, in order to fully model whether or not maternal obesity, or simply over-feeding on a fat- and sugar-rich diet, provided the programming stimulus leading to changes in offspring behaviour. To this end, cafeteria diet feeding prior to mating was used to model the effects of maternal obesity on anxiety related behaviour and exploration in adult offspring, whereas maternal cafeteria diet throughout pregnancy and lactation was used to model the effects of hyperenergetic diet during the latter periods in isolation, or in combination to maternal obesity. It was hypothesized that maternal obesity induced by cafeteria diet feeding prior to mating and/or cafeteria diet feeding during pregnancy and lactation would alter anxiety related behaviour in adult offspring, potentially in the form of a reduction in behaviours related to anxiety.

2.1 Experimental procedures

2.1.1 Animal procedures

All procedures were performed under licence from the Home Office, in accordance with The Animals (Scientific Procedures) Act 1986. Animals were maintained under a 12hr light-dark cycle (08:00-20:00 hrs, lights on 08:00 hrs), between 20-22°C temperature and at 45% humidity. Virgin female Wistar rats (Harlan, UK) were housed individually with sawdust as cage substrate and with *ad libitum* access to food and water, from 4 weeks of age. In addition to the standard laboratory chow diet (control), the experimental diet consisted of a range of highly palatable human foods (cafeteria diet; CD) as used in Akyol *et al.,* (2009) (Fig. 2.1). These included pork pie, pate, cocktail sausages, cheese, crisps, jam, fruit and nut chocolate, golden syrup cake, shortbread and peanuts. Four of these food items were provided daily and were placed in a bowl on the cage floor daily in excess quantities. Food items were changed each day to maintain variety and interest. A schematic representation of the feeding protocol is displayed in Figure 2.2.

Rats that were fed CD from weaning (CD pre-gestation; PG) exhibited excess weight gain and in keeping with the report by Akyol *et al.,* (2009) were deemed to be obese. In order to separate the effects of maternal cafeteria feeding from the effects of maternal obesity, rats from both pre-mating groups were divided into a chow feeding group or a CD group during gestation. Rats initiating CD only at mating represented a group that were overfed but not obese (CD gestation; G). After birth, offspring of dams from

each of the four pregnancy groups were further divided into two groups, either being fed chow or CD throughout lactation. Thus a set of non-obese animals exposed to CD in lactation only (L) were generated. A total of eight groups were included in the study (Figure 2). Litter size (8-12) was not significantly different between experimental conditions. After weaning (21 days post-partum) animals were group housed with littermates of the same sex, with all eight groups being maintained on chow for the duration of the behavioural study. Upon cessation of testing rats were humanely culled via exposure to CO_2 and cervical dislocation. The impact of the dietary protocols upon maternal food intake, macronutrient intake and body weight gain are reported elsewhere (Akyol *et al*., 2011).

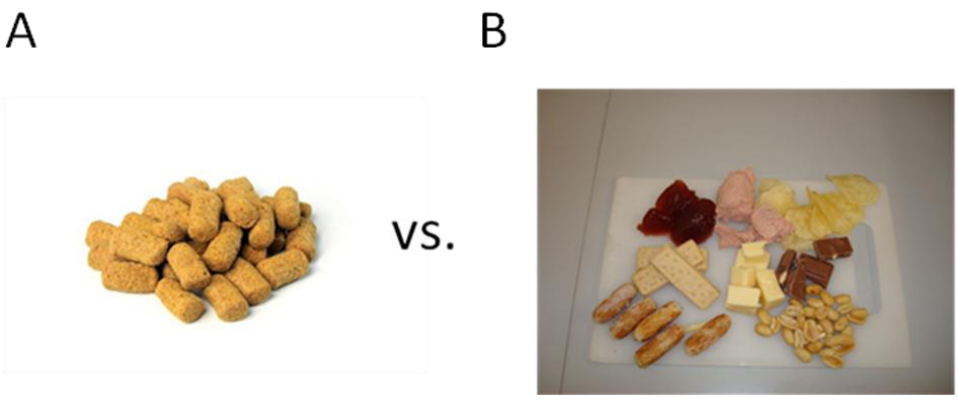

Fig.2.1. Control chow vs. cafeteria diet**.** A: Chow (B&K Universal Ltd., Hull UK) used as control diet (crude protein 24%, fat derived from either 4.7%, crude fibre 4.0%, energy density 3.0kcal/g, calories from protein 32%, calories from fat 14% & calories from carbohydrate 54%). B: Items from the hyperenergetic cafeteria diet including: strawberry jam, pate, potato crisps, cheddar cheese, shortbread biscuit, fruit and nut chocolate, sausages, peanut, pork-pie (not pictured), mars bar (not pictured) and golden syrup cake (not pictured). All items purchased from Tesco PLC, UK.

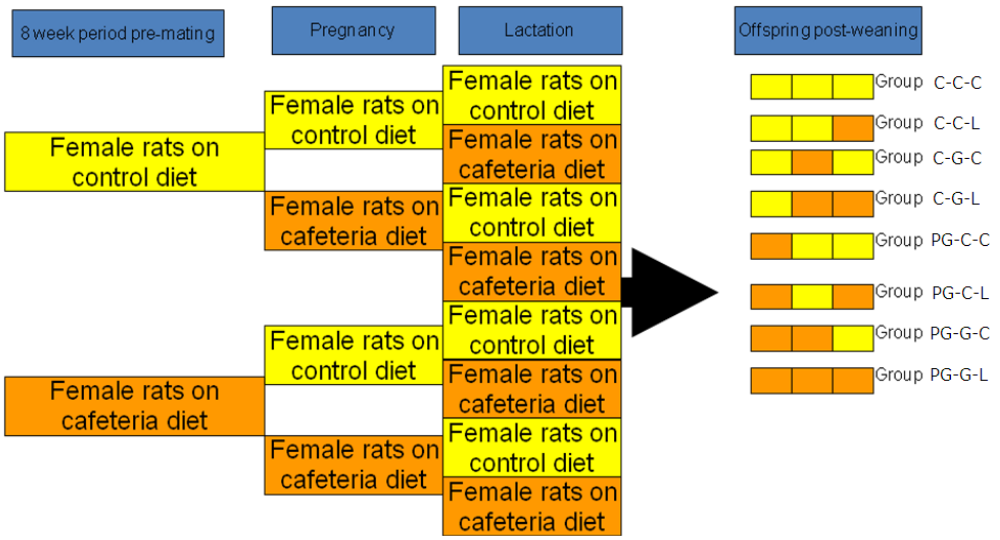

Fig. 2.2. Schematic presentation of experimental design. Rats were either fed on cafeteria diet (CD, darker boxes) or chow (C, lighter boxes) during various developmental stages. PG = pre-gestational CD, G = gestational CD, L = lactational CD, and C = chow. All offspring were fed chow after weaning. n between 5 and 12/group. As statistical analysis focused on these three nutritional conditions as main factors, these conditions and the related chow fed control groups were presented in the results, as well as data for the 7 experimental groups vs. the controls. For details refer to Experimental Procedures.

2.1.2 Weekly bodyweight and adipose tissue mass

The average weekly bodyweight was recorded for each animal from the first week post-weaning up to week ten. As seen in Fig. 2.3 after the offspring had been culled at the age of 5 months, abdominal adipose tissue (gonadal & perirenal adipose tissue) and intrascapular (white and brown) depositions of

adipose tissue were removed from the freshly culled carcasses and weighed. The weights of the fat pads were presented as percentage of bodyweight.

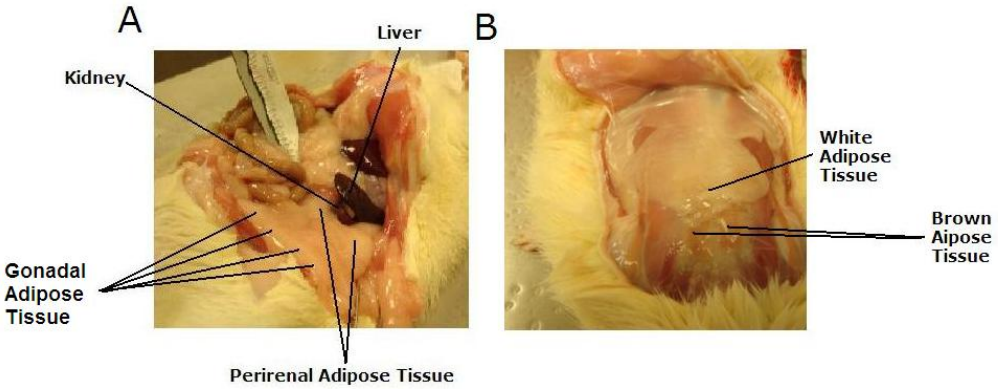

Fig. 2.3 Adipose tissue mass. A: Abdominal adipose tissue (gonadal & perirenal adipose tissue) B: Intrascapular adipose tissue (white & brown adipose tissue). See Experimental Procedure for details.

2.1.3 Offspring carcass composition

Offspring carcass composition was determined following the method used by Akyol *et al.,* (2009). The whole animal was oven-dried for approximately 2 weeks at 80°C to determine the water content of the carcass. The sample was then homogenised to a powder of uniform texture and consistency in order to perform a Soxhlet fat content analysis as described previously by Langley and York (1990). A 1g sample of homogenised carcass underwent chemical analysis so the fat from each sample could be isolated and weighed to calculate the percentage of fat content of each sample. The percentage of fat extracted from each sample was used to calculate the fat content of each animal.

2.1.4 Plasma leptin concentration

As most of the main effects of CD on adiposity and whole body fat content resulted from CD feeding during the lactation period, analysis of plasma leptin concentrations was performed upon offspring exposed to CD during the lactation period compared to controls (C-C-C vs. C-C-L). Offspring were culled at twenty weeks of age. Blood was extracted from the trunk of each carcass via cardiac puncture. Each sample was inserted into a 1.3ml LH Microtube (Sarstedt, Germany) for blood plasma separation and spun in a 22B Microfuge® centrifuge (Beckman-Coulter, USA) at room temperature for 15 minutes at 14000 RPM. The separated layer of plasma was removed and placed into a separate 1.5 polypropylene tube and stored at -40°C.

2.1.4.1 Leptin ELISA

Quantitative determination of plasma leptin concentration was performed using a Rat Leptin ELISA Kit (Crystal Chem Inc. USA), following the manufacturer's instructions. Each well of an antibody coated 96-well micro-plate was aspirated with 300 µl of wash buffer twice before 45 µl of sample diluent and 50 µl of guinea pig anti-mouse leptin serum were added to each well. A 5 µl sample of plasma from each animal, as well as working rat leptin standard was added to the microplate in duplicate, before being left to incubate overnight (16-20 hours) at 4°C. Each well was aspirated 5 times with 300 µl of wash buffer before 100µl of anti-guinea pig IgG enzyme conjugate was dispensed to each well.

The microplate was left to incubate for 3 hours at 4°C before each well was aspirated another 7 times with 300 µl of wash buffer. 100µl of enzyme substrate solution was added to each well before the microplate was left to incubate for 30 minutes with no exposure to light at room temperature. After the 30 minute incubation time, 100µl of enzyme stop solution was added to each well and using a microplate reader (Tecan Sunrise, Switzerland) the A_{450} and A_{630} were determined within 30 minutes. A_{630} values were subtracted from A_{450} values to quantify the plasma serum concentration of leptin of each sample and generate a standard curve.

2.1.5 *Analysis of behaviour*

All observation sessions were imaged using a CCD camera (Sanyo, Japan) mounted onto the roof of a specifically designated behavioural observation room, and recorded using Pinnacle (Pinnacle systems, UK) home movie software installed on a computer in a separate room. Light intensity for all the tests undertaken was 130lx. Elevated plus maze and open field testing took place between 0900 and 1300 hours with animals placed in the room of testing 30 minutes before the beginning of the observation period to become acclimatized to the testing area. Behaviour was tracked and analysed using Ethovision 3.1 (Noldus, Netherlands) software.

2.1.5.1 *Elevated plus maze*

Animals were tested on the elevated plus maze at week 10 post-partum. The elevated plus maze (EPM) consisted of 2 closed arms (60 cm x 10 cm x 35 cm), 2 open arms (60 cm x 10 cm), and a central zone (10 cm x 10 cm), elevated 70

cm above the ground. The elevated plus maze used in this study was made of black reflective Perspex to improve contrast. Light intensity in the open arms was 130lx and 70lx in the closed arms. Animals were placed within the elevated plus maze for 5 minutes with the experimenter absent from the test room. When placed in the apparatus animals were initially positioned within the central zone of the maze, providing the subject with equal choice of which arm to enter. After each animal was tested the plus maze was disinfected before the next animal was placed within the apparatus. The procedure followed was identical to that outlined by Pellow *et al.,* (1985).

The frequency and duration of open and closed arm entries was recorded, in order to calculate the percentage of open arm entries relative to enclosed and the percentage of time spent in the open arms relative to enclosed. Total distance travelled by the animals was also recorded, as was total distance travelled in the closed arms of the maze. Total distance upon the closed arms of the maze is seen as a more accurate measure of locomotor activity than overall total distance as conventionally the amount of time spent within the closed arms in greater than the open arms (Dawson *et al.,* 1995). Upon the elevated plus maze manipulations which increase the ratio of open arm entries to total entries and increase the percentage of time spent upon the open arms are indicative of anxiolysis and the reverse is indicative of anxiogenesis (Pellow *et al.,* 1985).

Four behaviours were manually coded including head-dips, grooming, rearing and stretch attend posture. Increased frequency of head-dips, defined as

when the animal lowers its head down over the side of the maze looking at the floor, has been reported to be indicative of anxiolysis (Pellow *et al.,* 1985; Almeida *et al.* 1996). Grooming has been defined as when the animal licks its paws, body or genitals, or scratches and rubs its fur or tail and has been purported to be related to anxiety (Dunn, *et al.,* 1981; Voigt *et al.,* 2005). Rearing is a parameter of vertical exploration when the animal stands on its hind legs with its front paws off the ground and in front of its body or on the walls of the maze (File, 1978a; 1978b). Stretch attend posture is when the animal stretches its body and head forward to scan the scene, usually from a closed arm or the central zone, towards an open arm and has been reported to represent a behavioural expression of risk assessment (Kaesermann, 1986; Blanchard *et al.,* 1990; Shepherd *et al.,* 1994).

2.1.5.2 *Open Field*

Animals were tested in an open field (OF) 11 weeks after birth. Open field test apparatus consisted of a grey open top PVC box (100 cm x 100 cm) virtually divided into 2 zones; an outer zone and an inner zone (50 cm x 50 cm). Light intensity in the open field was 130lx. Animals were exposed to the open field for 5 minutes, with the test apparatus being disinfected after each animal. Rearing and grooming behaviours were manually coded along with measures of total distance travelled and the frequency, duration and latency of inner zone entry. At the end of the 5 minute test period animals were returned to the home cage. In the open field, manipulations which increase the frequency of inner zone entry, the time spent inner zone and total

distance travelled, and reduce the latency of inner zone entry are indicative of anxiolysis, with the reverse indicative of anxiogenesis (Treit & Fundytus, 1988).

2.1.6 Statistical analysis

Data in figures are presented as mean + SEM throughout this report. $P< 0.05$ was considered statistically significant in all analyses. Weekly body weights were analysed using a general linear model repeated measures ANOVA with one within subject factor (Week of study) and one between subjects factor (feeding group). Tukey *post hoc* tests were used to facilitate individual comparisons. The main effects of pregestational, gestational and lactational diet, as well as gender on bodyweight at time of cull and adipose tissue mass were assessed using a general linear model ANOVA (four fixed factors - pregestational, gestational and lactational diet, as well as gender). Due to several significant maternal diet x gender interactions female and male offspring were from then on analysed separately.

The main effects of pregestational, gestational and lactational diet on whole body fat content and behavioural outcomes in the offspring were assessed using a general linear model ANOVA (three fixed factors - pregestational, gestational and lactational diet). Plasma leptin concentrations from both male and female offspring was analysed separately using independent measures *t*-tests. Data were analysed using the Statistical Package for Social Sciences (version 16; SPSS, Inc., Chicago, IL, USA).

2.2 Results

2.2.1 *Offspring bodyweight and adipose tissue mass*

As shown in Fig. 2.4, there was no effect of maternal diet during pregestation, gestation or lactation on bodyweight of the offspring when animals were chow fed after weaning. However, there were effects on adipose tissue mass. Table 2.1 displays bodyweight at the time of cull and adipose tissue weights for adult offspring fed either control chow or CD throughout each maternal feeding period. Both genders were included in the analysis at this stage. Adult offspring exposed to maternal CD during gestation had a smaller amount of white adipose tissue compared to offspring fed control chow during that period ($F_{1,84}$=7.12, P<0.01). Adult offspring exposed to maternal CD during lactation had significantly larger gonadal ($F_{1, 82}$=7.93, P<0.01) and white adipose tissue mass ($F_{1, 84}$=6.43, P<0.05) compared to offspring fed control chow throughout suckling (Table 2.1). As there were numerous significant diet x gender interactions male and female offspring were from now on analysed separately.

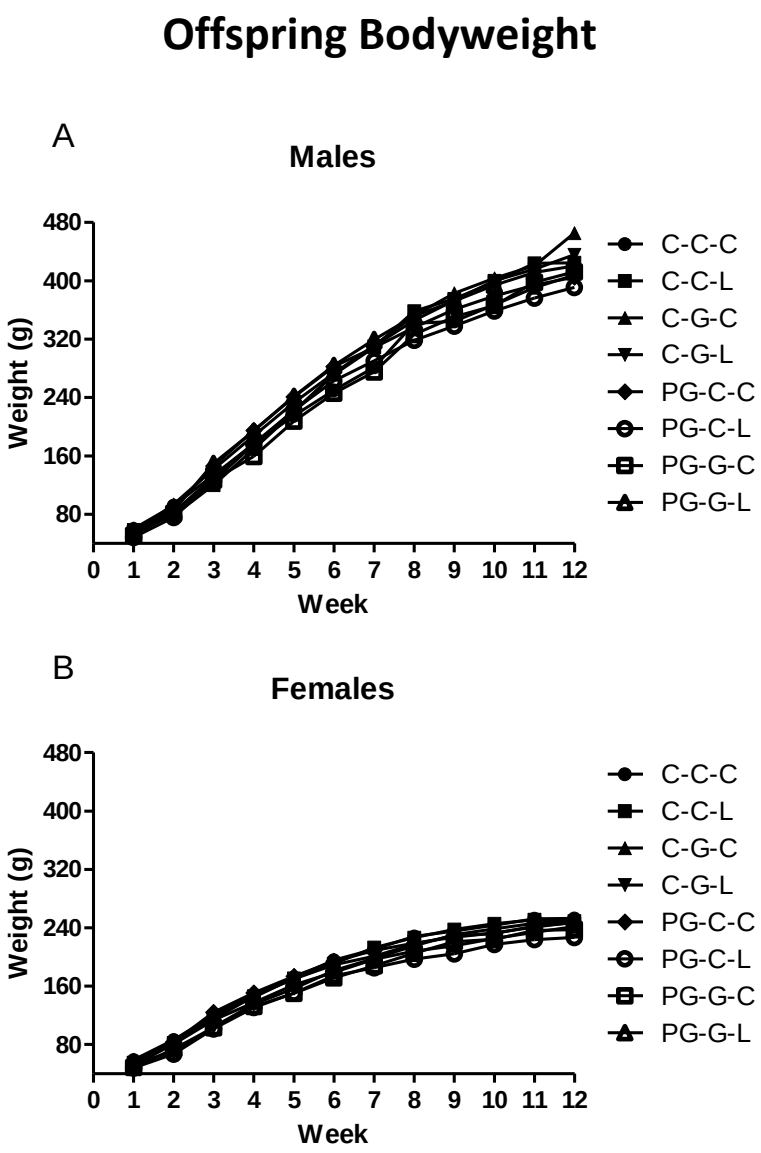

Fig. 2.4. The effect CD feeding upon post-weaning bodyweight in male and female offspring. A: Males B: Females. Males - C-C-C (*n*=10), C-C-L (*n*=11), C-G-C (*n*=9), C-G-L (*n*=10), PG-C-C (*n*=12), PG-C-L (*n*=7), PG-L-C (*n*=7), PG-G-L (*n*=12). Females - C-C-C (*n*=11), C-C-L (*n*=12), C-G-C (*n*=10), C-G-L (*n*=12), PG-C-C (*n*=12), PG-C-L (*n*=11), PG-G-C (*n*=7), PG-G-L (*n*=11). Data are shown as mean.

Table 2.1. Effect of pre-gestational (PG), gestational (G) and lactational (L) cafeteria diet on body weight and fad pats in female (F) and male (M) offspring as measured at 5 months of age. Chow feeding is indicated by "C." Mean ± SEM.

		Body weight (g)	**Gonadal fat pad (% body wt)**	**Perirenal fat pad (% body wt)**	**Intrascapular depot**	
	Gender (N)				**WAT (% body wt)**	**BAT (% body wt)**
PG. (C)	M (23)	513.57 ± 10.36	2.39 ± 0.19	2.57 ± 0.13	0.18 ± 0.01	0.12 ± 0.01
PG. (CD)	M (28)	445.66 ± 14.43	2.27 ± 0.10	2.45 ± 0.35	0.17 ± 0.02	0.12 ± 0.01
G. (C)	M (29)	474.99 ± 12.93	2.21 ± 0.15	2.61 ± 0.34	0.16 ± 0.01	0.11 ± 0.01
G. (CD)	M (22)	477.99 ± 17.04	2.48 ± 0.11	2.35 ± 0.13	0.18 ± 0.02	0.13 ± 0.01
L. (C)	M (25)	470.35 ± 14.14	2.06 ± 0.16	2.47 ± 0.39	0.16 ± 0.01	0.12 ± 0.01
L. (CD)	M (26)	482.00 ± 15.11	2.57 ± 0.10	2.53 ± 0.14	0.17 ± 0.02	0.14 ± 0.01
PG. (C)	F (20)	279.82 ± 3.82	2.64 ± 0.16	2.01 ± 0.13	0.20 ± 0.01	0.15 ± 0.01
PG. (CD)	F (29)	296.51 ± 19.55	2.61 ± 0.14	2.03 ± 0.15	0.22 ± 0.02	0.18 ± 0.03
G. (C)	F (32)	279.65 ± 12.51	2.69 ± 0.13	2.04 ± 0.13	0.25 ± 0.02	0.17 ± 0.01
G. (CD)	F (17)	308.60 ± 23.84	2.49 ± 0.16	1.99 ± 0.18	0.16 ± 0.01	0.16 ± 0.01
L. (C)	F (20)	292.80 ± 15.96	2.38 ± 0.16	1.72 ± 0.13	0.19 ± 0.01	0.16 ± 0.01
L. (CD)	F (29)	287.55 ± 16.54	2.78 ± 0.12	2.23 ± 0.14	0.24 ± 0.02	0.17 ± 0.01
P effect of PG. diet		NS.	NS.	NS.	NS.	NS.
P effect of G. diet		NS.	NS.	NS.	0.01	NS.
P effect of L. diet		NS.	0.01	NS	0.05	NS.
P effect of Sex		0.001	NS.	0.05	NS.	0.01
P effect of PG. x Sex interaction		0.001	NS.	NS.	NS.	NS.
P effect of G. x Sex interaction		0.05	NS.	NS.	0.001	NS.
P effect of L. x Sex Interaction		NS.	NS.	NS.	0.05	NS.

2.2.2 *Offspring carcass composition*

2.2.2.1 *Males*

There was no effect of maternal feeding on whole body fat content in male offspring (Fig. 2.5A).

2.2.2.2 *Females*

The effect of maternal CD feeding on whole body fat content in female adult offspring is displayed in Fig. 2.5B. The effect of maternal CD during lactation interacted with the effect of maternal CD during pregnancy to influence whole body fat content ($F_{1, 57}$= 4.21, P<0.05). This reflected an increase in whole body fat content in offspring subjected to CD during lactation, but only in animals also subjected to maternal CD during pregnancy.

Offspring exposed to pregestational CD feeding or CD feeding during pregnancy showed no difference in whole body fat content in isolation, however offspring exposed to CD feeding during both periods exhibited an increase in whole body fat content due to a significant interaction between the two feeding phases ($F_{1,57}$=9.40, P<0.01). Offspring of mothers exposed to CD during all three feeding periods showed an even greater increase in whole body fat content, as demonstrated by a further three way interaction ($F_{1,57}$=4.92, P<0.05).

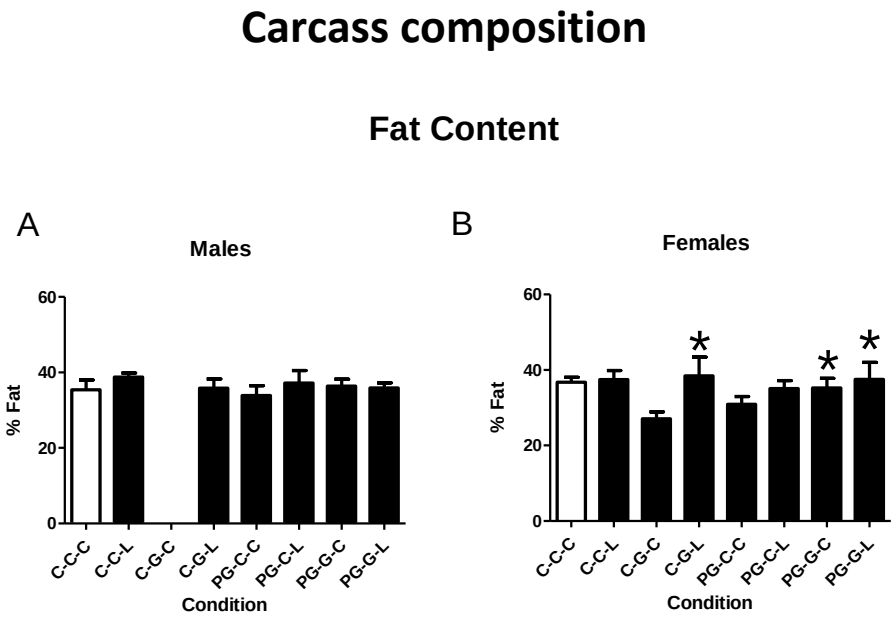

Fig. 2.5. The effect of pre-gestational (PG), gestational (G) and lactational (L) CD upon carcass composition in male and female offspring (fat content shown as wet weight). A: Males C-C-C (*n*=5), C-C-L (*n*=4), C-G-C (*n*=1), C-G-L (*n*=4), PG-C-C (*n*=5), PG-C-L (*n*=4), PG-G-C (*n*=5), PG-G-L (*n*=5). B: Females C-C-C (*n*=5), C-C-L (*n*=4), C-G-C (*n*=4), C-G-L (*n*=4), PG-C-C (*n*=5), PG-C-L (*n*=5), PG-G-C (*n*=4), PG-G-L (*n*=3). Significant interactions - B: Lactational and gestational CD, pregestational and gestational CD, as well as pregestational, gestational and lactational CD. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

2.2.3 Plasma leptin

Fig. 2.6 shows plasma leptin concentrations in adult male and female offspring exposed to either maternal CD, or control chow throughout lactation. Lactational exposure to CD had no effect upon plasma concentrations of leptin in male or female offspring.

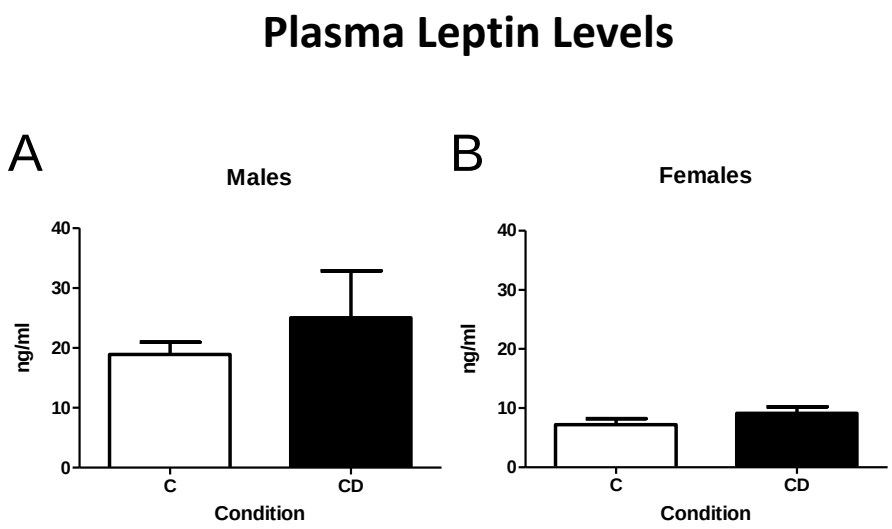

Fig. 2.6. The effect of lactational CD upon plasma circulation of leptin in adult offspring. A: Males Control (*n*=10), CD (*n*=8) B: Females. Control (*n*=10), CD (*n*=9). Control (*n*=10), CD (*n*=9). Data are shown as mean + SEM.

2.2.4 *Elevated plus maze*

2.2.4.1 *Males*

The effect of maternal CD on zone related parameters on the elevated plus maze (EPM) is shown in Fig. 2.7A-D. The effect of maternal exposure to CD during lactation interacted with the effect of pregestational CD feeding to influence the ratio of open arm entries to total arm entries on the EPM ($F_{1, 77} = 5.15$, $p < 0.05$). This reflected an increase in the ratio of open arm entries in animals maternally subjected to lactational CD, but only in offspring from mothers also exposed to CD during pre-gestation (2.7A).

As with the ratio of open arm entries, the effect of maternal exposure to CD during lactation interacted with pregestational CD feeding to influence the

ratio of time spent on the open arms of the EPM ($F_{1, 70} = 7.39$, $p < 0.01$). Again this reflected an increase in the ratio of time spent on the open arms in animals maternally subjected to lactational CD, but only in offspring from mothers also exposed to CD during pre-gestation (2.7B).

Despite there being no effect of maternal diet on frequency of total arm entries, there were main effects demonstrating distance travelled on the plus maze overall ($F_{1, 77} = 9.51$, $p < 0.01$) and on the closed arms of the maze ($F_{1, 68} = 6.29$, $p < 0.05$) was significantly lower in male offspring of obese dams fed the CD throughout pre-gestation compared to offspring of chow fed dams. These main effects were independent of any significant interactions with other periods.

Fig. 2.8 displays the effects of CD feeding on ethological parameters on the EPM in male offspring. The effect of maternal exposure to CD during pregestation interacted separately with the effects of gestational CD feeding ($F_{1, 70}=18.53$, $P < 0.001$) and lactational CD feeding ($F_{1, 70}=4.55$, $P < 0.05$) to influence grooming on the EPM. This reflected a decrease in grooming in offspring of mothers fed pregestational CD, but only in offspring from mothers also exposed to CD during either gestation or lactation (2.8A).

As seen in 2.8B, offspring from dams exposed to both pregestational and lactation CD feeding showed a reduced frequency of rearing, as demonstrated by an interaction between the two periods ($F_{1, 70} = 10.76$, $p < 0.01$). As seen in 2.8C, offspring of dams exposed both to pregestational and gestational CD feeding had an enhanced propensity to head-dip, as demonstrated by an

interaction between the two feeding phases ($F_{1, 70} = 10.45$, $p < 0.01$). As seen in Fig. 2.8D Maternal diet had no effect on stretch attend posture (SAP) in male offspring.

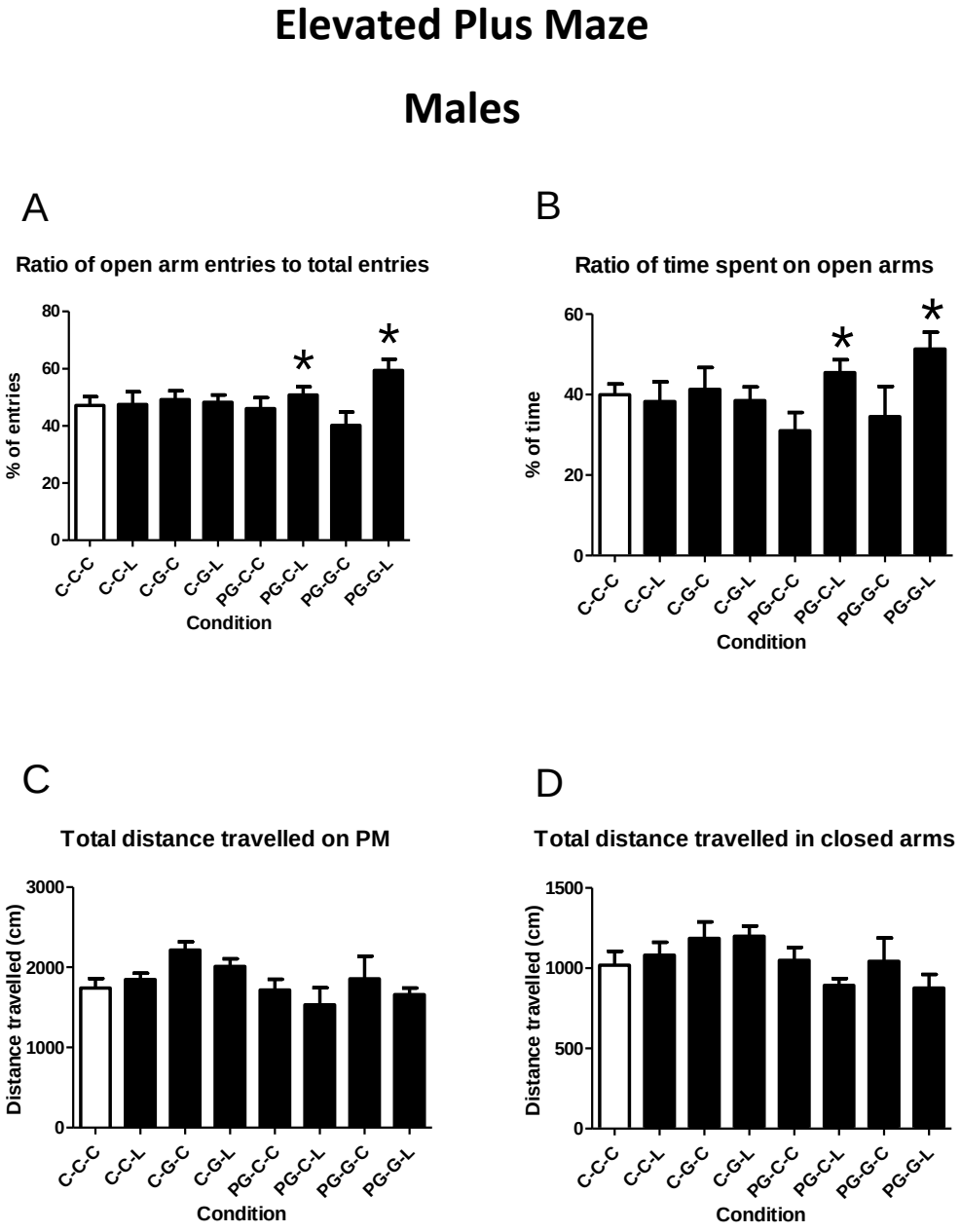

Fig. 2.7. The effect of CD feeding upon behaviour on the elevated plus maze in male offspring. A: Ratio of open arm entries B: Ratio of time spent on open arms C: Total distance travelled. D. Total distance closed arms. C-C-C (*n*=10), C-C-L (*n*=12), C-G-C (*n*=8), C-G-L (*n*=10), PG-C-C (*n*=12), PG-C-L (*n*=8), PG-L-C (*n*=6), PG-G-L (*n*=12). Significant interactions - A: Lactational and pregestational CD. B: Lactational and pregestational CD. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

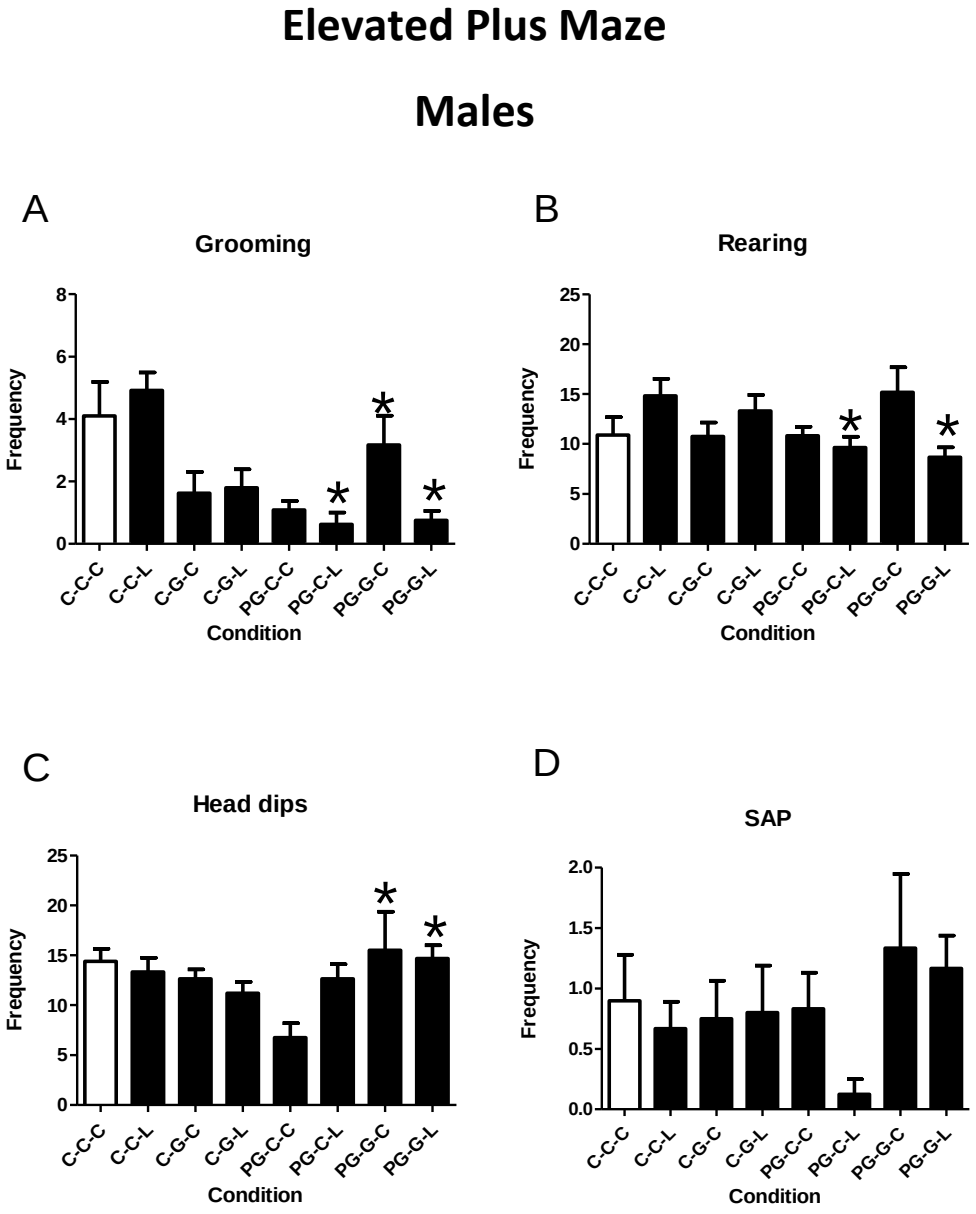

Fig. 2.8. The effect of CD feeding upon behaviour on the elevated plus maze in male offspring. A: Grooming B: Rearing C: Head-dips D: Stretch-attend posture (SAP). C-C-C (*n*=10), C-C-L (*n*=12), C-G-C (*n*=8), C-G-L (*n*=10), PG-C-C (*n*=12), PG-C-L (*n*=8), PG-L-C (*n*=6), PG-G-L (*n*=12). Significant interactions - A: Pregestation and gestation, as well as pregestation and lactation. B: Pregestation and lactation. C: Pregestation and gestation. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

2.2.4.2 *Females*

There was no effect of maternal diet on the ratio of entries into, or time spent on the open arms of the EPM among female offspring (Fig. 2.9A, B&D). The main effects of pre-gestational CD and lactational CD ($F_{1, 72}$=5.34, $P < 0.05$), as well as pregestational CD with CD feeding during both the latter periods ($F_{1, 72}$=4.03, $P < 0.05$) interacted to influence total distance travelled on the EPM. Offspring of dams exposed to pregestational CD travelled a shorter distance on the EPM, but only when also exposed to either lactational CD, or CD feeding throughout both the latter periods in conjunction (Fig. 9C).

Independent of any interactions, there was a main effect of lactational CD feeding for total distance travelled within the closed arms of the maze showing a significant reduction in locomotor activity in offspring exposed to maternal lactational CD compared to offspring of dams fed chow during that period ($F_{1, 69} = 4.11$, $P < 0.05$). Also independent of any interactions, there was a main effect of lactational CD feeding for total arm entries, demonstrating a significant increase in this parameter in offspring exposed to maternal CD during suckling compared to offspring of dams fed chow during that period ($F_{1, 72}= 5.06$, $P < 0.05$).

Fig. 2.10A-D shows the effects of maternal CD feeding on ethological parameters in female offspring on the EPM. The effect of maternal CD during pregestation interacted with maternal CD during gestation to influence grooming behaviour on the EPM ($F_{1\ 72} = 9.12$, $P<0.01$). This reflected a decrease in grooming in offspring of dams exposed to pregestational CD, but

only in animals also exposed to CD during pregnancy. Also the effect of maternal CD during lactation interacted with maternal CD feeding during both pregestation and gestation to influence grooming behaviour on the EPM ($F_{1, 72}$ = 6.79, P < 0.05). This also reflected a decrease in grooming in offspring of dams exposed to lactational CD, but only in animals from dams also exposed to both pregestational CD and CD during pregnancy (2.10A).

The effect of pregestational CD feeding interacted separately with gestational CD ($F_{1, 72}$ = 9.03, p < 0.01) and lactational CD ($F_{1, 72}$ = 14.2, p < 0.05) to influence rearing behaviour. This reflected an increase in this parameter of vertical exploration in offspring of dams fed CD during pregestation and throughout gestation (but not lactational CD), but a decrease in offspring of mothers fed pregestational CD in conjunction to CD feeding during lactation (2.10B).

A similar picture was presented for head-dips as pregestational CD feeding interacted separately with CD feeding during both gestation ($F_{1, 72}$ = 5.34, p < 0.05) and lactation ($F_{1, 72}$ = 5.76, p < 0.05). This reflected an increase in head-dips in offspring of dams fed CD during both pregestation and gestation (but not lactation), but a decrease in offspring of mothers fed pregestational CD in conjunction to CD feeding during lactation (Fig. 2.10D). Pregestational CD feeding interacted with CD feeding during lactation to influence the frequency of SAP in female offspring ($F_{1, 72}$ = 5.12, p < 0.05). This reflected an increase in SAP in offspring of dams fed CD during both periods (Fig. 2.10D).

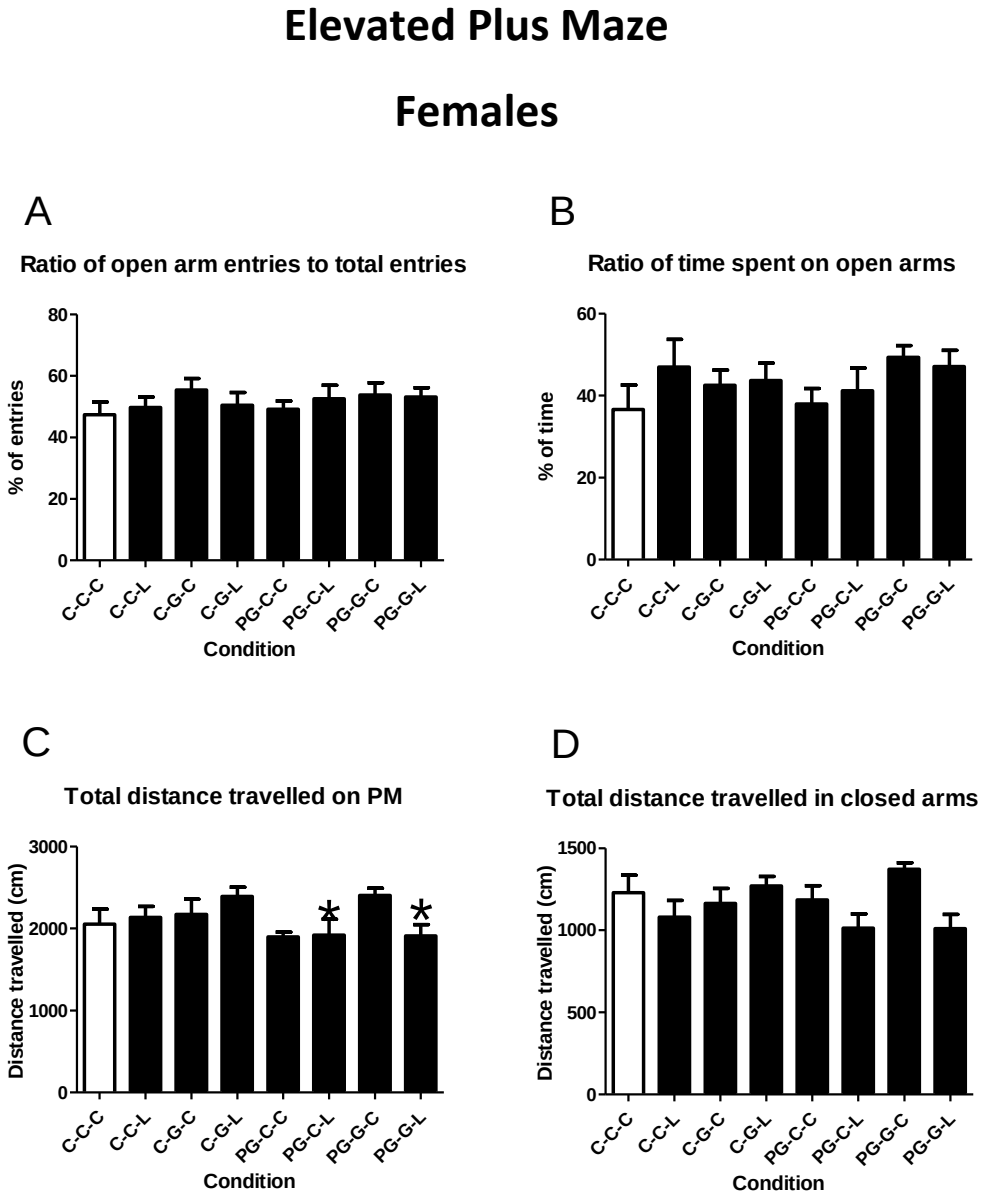

Fig. 2.9. The effect of CD feeding upon behaviour on the elevated plus maze in female offspring. A: Ratio of open arm entries B: Ratio of time spent on open arms C: Total distance travelled. D. Total distance closed arms. C-C-C (*n*=10), C-C-L (*n*=8), C-G-C (*n*=10), C-G-L (*n*=12), PG-C-C (*n*=14), PG-C-L (*n*=10), PG-G-C (*n*=6), PG-G-L (*n*=10). Significant interactions - C: Pregestation and lactation, as well as pregestation, gestation and lactation. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

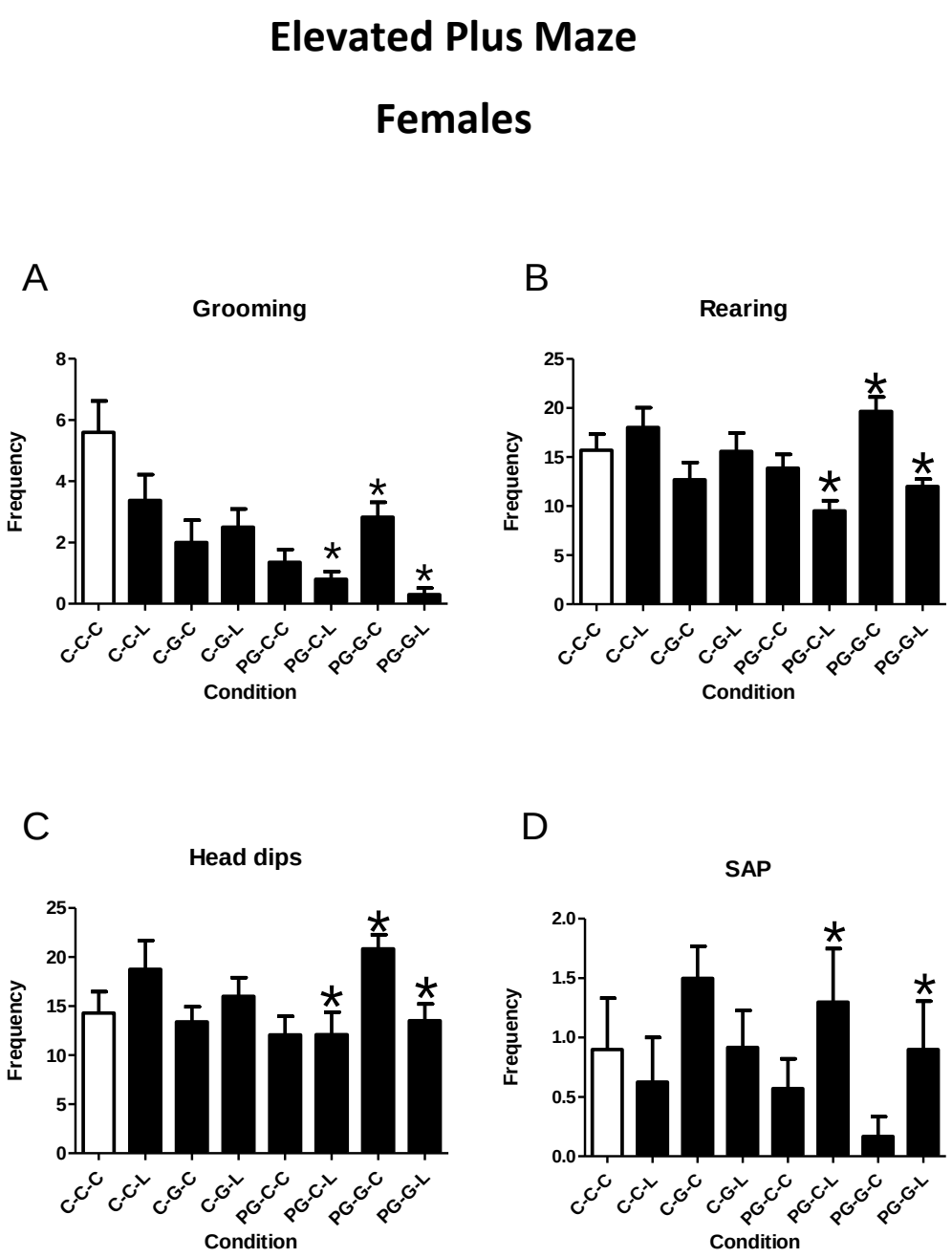

Fig. 2.10. The effect of CD upon behaviour on the elevated plus maze in female offspring. A: Grooming B: Rearing C: Head-dips D: Stretch-attend posture (SAP). C-C-C (*n*=10), C-C-L (*n*=8), C-G-C (*n*=10), C-G-L (*n*=12), PG-C-C (*n*=14), PG-C-L (*n*=10), PG-G-C (*n*=6), PG-G-L (*n*=10). Significant interactions - A: Pregestation and gestation, as well as pregestation, gestation and lactation. B: Pregestation and gestation, as well as pregestation and lactation. C: Pregestation and gestation, as well as pregestation, gestation and lactation. D: Pregestation and lactation. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

2.2.5 Open Field

2.2.5.1 *Males*

The effect of maternal CD on zone related parameters on the open field is displayed in Fig. 2.11A-D. Independent of any significant interactions, there was a significant main effect for CD feeding throughout lactation for the latency of time to enter the inner zone demonstrating a significant reduction in time elapsed in offspring of mothers exposed to maternal CD compared to those fed chow throughout the same phase ($F_{1, 63} = 7.38$, $P < 0.01$). Despite this the number of inner zone entries and time spent in the inner zone was unaffected by CD at any stage of the experiment (Fig. 2.11C&D).

Despite the shorter latency of inner zone entry and also independent of any significant interactions, there was a significant main effect of lactational CD for total locomotor activity in males on the open field ($F_{1, 65} = 7.44$, $P < 0.01$). This reflected a significant reduction in the average distance travelled in offspring of dams fed lactational CD compared to offspring maternally exposed to control chow throughout the same phase. Also pregestational CD interacted with CD during pregnancy to influence total locomotor activity in males on the open field ($F_{1, 65} = 8.66$, $P < 0.01$). Total distance travelled was increased in offspring of dams exposed to pregestational CD and CD during pregnancy (but not lactational CD), but reduced in offspring of dams exposed during both periods in conjunction to lactation (Fig. 2.11B).

The effect of maternal CD on ethological parameters in male offspring on the open field is displayed in Fig. 12A-B. Pregestational CD interacted with

maternal CD during gestation to influence grooming frequency in male offspring on the open field ($F_{1, 65} = 5.64$, $P < 0.05$). This reflected an increase in grooming in offspring from dams exposed to both pregestational and gestational (but not lactational CD), but a reduction in grooming in offspring exposed to CD throughout both earlier periods and also lactational CD (Fig. 2.12A). Additionally maternal CD during pregnancy interacted with maternal CD during lactation to influence rearing on the open field in male offspring ($F_{1, 65} = 7.39$, $P<0.01$). This reflected a decrease in this parameter of vertical exploration in offspring maternally exposed to CD during both pregnancy and lactation (2.12B).

Open Field

Males

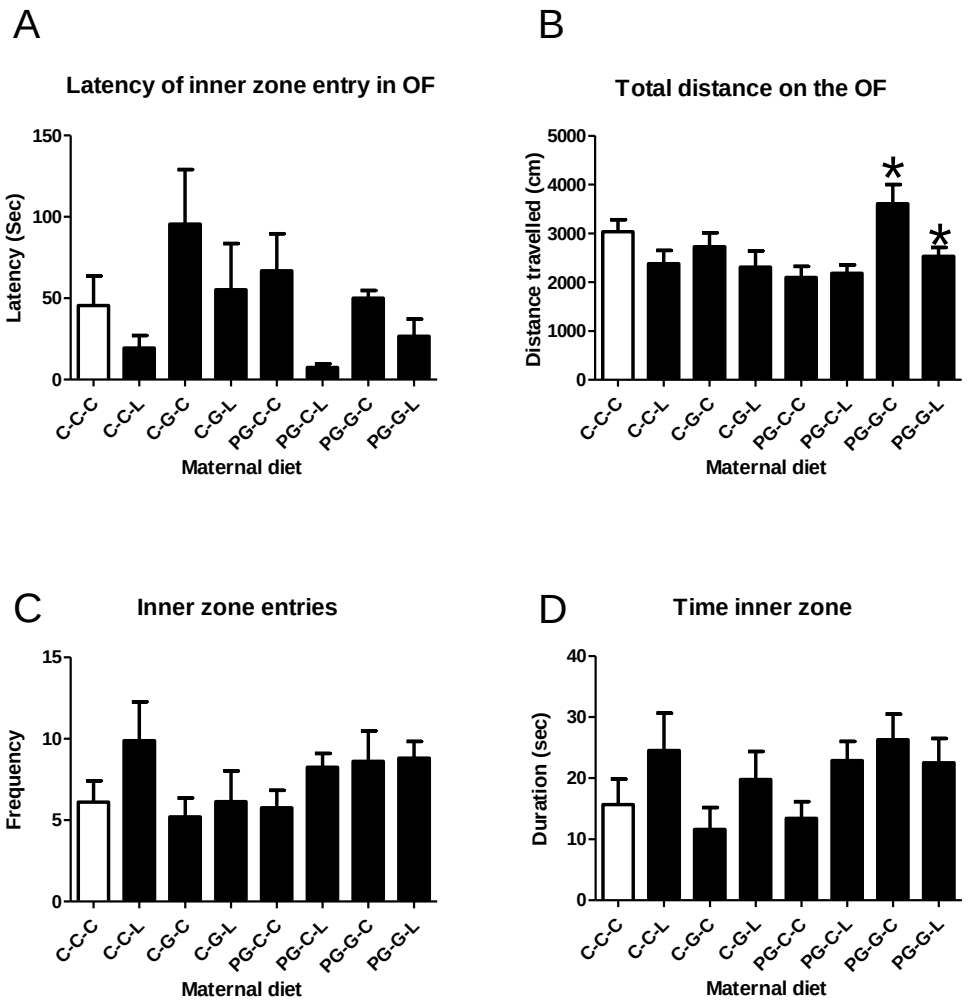

Fig. 2.11. The effect of CD upon behaviour on the open field in male offspring. A: latency B: distance travelled C: Inner zone entries D: time inner zone. C-C-C (*n*=9), C-C-L (*n*=8), C-G-C (*n*=5), C-G-L (*n*=9), PG-C-C (*n*=12), PG-C-L (*n*=8), PG-G-C (*n*=5), PG-G-L (*n*=10). Significant interactions - B: Pregestation and gestation. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

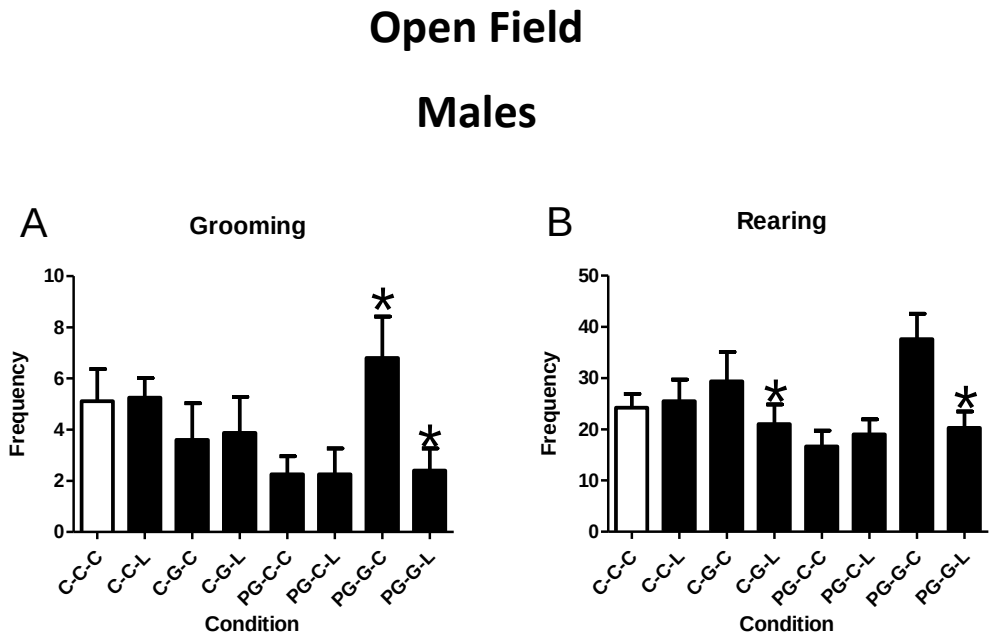

Fig. 2.12. The effect of CD upon behaviour on the open field in male offspring. A: Grooming B: Rearing. C-C-C (*n*=9), C-C-L (*n*=8), C-G-C (*n*=5), C-G-L (*n*=9), PG-C-C (*n*=12), PG-C-L (*n*=8), PG-G-C (*n*=5), PG-G-L (*n*=10). Significant interactions - A: Pregestation and gestation, as well as pregestation, gestation and lactation. B: Gestation and lactation. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

2.2.5.2 *Females*

The effect of maternal CD on zone related parameters on the open field in female offspring is displayed in Fig. 2.13A-D. Among female offspring there was no significant impact of maternal diet on latency to enter the inner zone, on time spent in the inner zone, or on total distance travelled (Fig. 2.13A-D). The effect of maternal CD on ethological parameters on the open field in female offspring is displayed in Fig. 2.14A-D. The effect of maternal CD during lactation interacted separately with both CD during gestation ($F_{1, 63}$ = 11.89, P <0.001), as well both the earlier feeding periods ($F_{1, 63}$ = 9.59, P <0.01) to influence grooming behaviour on the open field. This reflected a decrease in grooming in offspring maternally exposed to CD during lactation, but only in

offspring also exposed to either gestational CD in isolation, or offspring from dams exposed to both pregestational and gestational CD (Fig. 14.A).

Additionally pregestational CD interacted with gestational CD to influence the propensity to groom in females on the open field ($F_{1, 63} = 14.19$, $P < 0.001$). As in males, this reflected an increase in grooming in offspring from dams exposed to both pregestational and gestational CD (but not lactational CD), but a reduction in grooming in offspring exposed to CD throughout both earlier periods and also lactational CD (Fig. 2.14A). Maternal gestational and lactational CD interacted to influence rearing behaviour in females on the open field ($F_{1, 63} = 10.90$, $P < 0.01$), reflecting a reduction in this parameter of vertical exploration (Fig. 14.B). Independent of any significant interactions, there was a significant main effect of pregestational CD on rearing demonstrating a significant reduction in offspring exposed to CD during pregestation compared to those fed chow during the same phase ($F_{1,63}= 7.63$, $P < 0.01$).

Open Field

Females

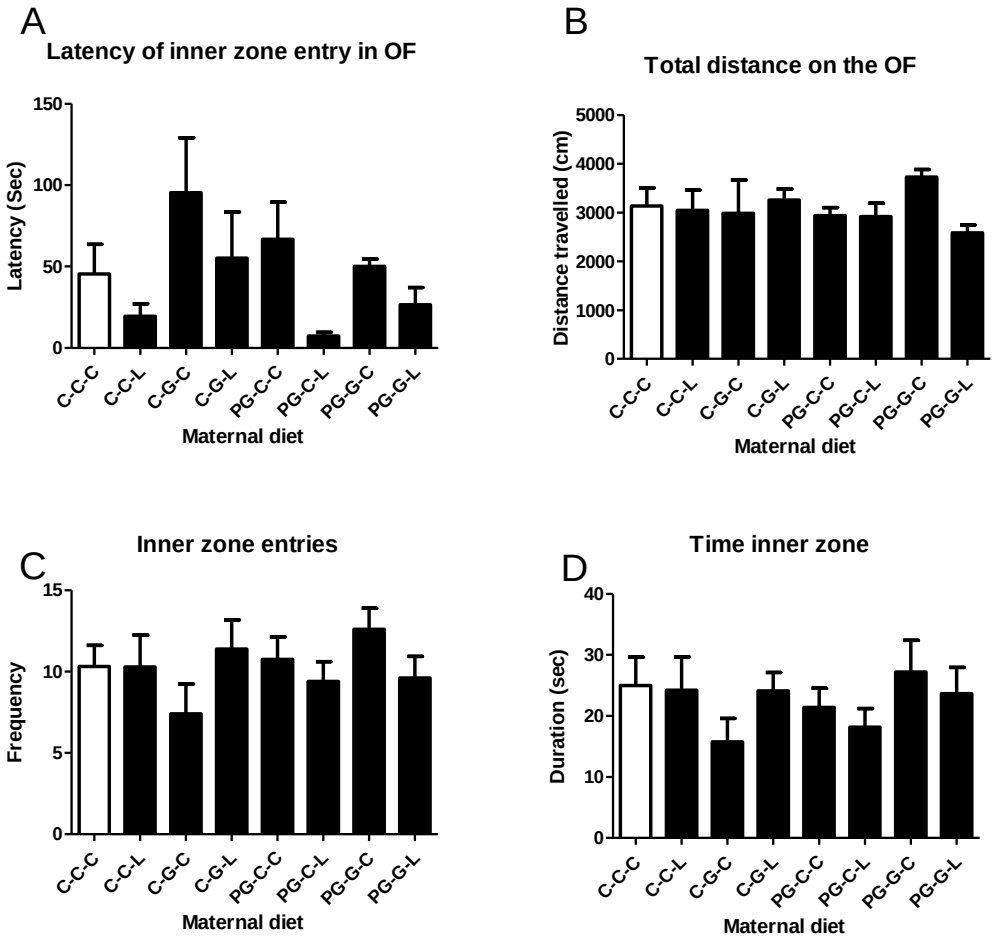

Fig. 2.13. The effect of CD upon behaviour on the open field in female offspring. A: Latency B: Distance travelled C: Inner zone entries D: Time inner zone. C-C-C (*n*=9), C-C-L (*n*=8), C-G-C (*n*=5), C-G-L (*n*=9), PG-C-C (*n*=12), PG-C-L (*n*=8), PG-G-C (*n*=5), PG-G-L (*n*=10). Data are shown as mean + SEM.

Open Field

Females

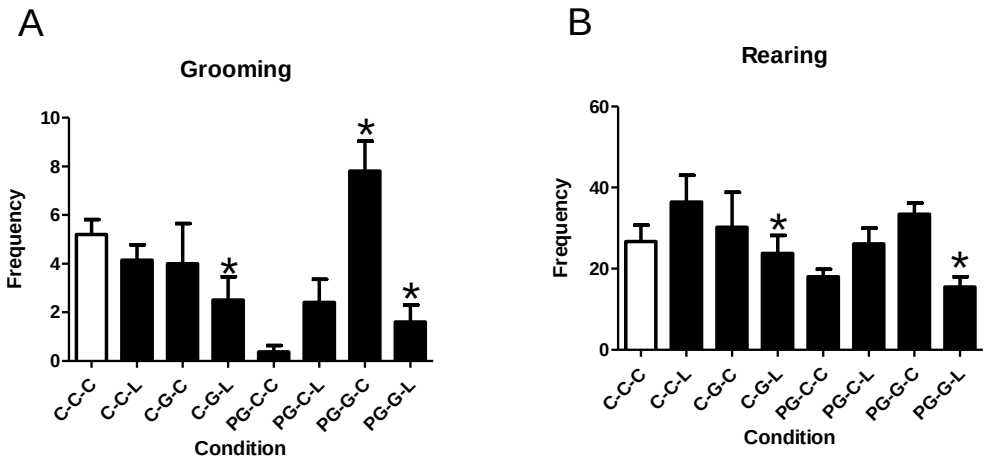

Fig. 2.14. The effect of CD upon behaviour on the open field in female offspring. A: Grooming B: Rearing. C-C-C (*n*=9), C-C-L (*n*=8), C-G-C (*n*=5), C-G-L (*n*=9), PG-C-C (*n*=12), PG-C-L (*n*=8), PG-G-C (*n*=5), PG-G-L (*n*=10). Significant interactions - A: gestation and lactation, as well as pregestation and gestation. B: Gestation and lactation. Data are shown as mean + SEM. * indicates group contributes to a significant interaction at P<0.05.

2.3 Discussion

The present study shows that maternal overnutrition prior to pregnancy, during gestation and in the suckling period had significant effects on anxiety-related and exploratory behaviour in rat offspring. Our experiment had a complex design, but this was necessary in order to fully model whether or not maternal obesity, or simply over-feeding on a fat- and sugar-rich diet, provided the programming stimulus leading to changes in offspring behaviour. As in the work of Akyol *et al.,* (2009), we found that feeding a cafeteria diet for 8 weeks prior to mating rendered young adult female rats obese (data reported in Akyol *et al*., 2011). As a result considering the offspring of rats exposed to CD pre-gestation allowed us to model the impact of maternal obesity during pregnancy independently of any influence of over-feeding. Introducing the cafeteria diet in pregnancy or lactation only, allowed consideration of the impact of over-feeding during fetal or neonatal brain development, whilst the other cafeteria fed groups enabled consideration of the interactive effects of maternal overweight and overfeeding. It was clear from our findings that cafeteria feeding at all stages of the experiment had some impact upon behaviour of the offspring.

Although pre- and postnatal CD did not significantly alter body weight later in life, small but significant effects on relative size of discrete fat pads indicated some programming of adiposity. The main effects resulted from CD feeding either during lactation, or during both gestation and lactation and were modest and confined to the gonadal and the intrascapular depot. Our findings

here differ from observations using other models of maternal obesity in rodents (Samuelsson *et al.,* 2008). Bayol *et al.,* (2008) employed an almost identical protocol but observed greater adiposity following maternal cafeteria feeding. These discrepancies are difficult to explain, but may relate to the Bayol study not using a pre-mating run-in to the introduction of cafeteria feeding, and employing a protocol where the variety in the cafeteria diet was greater than in the present study. Importantly our findings indicated that the influences of early life events on behaviour are not strongly related to obesity in adulthood.

Ostensibly this degree of relative adiposity was not represented by measures of whole body fat content. However, when comparing the contribution of each separate feeding phase of the experiment (PG vs. G vs. L) a modest increase in the body fat content of female offspring was noted, with main effects resulting from CD feeding during lactation, during both gestation and lactation, or when maternal obesity was combined with pregnancy or both the latter periods. The findings of the whole carcass composition analysis appear to be consistent with measures of adiposity, as there was some relative adiposity although this small was in magnitude compared to other studies. Bellinger *et al.,* (2004) reported that both male and female offspring subjected to protein restriction during pregnancy exhibited no difference in whole carcass fat content compared to controls. Although animals were culled at only twelve weeks of age and this experiment did not benefit from a complex design such as in the present instance. It is worth acknowledging

that the number of animals in each group for the whole body carcass analysis was very low making meaningful statistical analysis difficult.

Due to the fact that maternal CD during the lactation period programmed some relative adiposity in offspring, the effect of lactational CD feeding on plasma serum leptin concentrations was measured. The present study shows that maternal exposure to CD during lactation had no effect upon plasma serum concentrations of leptin in adult offspring of either sex compared to controls. The present control values for female offspring are consistent with those obtained from other laboratories using non-fasted Wistar rats (Mulet *et al.,* 2003; Gamaro *et al.,* 2008), however, the present control values obtained for male offspring were higher (approximately 90%) than control values obtained from other laboratories using non-fasted animals (Mulet *et al.,* 2003; Lamas *et al.,* 2004; Okere *et al.,* 2006; Yavuz *et al.,* 2004; Liu *et al.,* 2009). Male offspring exposed to CD also exhibited the same higher than average serum leptin concentration. Control values for male offspring obtained here are similar to plasma serum concentrations seen in rats fed CD directly over a prolonged period (De Shepper *et al.,* 1998; Lamas *et al.,* 2004).

This may be attributable to the fact the offspring in the present study were culled at an older age than in the studies mentioned above and so would have accumulated greater fat mass. In contrast to the present findings, Benkalfat *et al.,* (2011) demonstrated that adult offspring of Wistar rats exposed to maternal obesity induced by CD feeding prior to conception, as well as maternal CD feeding during gestation and lactation were hyperleptinaemic

compared to controls. This effect was exacerbated by direct CD feeding post-weaning. Hyperleptinaemia to such an extent is likely to co-occur with some degree of leptin resistance (De Shepper *et al.,* 1998). It is possible to infer from the findings above that increases in plasma serum leptin could have been observed in some of the groups not tested in the present study, perhaps in offspring that had been exposed to maternal obesity and/or CD feeding during pregnancy. Little can be inferred from the present finding other than that lactational CD did not alter plasma serum leptin concentrations in offspring and the observation that elevated concentration in males is difficult to explain. This is an indicator that the impact of maternal diet upon fat mass was inconsequential.

The complex experimental design made it possible to elucidate the contribution of each maternal feeding phase in considerably greater detail than considering each group individually. Based on this analytical approach, the behavioural results indicated a number of clear effects of maternal obesity as pre-gestational CD reduced the distance travelled and the frequency of grooming in males on the plus maze. Grooming was also reduced in females. Pre-gestational effects were frequently influenced by interactions with gestational and/or lactational CD, as shown by the reduced distance travelled by females on the EPM when exposed to pregestational and lactational CD and by the stimulatory effect on the open field locomotor activity, accompanied by reduced rearing seen in males.

Such pre-gestational and gestational interactions were also found for grooming in females upon EPM exposure, where both pregestational/gestational and pregestational/gestational/lactational CD decreased grooming, the latter combination also decreased grooming upon exposure to the open-field. In summary, the predominant effect of maternal obesity, either alone or in combination with later CD feeding periods, was a reduction of horizontal exploration/locomotor activity and a reduction of grooming, the latter possibly being a sign of reduced arousal or anxiety (Voigt *et al.,* 2005). Locomotor activity in the closed arms of the EPM, a more accurate measure of total locomotor activity independent of anxiety (Dawson*, et al.,* 1995; Collinson & Dawson, 1997), was also reduced by pregestational diet. Maternal obesity may therefore be a factor that can programme brain development independently of the diet per se. The mechanistic basis of this is unclear, but could relate to maternal interactions with offspring (Francis & Meaney, 1999; Champagne & Meaney 2006), or to numerous signals of maternal nutritional status that may be sensed during fetal development such as composition of maternal milk (Del Prado *et al.,* 1997; Pico *et al.,* 2007a; 2007b; Sanchez *et al.,* 2008). In the context of maternal undernutrition, disruption of the normal placental regulation of glucocorticoid transfer from mother to fetus may be one such signal (Langley-Evans, 2009) which may result in resetting of epigenetic marks, bringing about long-term changes to gene expression (Bogdarina *et al.,* 2010), or direct effects of glucocorticoids inducing alterations to brain structures (Langley-Evans *et al.,* 1996d).

Despite a clear impact of maternal obesity upon offspring behaviour, analysing the impact of individual maternal feeding regimens indicated that lactational CD was the most influential period in determining offspring anxiety-related behaviour. Overall, maternal CD during lactation led to an increased frequency and duration of open arm entries in male rats on the plus maze and a reduced the latency to enter the inner zone of the open field. The percentages of time spent on the open arms and entries into these arms have been validated as measures of anxiety-related behaviours (Pellow *et al.,* 1985). Pharmacological manipulations which increase the ratio of open arm entries to the total number of arm entries and increase the ratio of time spent in open arms relative to total time spent in the arms of the maze have been demonstrated to be anxiolytic (Pellow *et al.,* 1985). Although relatively small in magnitude, the increased ratio of open to closed entries seen after lactational CD was particularly interesting. The chow fed control rats here already showed an "anxiolytic" profile when compared with other plus maze studies using Wistar rats. Although such comparisons between laboratories can be obscured by environmental differences and breeding conditions (Rex *et al.,* 1996), previous studies (Bert *et al.,* 2001; Rex *et al.,* 1999; Thongsaard *et al.,* 1996) demonstrated between 31 and 36% open arm entries.

Rats with approximately 49% open arm entries have been described as "low anxiety" rats (Ho *et al.,* 2001.) The present high values for controls ranging between 46 and 48 percent (males) and 50 and 51 percent (females) could be explained by a high level of handling of these rats during lactation (regular monitoring of body weights and food intake). Handling has been shown to

have an anxiolytic effect on plus maze behaviour (Schmitt & Hiemke, 1998). As female rats on average do not prefer the closed arms of the plus maze, any anxiolytic effect would be very difficult to detect in females.

Evidence of an anxiolytic effect of the maternal CD during lactation, as shown in the plus maze test, was further supported by the reduced latency of rats to enter the inner zone of the open field. It has been suggested that this latency relates inversely to anxiety in the open field (Prut & Belzung, 2003), although other parameters might be considered as well. Locomotor activity is driven by exploration but may be reduced in rats with high baseline anxiety levels in the open field (Voigt *et al.,* 2005). In the present study, however, the reduced locomotor activity could possibly reflect reduced exploration as it accompanied reduced rearing, a parameter relating to vertical exploration. It is worth noting that there was an opposite effect of gestational and lactational CD on rearing frequency on the open field in male offspring demonstrating that the effect of maternal diet on exploratory behaviour may not always of been consistent across maternal feeding periods.

Bellinger *et al.,* (2006) reported a reduction in rearing in offspring of dams fed a low protein diet late in gestation compared to offspring fed a low protein diet in early and mid-gestation. Although there was relatively little impact of lactational CD on the behaviour of female offspring, a significant reduction in grooming behaviour was observed in both behavioural tests. Grooming is a self-directed behaviour. When shown upon exposure to aversive situations, as for example the plus maze and the open field, grooming can be interpreted as

a de-arousing activity. Anxious rats groom more often and anxiolytic drugs reduce grooming behaviour, whereas anxiogenic drugs facilitate grooming (Voigt *et al.,* 2005: Dunn *et al.,* 1981). Such an interpretation of the female grooming data would be in line with the observed anxiolytic effects of lactational CD on male offspring during the plus maze and open field tests. Male offspring from dams fed CD during lactation also groomed less upon exposure to the plus maze.

An anxiolytic effect of early postnatal overfeeding was also reported by Spencer and Tilbrook (2009). Raising rats in small litters not only induces obesity, but also reduces anxiety in the elevated plus maze and the open field. This effect was more obvious in females than in males. Whereas the findings by Spencer and Tilbrook generally support our results, a direct comparison with the present study is obscured by the different methods of overfeeding and the lack of obesity in the offspring studied in the present work. Further, it remains to be determined if litter size could impact on the behavioural outcome not only via nutritional factors, but also through changes in social interactions both between dam and pups and among the offspring themselves. Maternal behaviour itself is subject to influences of nutrition, and anxiolytic effects in the offspring could be down to modifications of maternal behaviour (Caldji *et al.,* 1998; Uriate *et al.,* 2007).

As discussed in the introduction, during the neonatal period environmental manipulations can alter the nature of mother-pup interaction, which can lead to epigenetic alterations and downstream effects upon gene expression in the

offspring (Meaney & Szyf, 2005; Zhang & Meaney, 2010; Caldji *et al.,* 2011). A study of dietary effects on maternal behaviour was beyond the scope of the present study, but would be an interesting point to cover in future investigations using procedures such as the pup retrieval test, or measuring the frequency and duration of maternal behaviours including LG-ABN. However, one could speculate that changes in maternal behaviour could contribute to the reduced anxiety as seen in the present study. Such a link has been shown, for example, following severe pre-gestational caloric restriction, however diet-induced augmentation of maternal behaviour does not necessarily translate into offspring behaviour if the dietary manipulation is modest (25% caloric restriction; Levay *et al.,* 2008). Nevertheless, a maternal behavioural contribution to the anxiolytic phenotype in the offspring cannot be excluded at present, but this would require confirmation by further investigations.

Apart from diet-induced changes in maternal behaviour, direct nutritional effects during lactation could programme development of offspring behaviour, a further hypothesis to be tested in future studies. Studies have reported that exposing mothers to high fat diet or CD during pregnancy and lactation can attenuate the protein content of milk but increase the fat content (Rolls *et al.,* 1986; Del Prado *et al.,* 1997). Additionally studies have shown that the administration of physiological doses of leptin during the lactation period can have a protective effect against obesity, and metabolic abnormalities in adult offspring, as well as altering food preferences (Pico *et al.,* 2007a; 2007b; Sanchez *et al.,* 2008). Factors such as macronutrient

composition or hormone content of maternal milk are obvious candidates for further investigation. Programmed anxiolysis has been shown in the past following severe perinatal protein malnutrition (Almeida *et al.,* 1996b) although impaired brain development also needs to be considered in such models (Morgane *et al.,* 1993).

It has been suggested that protein malnourished pups change their own behaviour through a reduced propensity to engage in rearing, climbing, feeding and locomotor behaviours, thereby modifying dam-pup interactions (Massaro *et al.,* 1977). The present findings are unlikely to be a consequence of protein malnutrition. Our analyses indicated that rats consuming CD were consuming approximately 13% protein in the diet by weight (Akyol *et al.,* 2008). The requirements for protein restriction during rat pregnancy are 12% by weight (Clarke *et al.,*1977). Compared to 18% protein content in chow, the CD protocol produced a modest protein reduction of approximately 28%. Previous studies showing anxiolysis induced by protein deficiency applied over a 60% reduction in protein intake (Almeida *et al.,* 1993; 1996b). The latter suggests that the reduced protein content in CD does not primarily account for the behavioural effect observed in the present study. As part of the same experiment Akyol *et al.,* (2011) ascertained that although mothers consuming CD did have a lower protein intake compared to mothers fed chow, the reduction was not significant. This is further supported by the fact that overconsumption of the highly palatable CD might even compensate for the reduction in protein intake due to dietary composition, thus bringing absolute

intake back to a level close to normal. The CD also increased maternal intake of salt, such that the animals consumed 20–30 mg sodium per day, compared to 10–12 mg/day consumed by chow fed animals. However, the impact on pup development would again be considered minimal as, although this level of sodium intake is marginally above the recommended minimum requirement for rat pregnancy and lactation (4–6 mg/day), it is well below the level of sodium fed to animals in "high salt diet" studies (typically 600 mg/day) (Coêlho *et al.,* 2006).

A recent study by Bilbo and Tsang (2010) seems to contrast with our results, as they indicated an anxiogenic effect of an obesogenic maternal diet. There are, however, key differences in experimental design. For example, these authors fed two different high fat diets (high-saturated-fat, high-trans-fat diet) and compared them to a group on low fat diet. Not only was the obesogenic diet not the same as our CD diet, the control diet was not equivalent to a standard chow diet. Furthermore Bilbo and Tsang presented limited evidence for an anxiogenic effect of maternal overfeeding as only a single behavioural parameter obtained from the plus maze was presented.

Despite the sparse experimental evidence from behavioural studies, there seems to be some coincidence with nutritional data. As mentioned before, both hypocaloric and hypercaloric pre-gestational, gestational and postnatal feeding can programme obesity in the offspring (Desai *et al.,* 1997a; 1997b; Wilson & Hughes, 1997; Bayol *et al.,* 2008; White *et al.,* 2009; Benkalfat *et al.,* 2011). These metabolic effects could possibly be paralleled by behavioural programming as previous reports showed anxiogenic effects of an energy

restricted maternal diet (Plagemann, 2005; Levay *et al.,* 2008). On the other hand we and others (Spencer & Tilbrook, 2009) could show anxiolysis after caloric overfeeding during sensitive phases of development. The balance of evidence appears that although both maternal caloric restriction and maternal over-feeding can programme obesity, their effects on anxiety-related behaviour are not necessarily the same. Such an interpretation would further suggest that the nutritional programming affects not only brain mechanisms involved in the control of body weight and feeding behaviour (McMillan *et al.,* 2005; Chang *et al.,* 2008), but also mechanisms involved in emotional behaviour. However, it remains a possibility that there is little causal relationship between these two nutritional programming effects. Although the present experimental approach provides a suitable model of maternal obesity (Akyol *et al.,* 2009), obesogenic diets can, at least in adult rats, change brain neurotransmitter turnover and behaviour independent of changes in body weight (Davis *et al.,* 2008).

Despite the fact that in isolation or, in varying combinations, maternal obesity and CD feeding during both the latter periods all contributed to the programming of emotional behaviour, it seems reasonable to assume that exposure to hyperenergetic diet during the lactation period had the biggest contribution. The anxiolytic effect was greatest in male offspring, although observed changes to grooming behaviour do suggest the effect may have extended to female offspring. The lactation period would be an obvious candidate for further investigations into effects of maternal CD feeding on offspring. Considering the literature demonstrating the programming of brain

neurochemistry in offspring by maternal or neonatal dietary perturbations (Plagemann, *et al.,* 1999; Davidowa & Plagemann, 2000; Davidowa *et al.,* 2002; Levin *et al.,* 2000; 2006) and the acknowledgment that neurotransmitter systems known to mediate emotional behaviour are also known to mediate other behaviours (Roth, 1994; Lucki, 1998), it may be reasonable to assume that other behaviours may be affected by maternal exposure to hyperenergetic diet during lactation. Behaviours related to appetite regulation and learning and memory would be obvious candidates for future investigation.

In summary, the present study demonstrates a nutritional programming effect on behaviour due to maternal obesity or overfeeding during early developmental phases. Pre-gestational, gestational and lactational high caloric maternal diet either alone or in interaction impact on the offspring's exploratory and anxiety related behaviours. The strongest effect was observed after feeding CD during lactation, as this reduced anxiety-related behaviour in the male offspring.

Chapter 3 - The impact of maternal cafeteria diet feeding during lactation upon feeding behaviour, learning and memory and brain turnover of 5-HT and DA in adult offspring

3.0 Introduction

The previous chapter not only demonstrated that feeding a cafeteria diet during all of the sensitive periods of early life had some effect upon offspring behaviour, but also that there is a complex relationship between maternal obesity and obesogenic diet during both the latter periods. Despite this, the findings underlined a dominant influence of maternal diet during the lactation period, relative to maternal obesity and gestational overfeeding in the programming of behaviour. With this in mind, the aim of the present chapter was to further investigate the consequences of maternal overnutrition during lactation on behaviour and to investigate potential mechanisms underlying such changes.

Literature described in *Chapter 1* suggests a possible role for maternal dietary manipulations during early critical periods of development in programming of feeding behaviour in adult offspring (Bellinger *et al.,* 2004; Bellinger & Langley-Evans, 2005; Bayol *et al.,* 2007; Orozco-Solis *et al.,* 2009). In the present study, in order to examine the consequences of maternal CD feeding during lactation upon the structural integrity of ingestive behaviour in adult offspring, the behavioural satiety sequence (BSS) was used. Described in greater detail in *Section 1.6.3 Chapter 1,* the BSS is widely utilized in behavioural science to investigate the impact of pharmacological, genetic or physiological manipulations on satiety mechanisms in rodents (Antin *et al.,*

1975; Halford *et al.,* 1998). The BSS integrates several behavioural parameters and analyses the predictable temporal transition from feeding through to grooming and to resting when an animal is exposed to a palatable test meal during a 1-hour test session. Using the BSS allows a detailed analysis of appetite related behaviour in rats enabling the identification of disturbances relating to post-prandial satiety mechanisms (Halford *et al.,* 1998).

A number of studies have also demonstrated impairments to performance on behavioural measures of learning and memory in adult offspring programmed by maternal undernutrition throughout pregnancy and lactation (Jordan *et al.,* 1981; Bedi *et al.,* 1991; Castro *et al*., 1989; Fukuda *et al.,* 2002). In order to investigate the consequences of maternal exposure to a hyperenergetic diet during lactation for learning and memory functions in adult offspring, we used an open field habituation test and a novel object discrimination (NOD) test.

Firstly, the open field habituation paradigm is a memory test known to measure habituation to a novel environment in rodents, by measuring changes to exploratory and locomotor behaviour with continued or repeated exposure to that environment (Bronstein, 1971; Rusell & Williams, 1973; Tamasy *et al.,* 1973). This can be across the same trial (intra-trial habituation), or across repeated exposures (inter-trial habituation) (Einon *et al.,* 1975; Leussis & Bolivar, 2006; Wilson & Linster, 2008). Although habituation has been purported to be a representation of simple memory in rodents, the mechanisms underlying habituation are thought to be complex. Perturbations to multiple neurotransmitter systems including serotonin, dopamine,

noradrenaline, acetylcholine and glutamate, as well as genetic manipulations have been reported to alter habituation to a novel environment (Leussis & Bolivar, 2006; Wilson & Linster, 2008). The NOD test has been used to investigate the impact of pharmacological, genetic or physiological manipulations on non-spatial working memory in rats and is dependent primarily upon activity in the hippocampus, rhinal cortices and frontal cortex (Ennaceur & Dalecour, 1988; Lehman *et al.,* 2007; Barker & Warburton, 2011). Performance in the NOD test has been reported to be sensitive to manipulations to a wide range of neurotransmitter systems including serotonin (King *et al.,* 2004; 2009), dopamine (Watson *et al.,* 2011), acetylcholine (Wooley *et al.,* 2003) and glutamate (King *et al.,* 2004; Kendall *et al.,* 2011).

The biogenic amine neurotransmitters serotonin (5-HT) and dopamine (DA) have been reported to mediate a wide range of behaviours making them both viable candidates for investigations into the mechanisms potentially underlying the altered behaviours reported in *Chapter 2*. It is known that acquisition of precursors essential for 5-HT and DA synthesis are entirely dependent on dietary provision of nutrients (Wurtman & Fernstrom, 1975; Fernstrom, 1976). 5-HT synthesis has been shown to be dependent upon dietary acquisition of the amino acid tryptophan (Green & Curzon, 1970; Knott *et al.,* 1974; Fernstrom & Wurtman, 1974), with DA synthesis dependent upon dietary acquisition of tyrosine (Wurtman & Fernstrom, 1975; 1976; Fernstrom, 1976). 5-HT and DA systems have been demonstrated to mediate a wide range of behaviours including those relating to: appetite

(Rodgers & Blundell, 1979; Blundell, 1977; 1984; 1992; Silverstone & Goodall, 1984; Halford *et al.,* 2004; 2007), anxiety (Handley & McBlane, 1993; Graeff *et al.,* 1996), depression (Graeff *et al.* 1996; Lucki, 1998) and learning and memory (McEntee & Crook, 1991; Palmer & DeKosky, 1993, Buhot *et al.,* 2000). In the present instance, we measured 5-HT and DA concentrations and turnover, at time of cull, in the hypothalamus, hippocampus and frontal cortex of adult offspring exposed to either maternal CD during lactation or a control chow. Such an undertaking was not to infer causation regarding changes to behaviour, but to examine the possibility of gross disturbance, or dysregulation of monoamine neurochemistry.

The hypothalamus was chosen for investigation due to its key contribution to energy homeostasis and appetite related behaviour in mammalian species (Brooks & Lambert, 1946; Miller *et al.,* 1950; Anand & Brobeck, 1951), the neurocircuitry of which has been demonstrated to be susceptible to programming manipulations such as maternal low protein diet, gestational diabetes, changes to litter size and plus others (Plagemann, *et al.,* 1999; Davidowa & Plagemann, 2000; Plagemann *et al.,* 2000a; Plagemann *et al.,* 2000b; Zippel *et al.,* 2000 Davidowa *et al.,* 2002; Muhlhausler, 2006; Muhlhausler & Ong, 2011).

The hippocampus was chosen for investigation due to its key contribution to learning and memory process in mammalian species (Drackman & Ommaya, 1964; Grossman & Mountford, 1964), the neurocircuitry of which has also been demonstrated to be susceptible to programming manipulations

including maternal malnutrition during gestation (Castro *et al.,* 1989; Bedi, 1991; Cordoba *et al.,* 1994; Fukuda *et al.,* 2002).

The frontal cortex was chosen for investigation due to its key contribution to executive function in mammalian species (Gerbner, 1972; Lukaszewska, 1974; Haas, 2001), the neurocircuitry of which has also been demonstrated to be susceptible to programming manipulations including maternal malnutrition during early life (Bedi *et al.,* 1980; Warren & Bedi, 1990).

Firstly, it was hypothesised that maternal CD during lactation would perturb feeding behaviour in adult offspring, leading to subtle alterations to the structural integrity of behaviours related to energy intake measured by the BSS, potentially resulting in a delay in the onset of satiety compared to controls. Secondly, it was hypothesised that maternal CD during lactation would lead to deficits in performance on measures of learning and memory in adult offspring. Thirdly, it was hypothesised that offspring of mothers fed CD during lactation would exhibit alterations in the concentrations of the neurotransmitters 5-HT and DA, as well as their metabolites and respective turnover in the hypothalamus, hippocampus and frontal cortex, when culled in adulthood.

3.1 Experimental Procedures

3.1.1 Animal procedures

Animals were maintained under identical conditions to those described in *Chapter 2*. Virgin female Wistar rats were housed individually with sawdust as cage substrate and with *ad libitum* access to food and water. Animals were matched with a male stud and mated at 12 weeks of age. At birth offspring were culled down to 8 pups per litter, split equally by sex, with litters being allocated to be fed either a standard laboratory chow diet (control), or fed the same chow in conjunction with CD. At the end of lactation (21 days after delivery) the offspring were weaned from their dams, group housed with littermates of the same sex and maintained on the chow control diet for remainder of the study. Offspring were humanely culled as young adults at 20 weeks of age post-partum via gradual exposure to CO_2 and cervical dislocation.

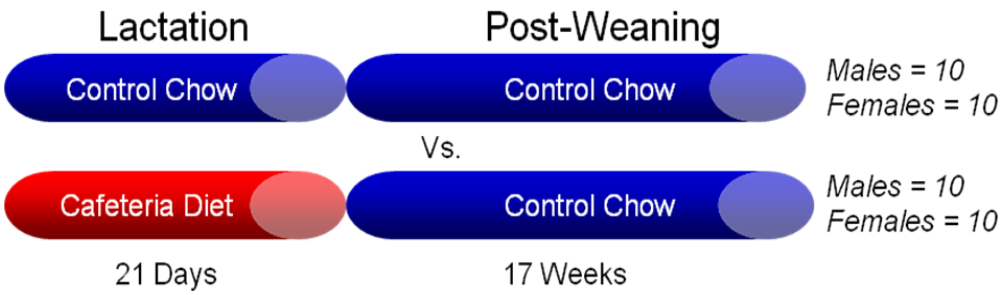

Fig. 3.1. Schematic representation of the experimental design. Rats were either fed on cafeteria diet (red box) or chow (blue boxes) during lactation. All offspring were fed chow after weaning (n = 10/ group). For details refer to Experimental Procedures.

3.1.2 Maternal macronutrient intake

Macronutrient intake of lactating mothers was recorded for ten days post-partum. The amount of protein, carbohydrate, sugar, unsaturated fat, saturated fat, fibre and salt consumed was calculated using the nutritional values for each product and by weighing each food item in and out of the cage each day. Intake was recorded for only the first the ten days post-partum of the twenty-one day lactation period due the fact that towards the end of the lactation period pups may start to consume food directly.

3.1.3 Ingestive behaviour

3.1.3.1 Behavioural satiety sequence (BSS)

Behavioural testing occurred at 13-15 weeks of age. The BSS analysis used here was adapted from the methodology described by Halford *et al.,* (1998). Experiments took place in a room that was separate from the normal holding area, maintained under the standard laboratory conditions described in section 2.15. All BSS testing was performed between 13:00-16:00 hours. Animals were habituated to the test arena and the test meal for 1 hour on the day immediately prior to day of test. The test arena consisted of a plastic box (54cm × 38cm × 40cm). Pilot investigations revealed a reluctance to eat when in a non-deprived state so on the day of testing, chow was removed from the cages of the animals being tested, 3 hours prior to being placed in the test arena. This facilitated food intake during the BSS experiment.

The test meal consisted of wet mash prepared 1 hour prior to the beginning of the test session. 60g of chow was left to soften in 60ml of boiling water. The food was then homogenized to a uniform texture and consistency using a pestle and mortar, 5 minutes before the start of the test session. Animal behaviour was observed using a video camera (Sanyo, Japan) and was tracked and analysed using Ethovision 3.1 (Noldus, Netherlands). The experimenter was absent from the test room throughout the duration of the test session. The amount of food consumed throughout the 1 hour test session was determined by weight. Behaviours essential for the BSS (eating, resting and grooming) were recorded, as well as the additional parameters rearing sniffing and locomotor behaviour. Frequency and duration of all behaviours were recorded, with latency recorded as an additional variable for eating and resting behaviours. Analysis of feeding bouts was performed by splitting the completed test session into 360 × 10 second time bins. The start of a feeding bout was defined as the presence of feeding behaviour in three or more consecutive 10 second time bins, with the end of a feeding bout defined as the absence of feeding behaviour in two or more consecutive 10 second time bins. Sporadic incidences of feeding behaviour were excluded from feeding bout analysis.

3.1.3.2 Micro-structural analysis and the BSS

To analyse behaviour across the 1 hour session the test session was divided into 12 × 5 minute time bins. This allowed measurement of the temporal transition between behaviours, throughout the observation period. Specific

attention was paid to the expression of eating, grooming and resting behaviour. The transition from eating to resting is seen as the onset of behavioural satiety (Halford *et al.,* 1998). To quantity the onset of satiety, a line graph was generated for each animal to measure the length of time elapsed throughout the 1-hour session before average duration of resting overtook eating as the dominant behaviour.

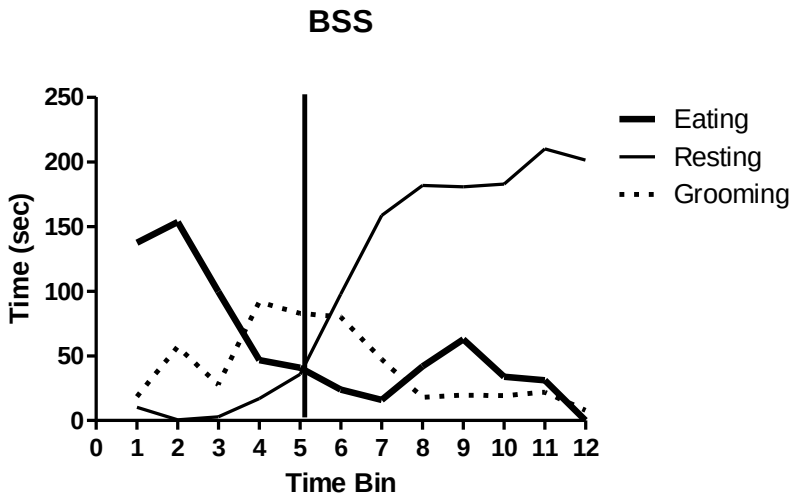

Fig.3.2. A conventional behavioural satiety sequence (BSS) across a 1-hour observation period from our laboratory. Throughout the initial time bins eating behaviour is the dominant behaviour declining as time progresses. In the latter half of the 1-hour observation session resting is the dominant behaviour with grooming behaviour acting an intermediary between the two. The vertical line emanating from the *x*-axis marks the transition between eating to resting and thus the onset of satiety.

3.1.4 Learning and memory

3.1.4.1 Open field habituation (OFH)

Habituation to a novel environment has been purported to be a reliable measure of simple memory in rodents (Bronstein, 1971; Rusell & Williams,

1973; Tamsay *et al.,* 1973; Enion *et al.,* 1975; Leussis & Bolivar, 2006; Wilson & Linster, 2008). Animals were tested in the open field 11 weeks after birth. The open field test apparatus was identical to that used in *Chapter 2*. Light intensity was reduced to 60lx to facilitate exploration. Animals were exposed to the open field for five minutes on day one and five minutes on day two, with the two exposures occurring exactly 24-hours apart. The test apparatus was disinfected after each animal. Total distance travelled (cm) and frequency of rearing was recorded. At the end of the 5 minute test period animals were returned to the home cage. In the open field test, significant reductions in parameters including total distance and vertical exploratory behaviour between day 1 and 2 have been postulated to be signs of habituation to the open field, thus implying that the animal is familiar with the object and is less likely to explore (Leussis & Bolivar, 2006; Wilson *et al.,* 2008).

3.1.4.2 Novel object discrimination (NOD)

The novel object discrimination task was originally devised by Ennaceur and Delacour (1988) and is based upon the principle that rodents have a natural propensity to explore a novel object relative to a familiar object when presented with a choice between the two. Experiments conventionally consist of a familiarisation trial when the animal is familiarised with two identical objects and then after an interval, a test trial when animals are presented with a familiar object and with a novel object. The amount of time the animal spends exploring the novel object relative to the familiar object during the test trial has been purported to represent memory of the familiar object and

is conventionally susceptible to decay over a 24-hour period (Ennaceur & Delacour (1988). The procedure is advantageous compared to operant measures or spatial measures of memory (some of which are described in *Section 1.2.2.3*) as it does not rely upon either positive or negative reinforcement processes, or a 'reference memory' component such as rule learning. NOD has been asserted to be a reliable and clean measure of non-spatial declarative working memory in rats (Ennaceur & Delacour, 1988).

The methodology used in the present study was based on the procedure reported by King *et al.* (2004). The test arena consisted of a plastic box (54cm × 38cm × 40cm). The objects within the test arena used for NOD were 150ml water-filled plastic bottles with three horizontal strips of either white or black masking tape. The objects were positioned 13cm from the length side of the arena, 11cm from the width side of the arena with colour of the object being randomly assigned for each animal during the training schedule. After each animal had been present in the test arena, the arena and objects were cleaned thoroughly with a 70% alcohol solution to eliminate any confounding olfactory cues which may affect behaviour.

The day before testing, each animal was placed within the observation arena for 1 hour with no objects present to habituate to the arena. On the day of testing animals received an additional 3 minute habituation session and were returned to the home-cage for 1 minute, before being placed into the observation arena with two identical objects for 3 minutes. Each animal was then returned to the observation arena after a 1, 2 or 4 hour interval in the

home-cage with a similar, but novel, object present. Video files were revisited and analysed manually using Ethovision 3.1 after the session, with rearing and duration of contacts being recorded. The duration of time spent with each object was calculated as the percentage of time spent exploring both objects.

3.1.5 5-HT and DA concentrations and turnover

3.1.5.1 Brain neurotransmitter content and metabolism

For neurotransmitter determination, rats exposed to maternal CD and their corresponding controls were culled at the age of 20 weeks. To eliminate circadian effects, culling occurred at the same time of day as the behavioural experiments were performed. Immediately after culling, brains were removed and placed upon a glass plate mounted on an ice filled container. The hypothalamus, hippocampus and frontal cortex were carefully dissected and placed in liquid nitrogen before being stored in a freezer at -80°C, for 1 month prior to analysis.

For sample preparation, tissue was weighed and placed in a perchloric acid working solution (0.05% PCA, 0.02% sodium metabisulphate, 0.01% EDTA). Tissue was homogenised on ice using a sonic probe (Soniprobe 150, output 20, 20-30 seconds). Each sample was then placed in 1.5ml centrifuge tube and centrifuged at 14000 rpm (approx 17500g in a Harrier 18/80 centrifuge) at 4°C for 20 min. Supernatant was removed and filtered through 0.45 µm PVDF 4 mm syringe filters immediately before analysis.

3.1.5.2 High-performance liquid chromatography with electrochemical detection

High performance liquid chromatography with electrochemical detection was used to measure 5-HT (5-hydroxytryptamine, serotonin), the 5-HT metabolite 5-HIAA (5-hydroxyindolacetic acid), DA (3, 4-dihydroxyphenethylamine, dopamine) and the two dopamine metabolites DOPAC (3,4-dihydroxyphenylacetic acid) and HVA (homovanillic acid). Samples were analysed using a CuO_4 detector connected to a VT-03 cell with a glassy carbon working electrode operated at 0.7V vs Ag/AgCl. (Antec, Netherlands), a PU980 pump (Jasco Pump PVT. Ltd, India), a Rheodyne injection valve (7125 injection valve; IDEX Corp, USA), a 4.6 x 150mm sphereclone, 5 µm ODS(2) Column (Phenomenex, UK) and a Chromjet integrator (Newport Spectra-Physics Ltd, UK). The mobile phase consisted of 0.05M KH2PO4 (Sigma), 0.1mM EDTA (Sigma), 0.32mM octane sulfonic acid (Sigma) and 13% methanol, adjusted to pH 2.8-3 with orthophosphoric acid (Sigma). Mobile phase was run at a flow rate of 1ml/min. Calibration standards of DA, DOPAC, HVA, 5-HT and 5-HIAA were run three times daily before, midway and after running brain samples.

3.1.6 Statistical analysis

All statistical analysis was performed using the Statistical Package for Social Sciences (version 16; SPSS, Inc., USA). Values are, if not otherwise stated, expressed as mean values with their standard errors. $P < 0.05$ was considered statistically significant for all tests. Measures of total maternal macronutrient intake, energy intake and the percentage of human food vs. control diet

consumed by CD mothers was analysed with a Students *t*-test. Maternal macronutrient and energy intake across the ten days post-partum was analysed using two-way repeated measures analysis of variance (day x diet). Differences in macronutrient intake at individual time points during the ten days post-partum were analysed with Student's *t*-tests adjusted for using the Bonferroni correction. For the offspring, with the exception of food intake during the test meal and brain neurotransmitter content, data from males and females were analysed independently. Post-weaning body weight was analysed using two-way repeated measures ANOVA (diet x time).

Behavioural measures of eating, grooming and resting were analysed with Student's *t*-test. Microstructure of behaviour and the BSS were analysed using two-way repeated measures ANOVA (diet × time). Where data sets did not pass Mauchly's Test of Sphericity, Greenhouse-Geisser significance levels are reported. Behaviour on day 1 and 2 of the OFH and percentage of time spent with each object on the training and test sessions of the NOD were analysed using a paired *t*-tests or the non-parametric equivalent adjusted using the Bonferroni correction. DA, DOPAC, HVA, 5-HT and 5-HIAA concentrations were analysed using a two-way independent measures ANOVA (diet x sex) with *post-hoc* Tukey-Test, or, when sphericity was not assumed, Games-Howell test.

3.2 Results

3.2.1 Maternal bodyweight and macronutrient intake

There were no differences in bodyweights of the two groups of dams across the lactation period, with exception of week one when control dams weighed significantly less than mothers exposed to CD (t=4.52, d.f=7, P<0.001) (Fig. 3.3A). As shown in Fig. 3.3B-C, mothers exposed to CD consumed significantly less protein (0.61 ± 0.2g/d vs. 0.95 ± 0.05g/d in controls, t=6.25, d.f=6, P<0.001), carbohydrate (1.75 ± 0.06g/d vs. 2.90 ± 0.15g/d in controls, t=7.01, d.f=6, P<0.001) and fibre (0.13 ± 0.01g/d vs. 0.19g/d vs. 0.01 in controls, t=8.27, d.f=6, P<0.001), compared to mothers fed just the control chow. Mothers exposed to CD consumed significantly more saturated fat (0.97 ± 0.03g/d vs. 0.30 ± 0.02g/d in controls, t= 19.19, d.f=6, P<0.001), unsaturated fat (0.39 ± 0.02g/d vs. 0.04 ± 0.01g/d vs. controls, t=17.32, d.f=6, P<0.001) and sugar (0.55 ± 0.01g/d vs. 0.24 ± 0.1g/d in controls, t=15.25, d.f=6, P<0.001), per day compared to those fed control chow (Fig. 3.3B-C).

As shown in Fig. 3.4A, mothers exposed to CD consumed significantly less protein (10.58 ± 0.35% vs. 19.10 ± 0.02%, t=24.06, d.f=6, P<0.001) and more fat (9.50 ± 0.28% vs. 4.84 ± 0.11%, t=19.68, d.f=6, P<0.001) and sugar (23.53 ± 0.8% vs. control 7.03 ± 0.01%, t=15.28, d.f=6, P<0.001), when expressed as a percentage of total macronutrient intake, compared to mothers fed control chow. Fig. 3.4B shows the percentage of palatable human foods compared to rat chow consumed by the mothers on the CD feeding regimen, showing a significantly greater percentage of human food (77.50 ± 3.31% vs. 24.68 ±

2.40% control chow) being consumed over the first ten days post-partum compared to the chow diet (t=12.92, d.f=6, P<0.01).

Fig. 3.5A shows the energy intake of the mothers across the first ten days post-partum. The amount of energy consumed increased from day one post-partum to day ten in all animals ($F_{1.64\ 9.85}$=4.90, P<0.05), although no day x diet interaction occurred. Fig. 3.5B shows that there was no significant difference in the total energy consumption, between groups during the first ten days post-partum.

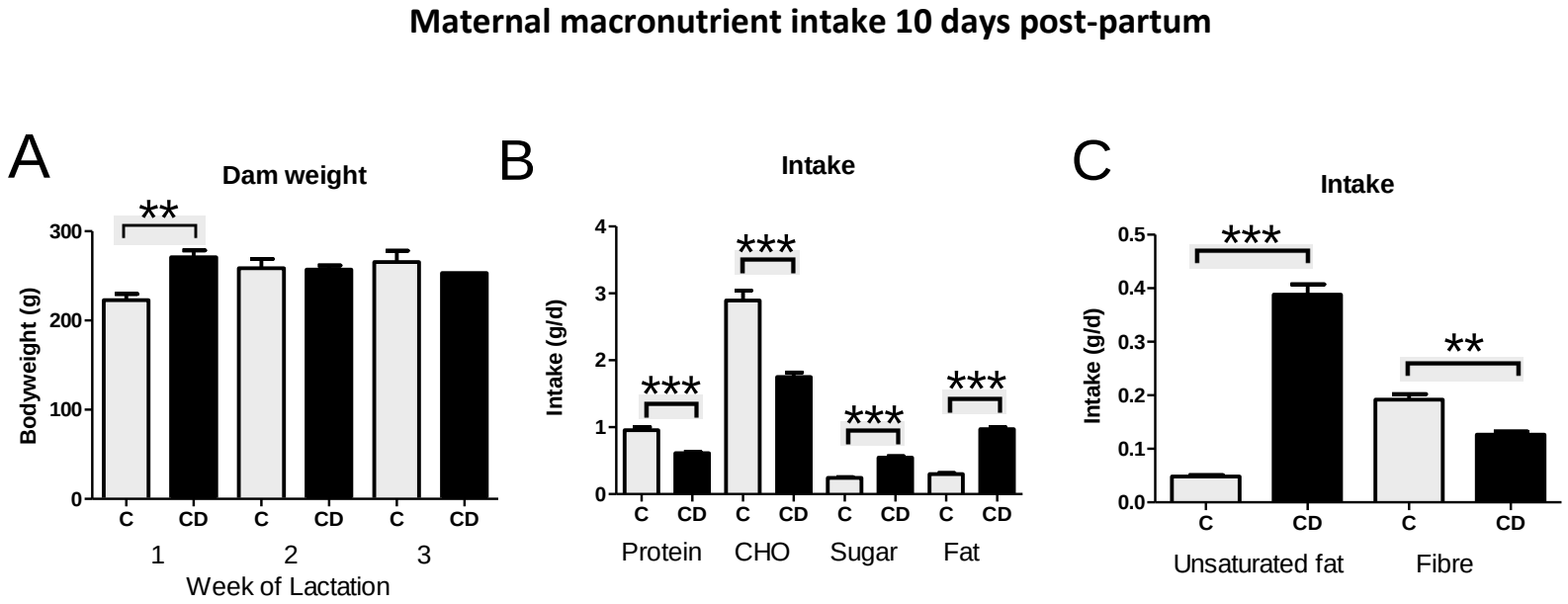

Fig. 3.3 Bodyweight of dams during the three week lactation period and macronutrient intake in mothers either fed cafeteria diet (CD) or control chow (C) during the first 10 days post-partum. A. Bodyweight of mothers during lactation B. Protein, carbohydrate (CHO), sugar and Saturated (CD n= 4, C n=4). C. Unsaturated fat and fibre (CD n= 4, C n=4). **= P<0.01, ***=P<0.001.

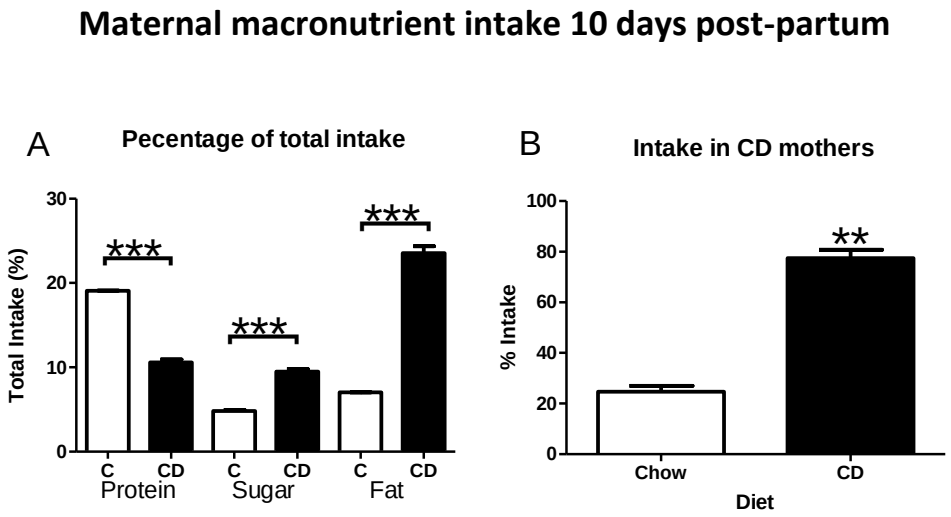

Fig. 3.4 Macronutrient intake in mothers either fed cafeteria diet (CD) or control chow (C) during the first 10 days post-partum and the amount of human food vs. chow diet consumed by CD mothers. A. Percentage of protein, sugar and fat of total intake of all macronutrients (carbohydrate, fibre and salt not shown) (CD n= 4, C n=4). B. The percentage of human food vs. chow consumed in mothers exposed to CD (CD n= 4, C n=4). **= P<0.01, ***=P<0.001.

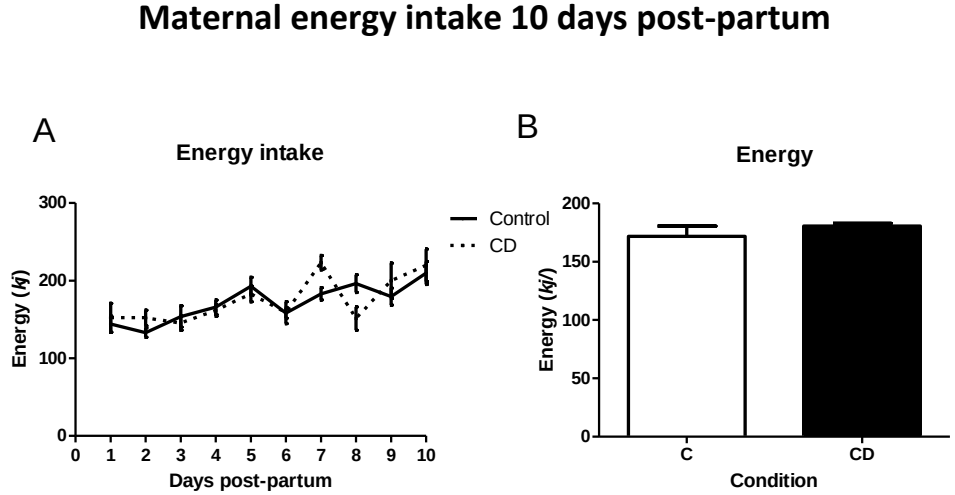

Fig. 3.5 Energy intake in mothers either fed cafeteria diet (CD) or control chow (C) during the first 10 days post-partum. A. Maternal energy intake across from day one to ten post-partum (CD n= 4, C n=4). B. Total energy intake consumed during ten days post-partum (CD n= 4, C n=4). *= P<0.05.

Fig. 3.6 shows maternal macronutrient intake post-partum. As seen in Fig. 3.6A, the average amount of protein consumed throughout the first ten days post-partum in both groups increased ($F_{3.80\ 22.79}$ =20.97, P<0.001). The

treatment x day interaction reached significance ($F_{3.80\ 22.79}$=3.40, P<0.05) with mothers exposed to CD consuming less protein during days one (*t*=5.06, d.f.=6, P<0.05), two (*t*=6.36, d.f.=6, P<0.05), four (*t*=4.07, d.f.=6, P<0.5), five (*t*=5.17 d.f.=6, P<0.05), seven (*t*=5.26, d.f.=6, P<0.05) and eight (*t*=7.17, d.f.=6, P<0.01) post-partum compared to controls (Fig. 3.6A). For carbohydrate there was no significant effect of day, or significant treatment x day interaction demonstrating that intake did not differ over time or between groups across the first ten days post partum (Fig. 3.6B).

As seen in Fig. 3.6C, the amount of unsaturated fat consumed throughout the first ten days post-partum increased in both groups ($F_{1.64\ 9.83}$=5.35, P<0.05), although changes in consumption were not influenced by maternal diet as there was no significant treatment x day interaction. There was no significant effect for day, or treatment x day interaction for the amount of saturated fat or sugar consumed across the first the days post partum (Fig. 3.6D-E). Despite a significant increase in salt consumption in both groups during the first ten days post-partum ($F_{1.78\ 10.70}$=4.46, P<0.05), the treatment x day interaction did not reach significance demonstrating the increase in consumption was independent of maternal diet (Fig. 3.6F).

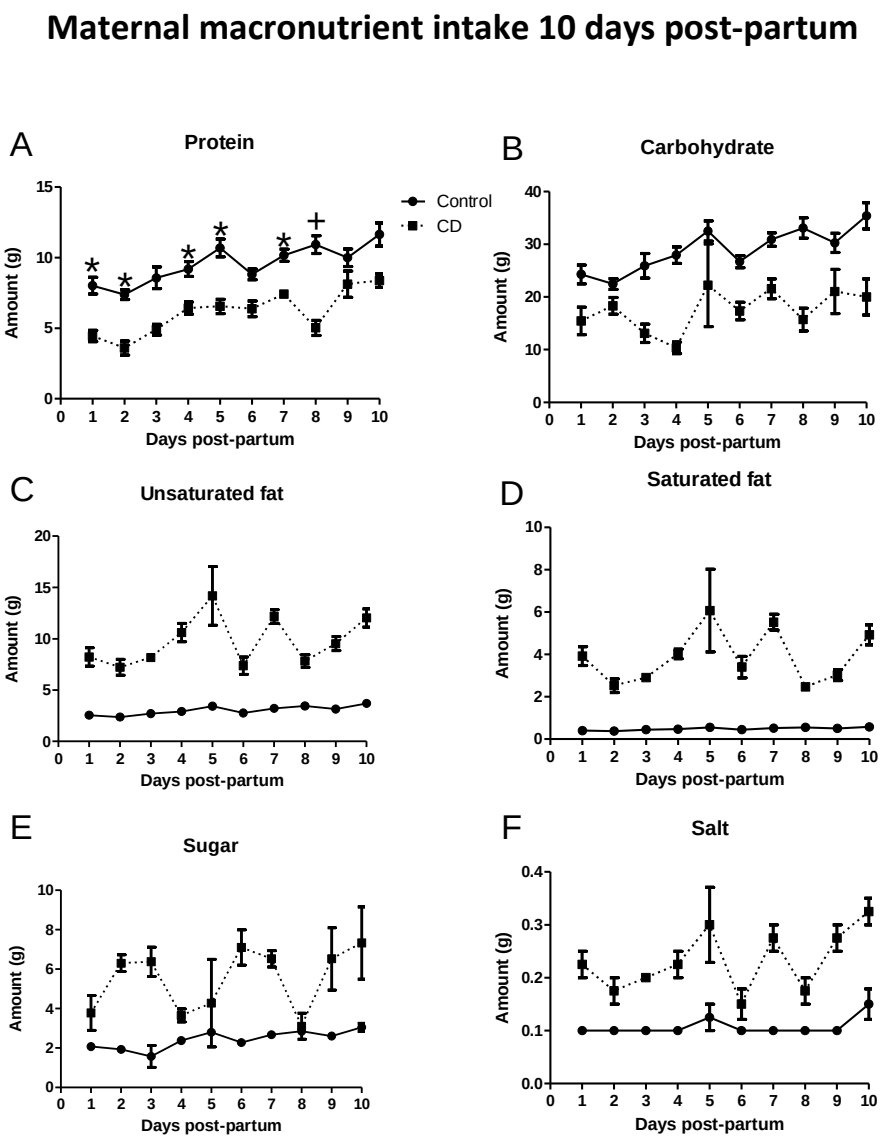

Fig. 3.6 Macronutrient intake in mothers either fed cafeteria diet (CD) or control chow (C) during the first 10 days post-partum. A. Protein (CD n= 4, C n=4). B. Carbohydrate (CD n= 4, C n=4). Unsaturated fat (CD n= 4, C n=4). D. Saturated fat (CD n= 4, C n=4). E. Sugar (CD n= 4, C n=4). F. Salt (CD n= 4, C n=4) * = P<0.05, + = P<0.01.

3.2.2 Offspring body weight

There was no effect of maternal diet upon body weight of offspring throughout post-weaning weeks one to twelve (Fig. 3.7A and B). There were also no differences in food intake between offspring maternally exposed to chow or cafeteria diet (CD) (data not shown).

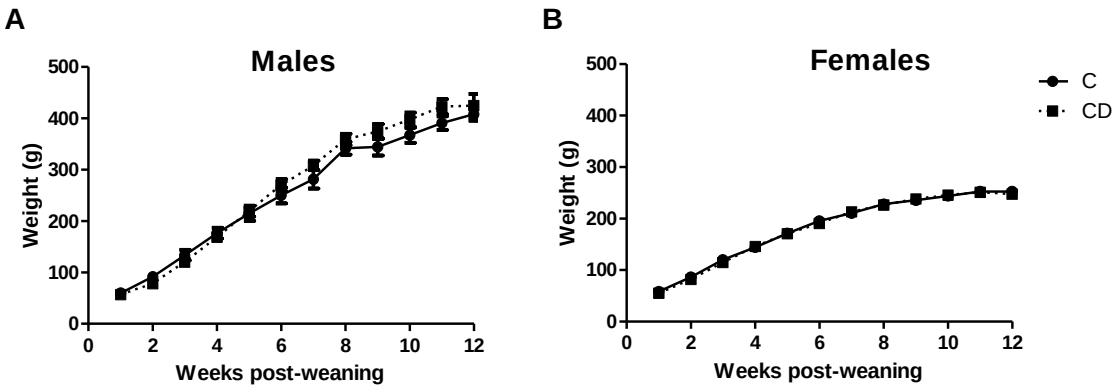

Fig. 3.7 The effect of maternal cafeteria diet (CD) upon post weaning bodyweight development. Control rats (C) were chow fed during lactation. A. Male offspring (n= 10/group). B. Female offspring (CD n= 12, C n=11).

3.2.3 Behavioural satiety sequence

3.2.3.1 Test food intake and 1 hour behavioural scores

Fig. 3.8A shows that there was no effect of the maternal diet upon total food intake of the animals during the BSS test session. The higher mash consumption in males compared to females ($F_{1, 22}$=6.90, P<0.05) was in line with the overall higher and body weight-related food intake in males. There was also no significant effect of maternal CD on the latency to feed in either sex. Male controls started feeding on average after 81.2± 33.3 seconds of the test, compared to 26.2 ± 19.0 in the CD group. Females started to feed after 41.1±13.0 seconds (control = C) or CD: 61.4 ± 14.6 (CD), respectively (Figure 3.8B).

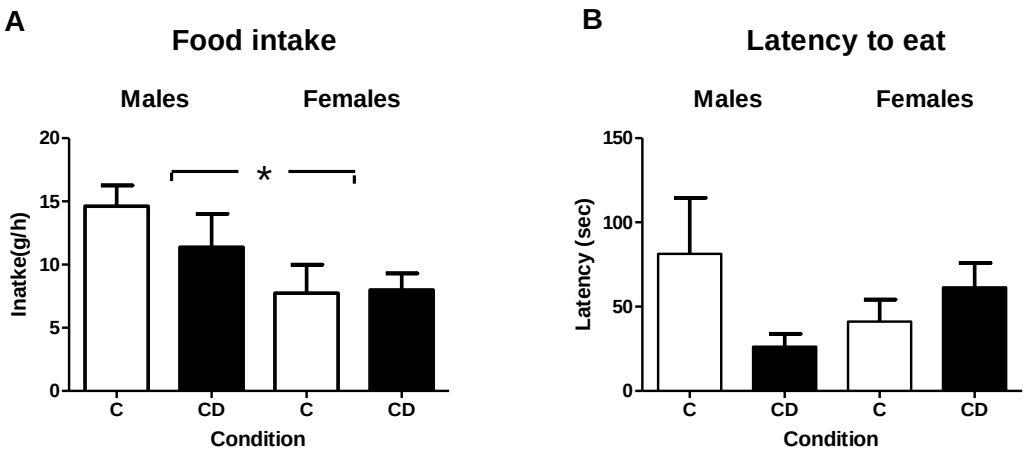

Fig. 3.8 The effect of maternal CD (black columns) upon intake of test mash (A) and latency to start eating (B) Male offspring (CD n=8, C n= 6). Female offspring (n=7/group). * P<0.05 between males and females.

3.2.3.2 Behavioural measures of eating, grooming and resting (males)

Among the male offspring there was evidence that the rats exposed to maternal CD during lactation engaged in a greater frequency of eating during the 1 hour BSS test, with more than double the number of feeding episodes seen in controls (Fig. 3.9A, *t*=2.33, d.f. =12, P<0.05). Despite this difference the overall amount of time spent feeding was not significantly different to the control animals (Fig. 3.9B). This might be explained by the findings of the analysis of feeding bouts (Fig. 3.9C-D). Although the overall bout frequency was higher in the CD group (Fig. 3.9C, *t*=4.4, d.f.=12, P<0.001), the mean duration of feeding bouts was shorter (Fig. 3.9D, *t*=2.28, d.f.=12, P<0.05). CD males showed an increased latency to rest (*t*=4.42, d.f.=12, P<0.001, Fig. 3.10A). The duration and frequency of time spent grooming or resting were unaffected by maternal diet in male offspring (Figs. 3.10B &C).

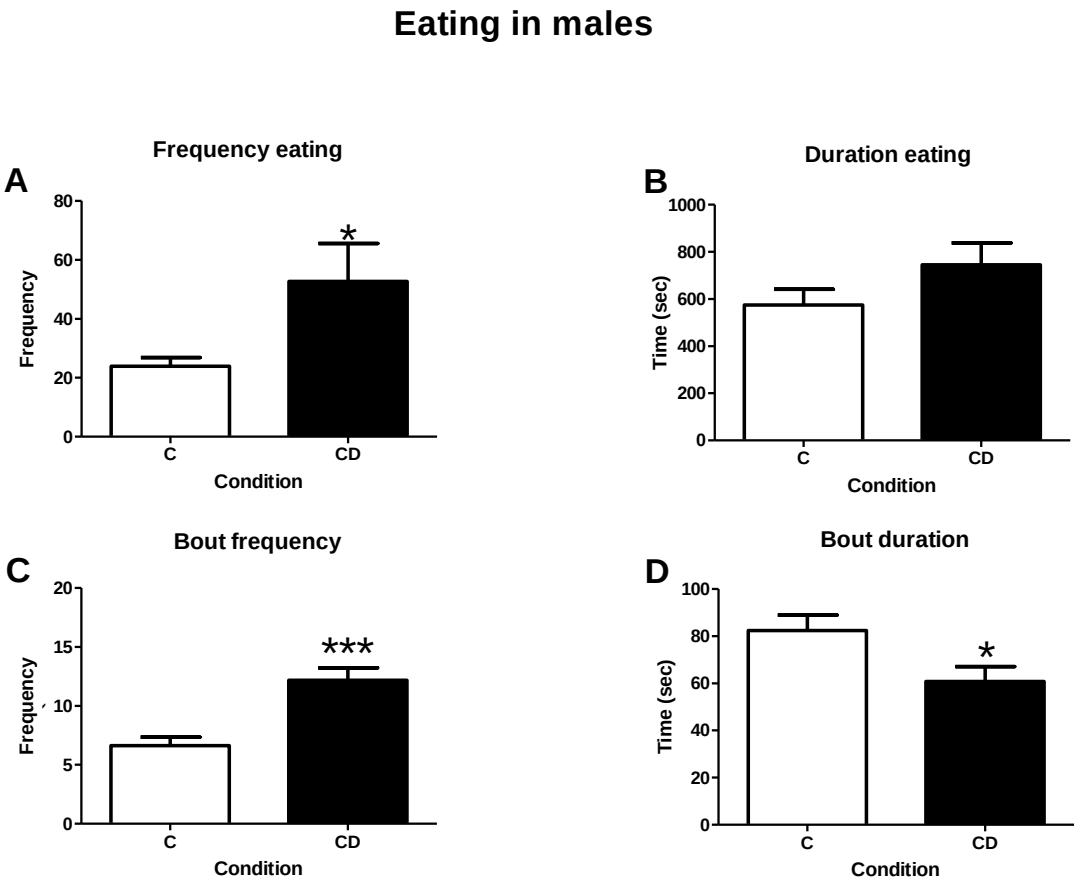

Fig. 3.9 The effect of maternal CD upon eating parameters in males. Frequency (A). Duration (B). Bout frequency (C). Bout duration (D). (CD n=8,C n=6). * P<0.05 *** P<0.001.

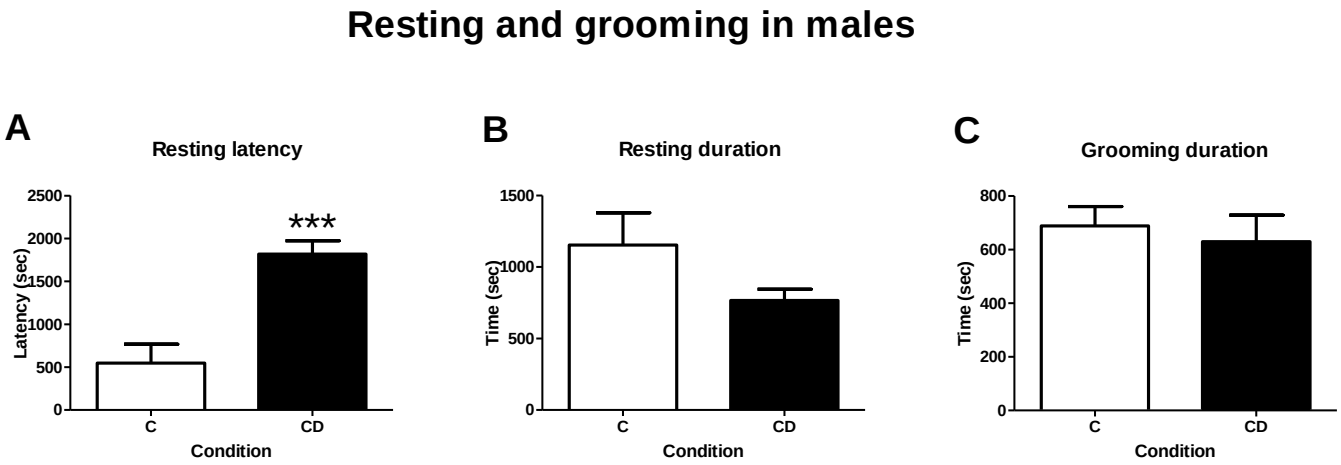

Fig. 3.10. The effect of maternal CD upon resting latency (A) resting duration (B) and grooming duration (C) in males. (CD n=8, C n=6). *** P<0.001.

3.2.3.3 Behavioural measures of eating, grooming and resting (females)

In contrast to males, female offspring exposed to maternal CD during lactation showed no change in the frequency of eating episodes (Fig. 3.11A). These animals spent significantly more time eating (Fig. 3.11B, t=2.72, d.f=12, P<0.05) and less time resting (Fig. 3.12B, t=3.32, d.f=7.88, P<0.01) than control females during the 1 hour BSS test. This effect of maternal diet was associated with a greater number of feeding bouts (Fig. 11C, t=2.37, d.f=12, P<0.05), but no difference in bout duration (Fig. 3.11D). As in males, no differences in the frequencies of resting or grooming were noted between the two groups of female offspring (data not shown), but resting latency was increased in CD fed females (Fig. 3.12A, t=2.42, d.f=6.77, P< 0.05). Duration of grooming was unaltered by maternal diet (Fig. 3.12C).

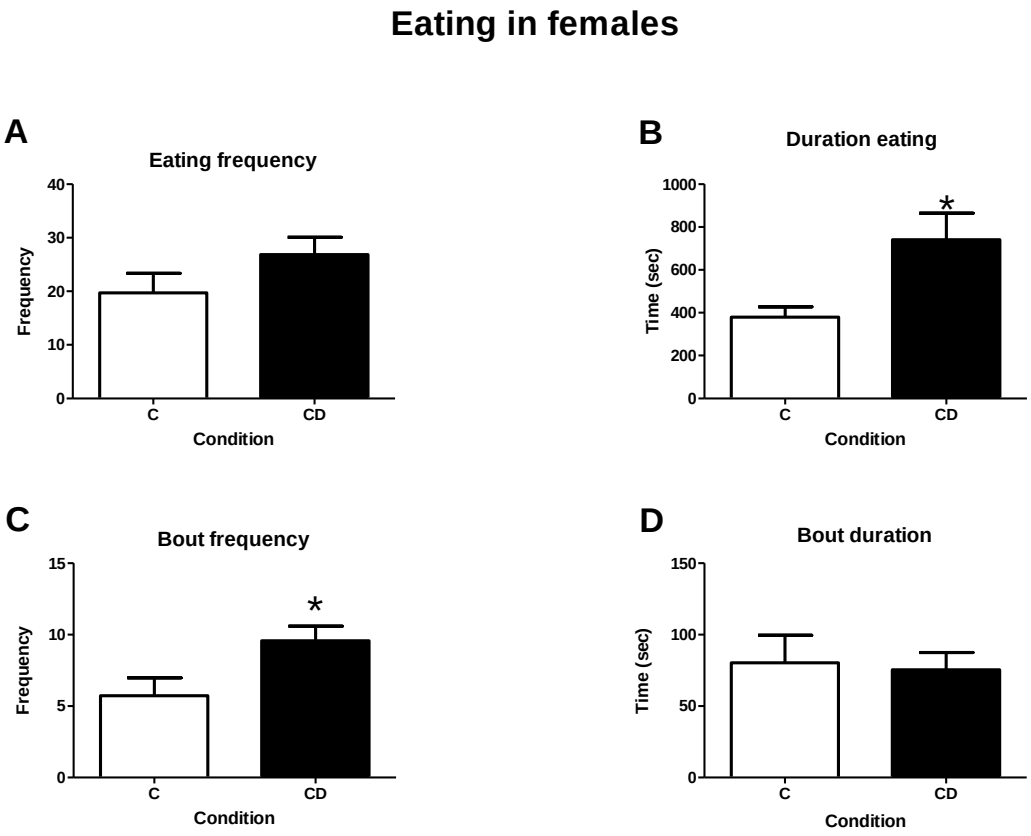

Fig. 3.11. The effect of maternal CD upon eating parameters in females. Frequency (A). Duration (B). Bout frequency (C). Bout duration (D). (n=7/group). *P<0.05.

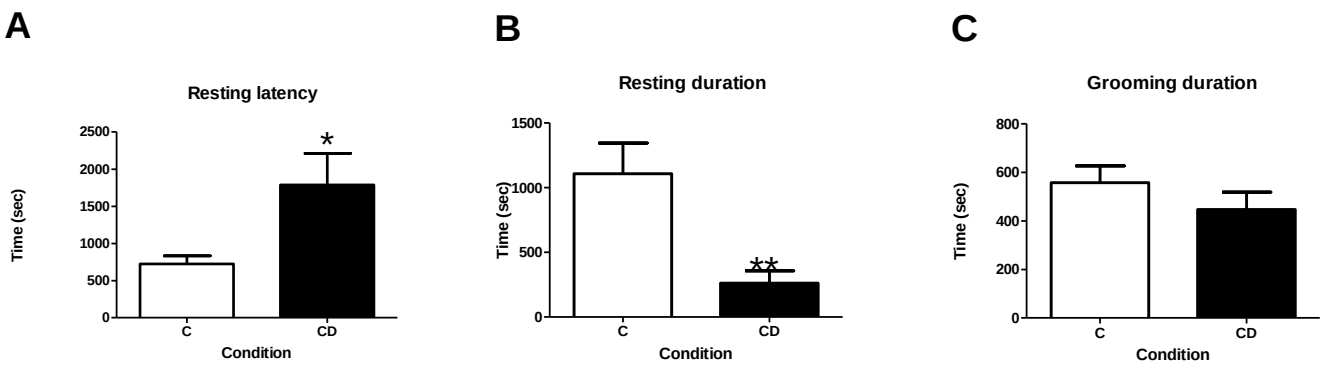

Fig. 3.12. The effect of maternal CD upon resting latency (A) resting duration (B) and grooming duration (C) in females. (n=7/group). *P<0.05 **P<0.01.

3.2.3.4 Microstructural analysis and BSS

Fig. 3.13A-B shows a decrease in the frequency of eating across the twelve 5-minute time bins of the test in both groups of males ($F_{3.48, 132}$ = 4.58, P < 0.01) and females (Fig. 3.13B, $F_{3.60, 88}$=3.88, P<0.05). Despite such a decrease no diet x time interactions were observed indicating maternal diet during lactation had no effect on the frequency of eating behaviour across the 1-hour observation period in offspring of both sexes. As shown in Fig. 3.14A and B, in both control and CD males, eating duration decreased over time ($F_{5.40, 132}$ =7.96, P<0.001), whereas resting increased ($F_{4.82,121}$=9.36, P<0.001) and grooming increased and then decreased ($F_{5.03,132}$=3.57, P<0.001). Despite these changes there was an absence of treatment x time interactions for any of these parameters in male offspring, indicating that maternal diet during lactation had no effect upon any of these parameters across the 1-hour observation period.

The transition between eating and resting occurred at 27.6 ± 1.8 minutes in male controls and 31.5 ± 8.9 minutes in the CD offspring. This transition point represented the onset of satiety and is indicated by the vertical bars in Fig. 3,14A and B. There was no difference in the onset of satiety between the two groups of male offspring.

As in males, eating decreased over time in female rats ($F_{3.81, 77}$=3.37, P<0.05) (Fig. 3.15A&B). However, in contrast to males, there was also a significant diet x time interaction ($F_{3.8,1\ 77}$=2.96, P < 0.05) indicating that maternal diet altered the progression of eating across the 1-hour observation period. Resting

tended to increase over time (P<0.01) and grooming was not affected by time. Importantly we also noted a significantly delayed onset of satiety in CD exposed females. This occurred after 21.1±2.8 minutes in control females and after 49.0 ± 8.2 minutes in CD offspring (*t*=6.72, d.f.=11, P < 0.001) (Fig. 3.15A&B).

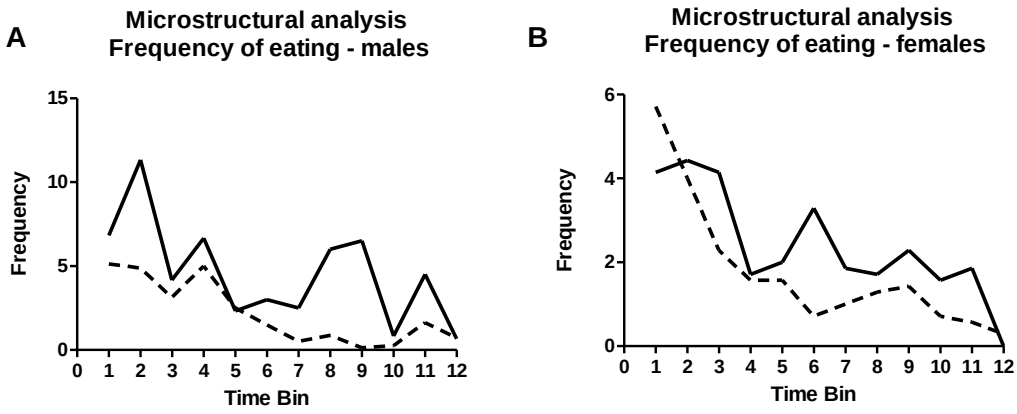

Fig. 3.13. The effect of maternal CD upon the frequency of eating behaviour in males (A) and females (B) across the 1-hr test session. Data are shown as means for 12 periods, each representing a 5-minute time bin across the 1-hr in the test period. Males: CD = solid line, n = 8, C = dotted line, n = 6. Females: CD = solid line, C = dotted line, n = 7/group.

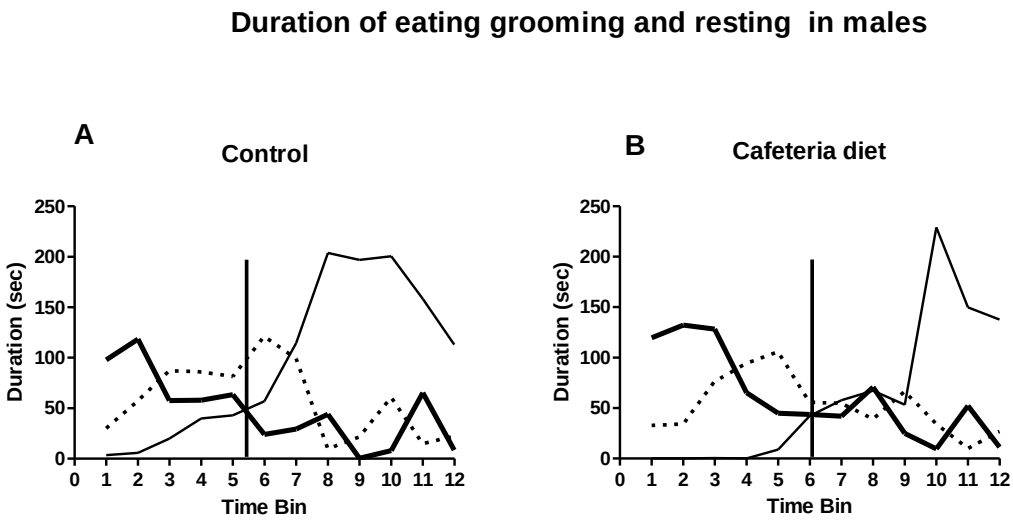

Fig. 3.14. The effect of maternal CD upon duration of eating (thick line), resting (thin line) and grooming behaviours (dotted line) in males. Data are shown as means for 12 periods, each representing a 5-minute time bin across the 1-hr in the test period. The vertical line emanating from the x-axis marks the transition between eating to resting. A. C (n=8). B. CD (n=6).

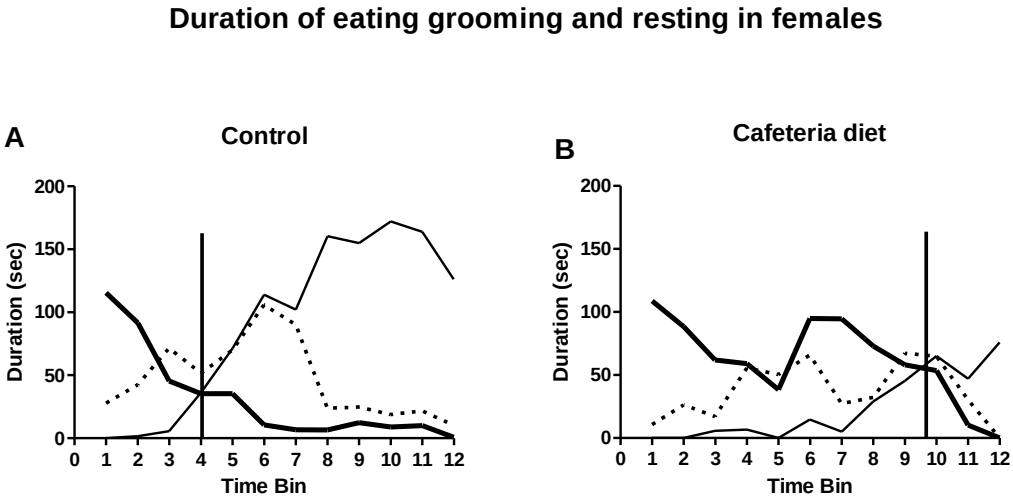

Fig. 3.15. The effect of exposure to CD throughout lactation upon duration of eating (thick line), resting (thin line) and grooming behaviours (dotted line) in females. Data are shown as means for 12 periods, each representing a 5-minute time bin across the 1-hr in the test period. The vertical line emanating from the x-axis marks the transition between eating to resting. A and B. (n = 7/group) * P<0.05. Eating during periods 7, 8 (CD vs. C).

3.2.3.5 Behavioural measures of rearing, locomotor and olfactory behaviour (Males)

Fig. 3.16 displays the frequency and during of rearing, locomotor behaviour and olfactory behaviour in offspring throughout the 1-hour observation session. Maternal exposure to CD during lactation had no effect upon the frequency or duration of vertical exploration or locomotor behaviour in male offspring (Fig. 3.16A&B). However as shown in Fig. 3.16B, maternal exposure to the CD did increase the duration of sniffing compared to male control offspring (t=4.52 d.f.=12, P<0.01). There were no other significant differences between offspring exposed to maternal CD and control offspring.

3.2.3.6 Behavioural measures of rearing, locomotor and olfactory behaviour (Females)

As shown in Fig. 3.16D, maternal exposure to CD significantly increased the duration of vertical exploration (780.74 ± 183.56 seconds vs. 215.86 ± 74.83 seconds in controls) compared to control offspring (t=2.85, d.f.=12, P<0.05). Maternal exposure to CD also significantly reduced the duration of locomotor behaviour (Fig 3.16D, 148.63 ± 29.09 seconds vs. 277.00 ± 45.80 seconds in controls) compared to control offspring (t=2.37, d.f.=12, P<0.05). There were no other significant differences in behaviour.

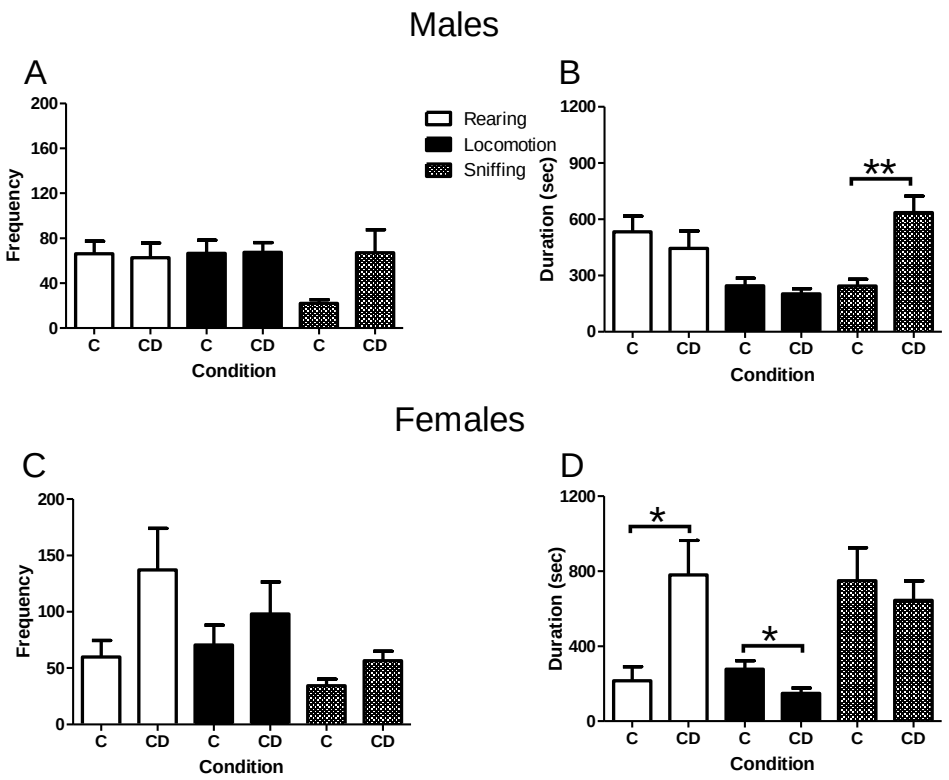

Fig. 3.16. The effect of maternal CD upon frequency and duration of rearing, locomotor and olfactory behaviour in adult offspring. A & B: Males Control (*n*=6), CD (*n*=7). C & D: Females. C & D Control (*n*=8), CD (*n*=7). Data are shown as mean + SEM. * and ** indicate P<0.05, P<0.01 significance between control and CD groups.

3.2.3.7 Rearing, locomotor and olfactory behaviour microstructure (Males)

Time spent engaging in vertical exploration, locomotor behaviour and olfactory behaviour in male offspring across the 1-hour observation session is displayed in Fig. 3.17. Time spent rearing by male offspring (Fig. 3.17A) decreased steadily in both groups across the 1-hour test session ($F_{4.30, 51.59}$=3.98, P<0.01). Maternal exposure to CD during lactation had no major effect upon the duration of rearing across the 1-hour test session as the time x treatment interaction failed to reach significance.

Time spent engaging in locomotor behaviour in male offspring (Fig. 3.17B) decreased steadily in both groups throughout the first half of the observation session, then increased sharply throughout the latter half ($F_{11, 132}$ =4.40, P<0.001). Again, maternal exposure to CD during lactation had no major effect upon the duration of locomotor behaviour across the 1-hour test session as the time x treatment interaction failed to reach significance.

Time spent engaging in olfactory behaviour in male offspring (Fig. 3.17C) increased steadily in both groups across the first half of the observation session and then was attenuated throughout the latter half ($F_{3.70, 40.68}$=2.84, P<0.05). However, maternal exposure to CD during lactation had no major effect upon the duration of olfactory behaviour across the 1-hour test session as the time x treatment interaction failed to reach significance.

3.2.3.8 Rearing, locomotor and olfactory behaviour microstructure (females)

Time spent engaging in vertical exploration, locomotor behaviour and olfactory behaviour in female offspring across the 1-hour observation period is shown in Fig. 3.18. Time spent rearing by female offspring (Fig. 3.18A) decreased steadily in both groups across the 1-hour test session ($F_{4.60, 27.62}$=4.74, P<0.01), but maternal exposure to the CD during lactation had no major effect upon the duration of rearing across the 1-hour test session as the time x treatment interaction failed to reach significance. Both locomotor behaviour and sniffing behaviour in female offspring did not change significantly across the 1-hour test session, nor were they affected by maternal exposure to maternal CD (Fig. 3.18B&C).

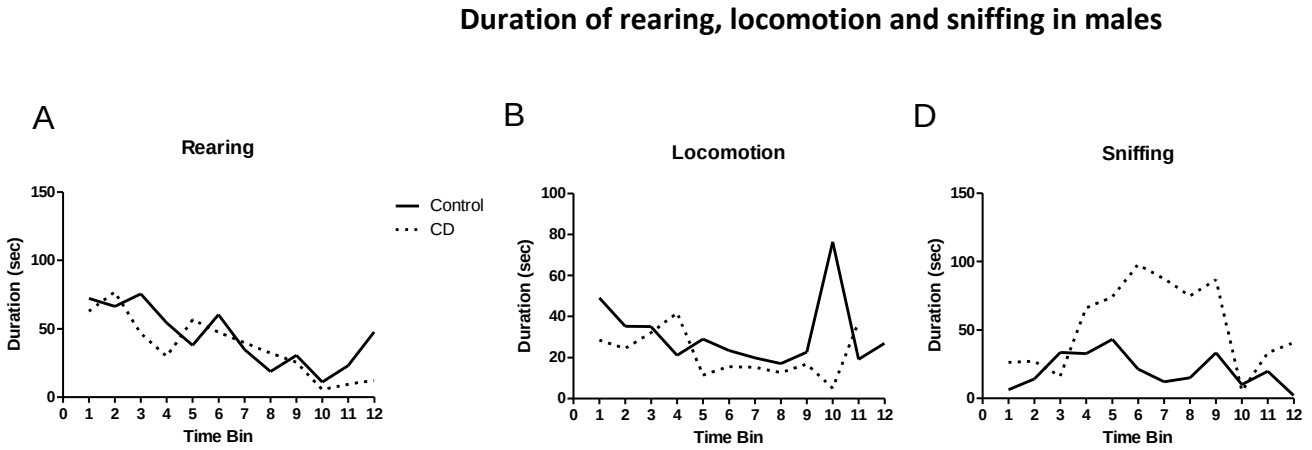

Fig. 3.17. The effect of maternal CD upon rearing, locomotor and olfactory behaviour in adult male offspring across the 1-hour test session. A: Rearing B: Locomotion C: Sniffing. Control (*n*=6), CD (*n*=7) B: Females. Control (*n*=8), CD (*n*=7). Data are shown as mean.

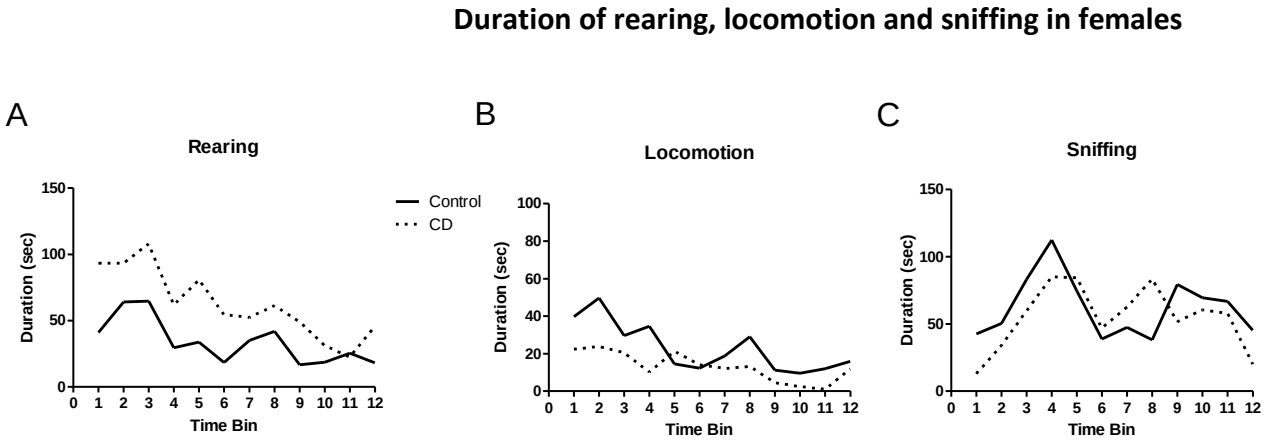

Fig. 3.18 The effect of maternal CD upon rearing, locomotor and olfactory behaviour in adult female offspring across the 1-hour test session. A: Rearing B: Locomotion C: Sniffing. Control (*n*=8), CD (*n*=7). Data are shown as mean.

3.2.4 Open Field Habituation

3.2.4.1 Males

As seen in Fig. 3.19A-B, both CD and control male offspring failed to show signs of habituation in the open field. There was no significant difference between day 1 and 2 for either of the maternal feeding groups for distance travelled and frequency of rearing (Fig 3.19A-B).

3.2.4.2 Females

As seen in Fig. 3.19C-D, both CD and control female offspring failed to show signs of habituation upon the open field. Distance travelled upon day 2 was significantly greater than upon day 1 in control offspring (t=4.05, d.f.=7, $p < 0.01$) but not in offspring exposed to maternal CD (Fig. 3.19C).

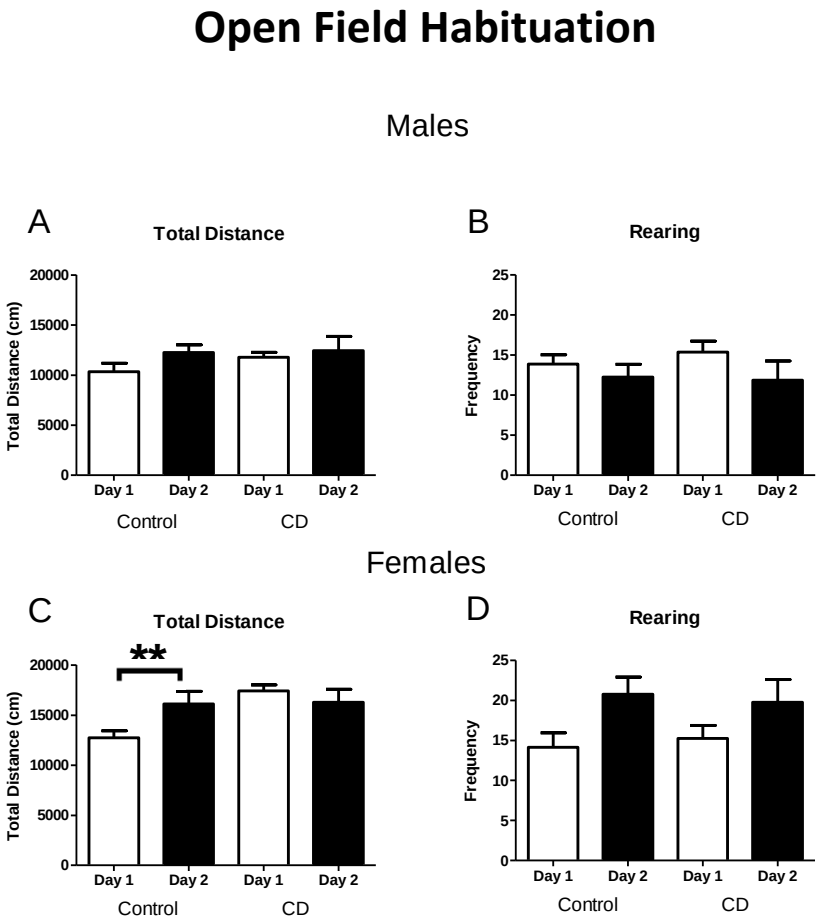

Fig. 3.19. The effect of maternal CD upon habituation in the open field. A: Total distance B: Rearing. Males - Control (*n*=8), CD (*n*=8). Females - Control (*n*=8), CD (*n*=8) Data are shown as mean + SEM. ** indicates P<0.01 significance between control and CD groups.

3.2.5 Novel Object Discrimination

3.2.5.1 Males

Fig. 3.20 shows the effect of maternal CD upon novel object discrimination in male offspring. During the training period, both groups spent an equal amount of time exploring the 2 identical objects, indicating that object preference did not play a role in determining exploratory behaviour of animals in the arena. After the 1-hour interval male offspring of mothers from

dams fed CD (Novel 76.53 ± 22.15% vs. Familiar 23.47 ± 22.15% - *t*=6.19, d.f.= 9, P< 0.001) and control offspring (Novel 67.04% ± 2.75 vs. Familiar 32.96 ± 2.75% - *t*=2.667, d.f.=6, P<0.05) were both able to distinguish the novel object from the familiar as demonstrated by percentage of time spent exploring the novel object relative to the familiar. At the 2-hour interval male offspring maternally exposed to CD distinguished the novel object (72.16 ± 6.77%) from the familiar object (27.84 ± 6.77%) (*t*=3.27, d.f.=9, P<0.05) with controls showing no behavioural signs of memory, confirming that maternal exposure to CD during lactation attenuated the delay dependent decay of memory in male offspring. Neither group spent more time exploring the novel object or the familiar object after the 4-hour interval.

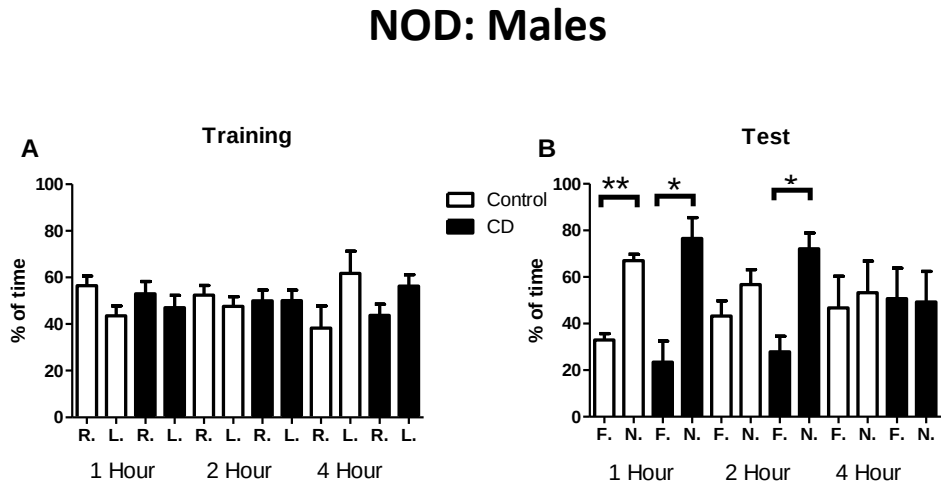

Fig.3.20. The effect of maternal CD upon novel object discrimination in males. A: Training B: Test. Control (*n*=10) CD (*n*=10). R (Right), L (Left), F (Familiar), N (Novel). Data are shown as mean + SEM. * and ** indicate P<0.05 and P<0.01 significance between control and CD groups.

3.2.5.2 Females

Fig. 3.21 shows the effect of maternal CD upon novel object discrimination in female offspring. During the training sessions both groups spent an equal amount of time exploring the 2 identical objects regardless of the interval, indicating that object preference did not play a role in determining exploratory behaviour of animals in the arena. After the 1-hour interval both offspring of mothers from dams fed maternal CD (Novel 62.93 ± 2.47% vs. Familiar 37.07 - t=5.23, d.f.=7, P<0.01) and offspring of mothers fed control chow throughout that period (Novel 62.21 ± 4.5% vs. Familiar 37.79 ± 4.5% - t=2.72, d.f.= 7, P< 0.05) spent more time exploring the novel object compared to the familiar object demonstrating a strong recognition memory effect.

It was observed that at the 2-hour interval female offspring exposed to maternal CD did not spend more time with the novel object compared to the familiar object, with a strong memory effect in control female offspring (Novel 72.46 ± 5.67% vs. Familiar 27.54 ± 5.67% - t=3.96, d.f.=9, p < 0.05). This suggests that maternal exposure to CD during lactation accelerated the delay dependent decay of memory in female offspring. Both groups failed to show behavioural signs of memory after the 4-hour interval, with neither being able to discriminate the novel object from the familiar object.

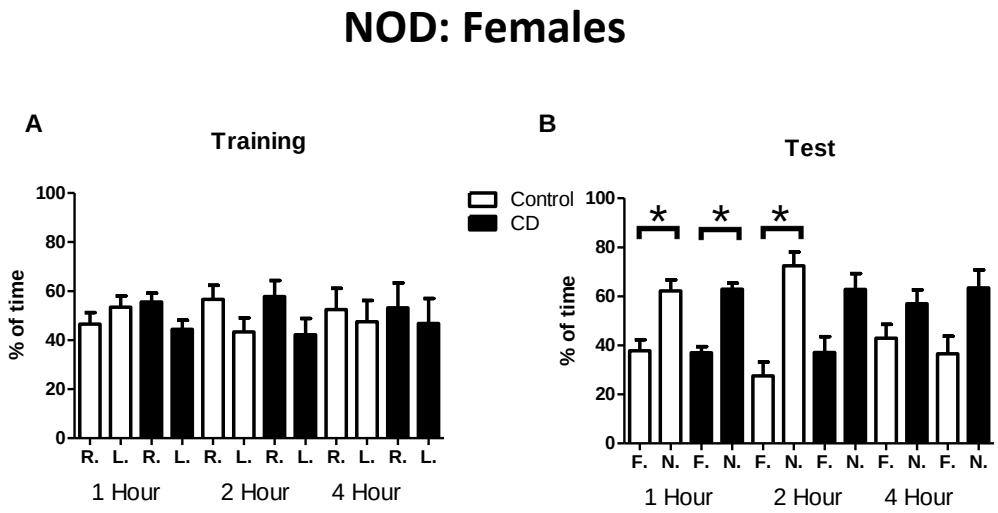

Fig. 3.21. The effect of maternal CD upon novel object discrimination in females. A: Training B: Test. Control (*n*=10) CD (*n*=10). R (Right), L (Left), F (Familiar), N (Novel). Data are shown as mean + SEM. * indicate P<0.05 significance between control and CD groups.

3.2.6 Brain neurotransmitter determination

The effects of maternal diet upon DA and 5-HT concentrations and turnover in the hypothalamus are shown in Table 3.1. Maternal consumption of CD had a significant effect on hypothalamic 5-HT concentrations ($F_{1, 30}$=20.65, P<0.001), which were increased in both male (P<0.05) and female offspring (P<0.01). Unchanged concentrations of 5-HIAA, indicated significantly reduced 5-HT metabolism in offspring exposed to maternal CD ($F_{1, 29}$=42.86, p < 0.001). The 5-HIAA/5-HT ratio was reduced in both males (P<0.001) and females (P<0.01). There was an overall effect of maternal CD exposure on hypothalamic DA, evidenced by an increase in the neurotransmitter concentration ($F_{1,30}$=9.74, P < 0.01). Hypothalamic DA metabolism (DOPAC+HVA/DA ratio) was lower

overall in females compared to males ($F1_{1,29}$=11.01, P<0.01). There was no effect of maternal CD on 5-HT, DA concentrations and their metabolism in the hippocampus (Table 3.2), and the fontal cortex (Table 3.3). However, there were several gender effects on dopamine metabolism. HVA concentrations in the frontal cortex were greater in female offspring compared to males ($F_{1, 13}$=6.36, P<0.05), as was the DOPAC+HVA/DA ratio ($F_{1,26}$=15.26,P<0.01).

Table 3.1 The effect of exposure to cafeteria diet throughout lactation upon dopamine (DA) and serotonin (5-HT) content and turnover in the hypothalamus. Mean ± SEM. (males: C n = 7, CD n = 10; females: C n = 9, CD n = 7). * P<0.05, ** P<0.01, *** P< 0.001.

Hypothalamus	Picomoles per mg of tissue	Picomoles per mg of tissue	Picomoles per mg of tissue	Turnover	Picomoles per mg of tissue	Picomoles per mg of tissue	Turnover
	DA	DOPAC	HVA	DOPAC+HVA/ DA	5-HT	5-HIAA	5-HIAA/5-HT
Control (M)	1.52 ± 0.45	0.50 ± 0.11	0.21 ± 0.5	0.60 ± 0.12	1.81 ± 0.22	3.08 ± 0.31	1.73 ± 0.07
CD (M)	2.65 ± 0.49	0.64 ± 0.22	0.42 ± 0.19	0.50 ± 0.06	3.44 ± 0.68 *	3.01 ± 0.19	1.01 ± 0.09 ***
Control (F)	1.05 ± 0.27	0.30 ± 0.05	0.2 ± 0.02	0.35 ± 0.08	2.06 ± 0.22	2.92 ± 0.13	1.53 ± 0.14
CD (F)	1.80 ± 0.21	0.18 ± 0.02	0.13 ± 0.02	0.26 ± 0.01	4.58 ± 1.24 **	3.15 ± 0.36	0.85 ± 0.07 **
P (effect of diet)	<0.01	NS	NS	<NS	< 0.001	NS	< 0.001
P (effect of gender)	NS	< 0.05	NS	< 0.01	NS	NS	NS
P (diet x sex)	NS	NS	NS	NS	NS	NS	NS

DA = dopamine, DOPAC = 3,4-dihydroxyphenylacetic acid, HVA = homovanillic acid, 5-HT = 5-Hydroxytryptamine / serotonin, 5-HIAA = 5-hydroxyindolacetic acid

Table 3.2 The effect of exposure to cafeteria diet throughout lactation upon dopamine (DA) and serotonin (5-HT) content and turnover in the hippocampus. Mean ± SEM. (males: C n = 7, CD n = 10; females: C n = 9, CD n = 7). * P<0.05.

Hippocampus	Picomoles per mg. of tissue	Picomoles per mg. of tissue	Picomoles per mg. of tissue	Turnover	Picomoles per mg. of tissue	Picomoles per mg. of tissue	Turnover
	DA	DOPAC	HVA	DOPAC + HVA/DA	5-HT	5-HIAA	5-HIAA/5-HT
Control (M)	0.18 ± 0.02	0.11 ± 0.01	0.34 ± 0.04	NA	0.36 ± 0.06	4.00 ± 0.40	13.90 ± 2.56
CD (M)	0.35 ± 0.09	0.43 ± 0.16	0.36 ± 0.04	2.16 ± 0.18	0.37 ± 0.07	4.00 ± 0.26	15.58 ± 2.43
Control (F)	0.29 ± 0.16	0.53 ± 0.35	NA.	NA	0.41 ± 0.10	5.09 ± 0.29	15.68 ± 3.84
CD (F)	0.2 ± 0.03	0.35 ± 0.11	NA.	3.19 ± 0.31	0.34 ± 0.05	4.33 ± 0.55	14.23 ± 1.97
P effect of diet	NS	NS	NS	NS	NS	NS	NS
P effect of gender	NS	NS	NS	NS	NS	NS	NS
P diet x sex	NS	NS	NS	NS	NS	NS	NS

Abbreviations: DA, Dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, Homovanillic acid; 5-HT, 5-Hydroxytryptamine; 5-HIAA, 5-Hydroxyindoleacetic acid; NA, Not available; NS, Not Significant.

Table 3.3 The effect of exposure to cafeteria diet throughout lactation upon dopamine (DA) and serotonin (5-HT) content and turnover in the frontal cortex. Mean ± SEM. (males: C n = 7, CD n = 10; females: C n = 9, CD n = 7). * P<0.05.

Frontal cortex	Picomoles per mg. of tissue	Picomoles per mg. of tissue	Picomoles per mg. of tissue	Turnover	Picomoles per mg. of tissue	Picomoles per mg. of tissue	Turnover
	DA	DOPAC	HVA	DOPAC + HVA/DA	5-HT	5-HIAA	5-HIAA/5-HT
Control (M)	0.46 ± 0.23	0.3 ± 0.14	NA.	1.34 ± 0.12	0.57± 0.06	3.54 ± 0.22	6.75 ± 0.74
CD (M)	0.27 ± 0.02	0.17 ± 0.03	NA.	NA	0.55 ± 0.1	3.47 ± 0.33	6.94 ± 0.68
Control (F)	0.39 ± 0.12	0.32 ± 0.07	1.04 ± 0.23	3.79 ± 0.48	0.72 ± 0.08	4.17 ± 0.44	6.16 ± 0.1
CD (F)	0.28 ± 0.06	0.3 ± 0.04	0.72 ± 0.19	2.90 ± 0.32	0.68 ± 0.2	4.01± 0.48	8.74 ± 1.63
P effect of diet	NS	NS	NS	NS	NS	NS	NS
P effect of gender	NS	NS	< 0.05	<0.05	NS	NS	NS
P diet x sex	NS	NS	NS	NS	NS	NS	NS

Abbreviations: DA, Dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, Homovanillic acid; 5-HT, 5-Hydroxytryptamine; 5-HIAA, 5-Hydroxyindoleacetic acid; NA, Not available; NS, Not Significant

3.3.0 Discussion

There is an emerging body of evidence which suggests that nutritional exposures in early life determine long-term and possibly lifelong behaviours. Maternal undernutrition during pregnancy, for example, establishes a preference for high fat foods in young adult offspring, whilst feeding a cafeteria diet in pregnancy and lactation establishes a preference for similar foods in the offspring. Maternal undernutrition has also been demonstrated to programme deficits in performance on behavioural measures of learning and memory in adult offspring. has We have previously demonstrated that targeting the feeding of a hypercaloric cafeteria diet to the suckling period had subtle effects on adiposity, but more importantly that rats exposed to maternal CD showed reduced anxiety when tested as adults (*Chapter 2*). In an attempt to explore the wider implications of such nutritional programming of behaviour, here we studied the structure of feeding behaviour, habitation to the open field, recognition memory and brain monoamine turnover in the adult offspring.

3.3.1 Maternal macronutrient intake

During the first ten days of the suckling period lactating mothers fed the CD consumed significantly more sugar, fat and salt, and significantly less protein than mothers fed the control chow. The fact that lactating mothers fed CD consumed significantly less protein and significantly more fat and sugar compared to control chow is consistent with previous results obtained from our laboratory using unmated and pregnant female Wistar rats (Akyol *et al.,*

2009). However, despite this the amount of energy consumed per day between groups did not differ, which was greater in CD dams in the above study. As with the present findings, Akyol *et al.,* (2011) observed mothers fed CD during the lactation period consumed significantly more fat and significantly less carbohydrate, across the lactation period.

Unlike in Akyol *et al.,* (2011), the mothers in the current study consumed significantly less protein across the lactation period, although it is worth noting that in the present instance we only measured macronutrient intake during the first ten days of lactation. The average daily consumption of protein in CD mothers was around 10% of total macronutrient intake compared to fewer than 20% in chow mothers. It has been reported that diets consisting of 9% and less protein during pregnancy was enough to effect fetal growth and induce hypertension in weanling and adult offspring (Langley & Jackson, 1994; Langely-Evans *et al.,* 1996b; Langley-Evans *et al.,* 1999b; Manning & Verhaskari 2001; Verhaskari *et al.,* 2001). It is possible that some of the behavioural effects of CD during lactation on offspring may be partially attributable to effects of protein restriction. Interestingly, a maternal diet of 9% protein was enough to programme macronutrient selection and locomotor behaviour in adult offspring compared to controls (Bellinger *et al.,* 2004; Bellinger & Langley-Evans, 2005; Bellinger *et al.,* 2006).

3.3.2 Ingestive behaviour

The present study demonstrated that maternal exposure to maternal hyperenergetic diet can programme aspects of ingestive behaviour in adult

offspring. Impairments to the BSS were most pronounced in female offspring. Although there was no programming effect on total food intake during the 1-hour test meal and on the latency to start eating, a more detailed analysis revealed an altered eating pattern in CD males. The most obvious effects were an increased number of feeding bouts and a reduced average bout duration. A comparable pattern of reduced duration and increased frequency of feeding episodes was observed when a test meal was adulterated with quinine (Ishii *et al.,* 2003a). Interestingly in the aforementioned study, a higher concentration of quinine adulteration (0.04%) suppressed food intake in conjunction to altering feeding bout frequency and duration, whereas in an almost identical manner to the present study a lower concentration of quinine adulteration (0.015%) spared the effect on food intake but still altered bout frequency and duration.

A normal BSS profile was observed in control animals. This profile is characterised by an initially high but progressively decreasing duration of feeding with an inverse relationship with grooming and resting, both of which increase at the expense of feeding towards the end of the test (Halford *et al.,* 1998; Rodgers *et al.,* 2010). Although CD offspring started to rest later, the BSS profile was very similar in both male groups and the transition between eating and resting was not significantly affected. This finding marks another difference to the study by Ishii *et al.,* (2003a) where quinine delayed satiation as expressed by a delayed transition from eating to resting. To our knowledge the only study reporting a programming effect on the BSS (Orozco-Solis *et al.,* 2009) looked at male offspring of rats fed a low protein diet (8%) during

pregnancy and lactation. 35 day old offspring, exposed to protein restriction had slightly delayed satiety, a reduced latency to feed and greater meal size. By 180 days only the greater meal size remained. As we tested only a single age group, the persistence of the present effects still needs to be investigated. From the available data it seems likely that both early protein deficiency and early CD diet can re-programme the BSS, even if this effect does not seem to be long-lasting when fed on a low protein diet. However, there is also a similarity in the programming effect of both diets as expressed by the delayed onset of satiety during the test meal, although the delay in the onset of satiety in the present instance was observed in female offspring. This could be attributable to protein restriction during lactation as in the present study protein consumption of mothers fed CD (10%) was similar to the protein content of the low protein diet used by Orozco-Solis *et al.,* (2009).

Exposure to CD during lactation had a greater impact on feeding behaviour in females than in males. Overall, females did not show any changes in eating frequency. Compared to male CD offspring, females spent a longer time eating. Like their male counterparts, CD females started to rest later than controls, but only the females spent also less time resting. Whereas control females showed a normal BSS pattern, the most striking effect in female CD offspring was the greatly delayed transition from eating to resting which is indicative of a delayed onset of satiety (Halford *et al.,* 1998). This delay in the onset of satiety in female offspring as shown in the present study is comparable to the delay observed in animals subjected to several hours fasting relative to control animals (Ishii *et al.,* 2003b).

As low doses of the orexigenic neuropetide orexin impair the BSS to the extent that satiety did not occur at all throughout a 1-hour test meal (Rodgers *et al.,* 2000) our findings would support an interpretation that suggests postponed satiety in female offspring. However, the lack of maternal dietary effects on total food intake seems to contradict such an interpretation. Considering that a 1-hour test meal, as applied here, is established as a standard procedure for BSS-analysis (Ishii *et al.,* 2003a; Rodgers *et al.,* 2010), it could be speculated that either manipulation of the palatability of the test meal or a prolonged testing time may be useful to further investigate this discrepancy. In our experiments rats were food deprived for 3-hours and this had little effect on the eating–resting transition point and no effect on the structure of the BSS compared to other studies (Ishii *et al.,* 2003b). Nevertheless, the food deprivation could have imposed a ceiling effect, masking existing differences in total food intake, at least when measured during a testing period of only 1-hour.

It is possible that the effect of early nutritional programming upon ingestive behaviour is manifested as a changed response to the palatability or novelty of the mash. This assumption is supported by the fact that rats whose mothers were fed on CD have similar post-weaning bodyweights and chow consumption compared with offspring of rats fed on chow. It is unlikely that habitual feeding is affected in adult offspring. This would be in line with the report that maternal CD programmes a preference for this diet later in life (Bayol, *et al.,* 2007). Interestingly maternal protein restriction also programmes preferences for foods with a high fat content, in a sex-specific

manner (Bellinger *et al.,* 2004). Bellinger & Langley-Evans, (2005) reported that in conjunction with alterations to macronutrient selection, maternal protein restriction can also remodel hypothalamic neural circuitry and alter gene expression of neuropeptides and taste receptors which may determine food palatability.

Conventionally, in instances when the test meal is made severely unpalatable, increases in bout frequency and reductions in bout duration are accompanied by increases in the duration of sniffing behaviour during the first half of the test period, which also delay the onset of satiety (Halford *et al.,* 1998). Despite alterations to bout frequency and duration in male offspring suggestive of changes in response to the palatability or novelty of the mash (such as those seen in Ishii *et al.,* 2003b), maternal diet had no effect upon the duration of sniffing across the 1-hour test period. Maternal CD during lactation had even less of an effect upon sniffing in females. Such observations suggest that whilst palatability mechanisms may have been altered, the effect was not severe enough to induce changes to olfactory behaviour.

Manipulations which induce severe hyperactivity in animals, such as *d*-amphetamine, have been demonstrated to disrupt the BSS through fragmentation of feeding episodes and the induction of a delay the onset of resting due to increased locomotor behaviour (Blundell & Latham, 1979; Halford *et al.,* 1998). Locomotor behaviour was unaffected by maternal diet across the 1-hour observation period in males.

Instances of severe hyperactivity are known to involve repeated bouts of active locomotor behaviour at the expense of eating and resting, often inducing the fragmentation of eating behaviour into multiple episodes and the induction of a sustained delay to the onset of resting (Halford *et al.,* 1998). In this instance levels of locomotor behaviour were far below what could be considered hyperactive (Halford *et al.,* 1998). Changes to the BSS in female CD offspring was accompanied by no change in locomotor behaviour across the 1-hour test period. However, maternal CD reduced the duration of locomotor behaviour throughout the overall session, which is likely to have been a consequence of prolonged eating behaviour. Vertical exploration measured by duration of rearing behaviour was also increased in female offspring exposed to maternal CD throughout the total 1-hour observation session, which also could have contributed to the reduction in locomotor behaviour and possibly the fragmentation of eating behaviour and the delay in the onset of resting. Additionally, rearing behaviour was also unaffected by maternal diet across the observation period in females.

In summary, such observations suggest that the behavioural programming effects outlined in the previous chapter extend from anxiety and exploration, to also impact upon aspects of ingestive behaviour. The results emphasize further the contribution of maternal exposure to overnutrition during lactation to programming of behaviour in offspring. Changes to the BSS and related parameters were observable in offspring of both sexes but were the most pronounced in female offspring.

3.3.3 Learning and memory

The present study shows that maternal exposure to a hyperenergetic diet during the suckling period had a significant effect upon recognition memory in adult offspring. It was noted that maternal overnutrition improved object discrimination in male offspring, attenuating the delay dependent decay in the ability to distinguish a novel object from a familiar object compared to controls. Conversely, maternal overnutrition impaired object recognition in female offspring, accelerating the delay dependent decay in the ability of rats to distinguish a novel object from a familiar object compared to controls. Neither CD offspring nor controls exhibited any signs of habituation to a novel environment in the open field habituation paradigm.

Performance in the novel object discrimination test by male offspring was comparable to the performance of untreated Wistar rats from other laboratories. In the present study, male control offspring did not spend more time with the novel object relative to the familiar object after the 2-hour interval. This level of performance was exceeded by control Wistar males in the study by Karasawa *et al*.,(2008), but not by control Wistar males in a study by Salomon *et al.,* (2011), which ceased spending more time with the novel object after just a 40 minute interval. The ratio of time spent exploring the novel object relative to the familiar object by male controls, after a 1 and 2-hour interval was also consistent with values obtained from other laboratories using Wistar rats and other strains (Karasawa *et al.* 2008; Salomon *et al.* 2011; King *et al.,* 2004).

Performance in the NOD test by female offspring was also comparable to the performance of Wistar controls from other laboratories. In the present study, female control offspring stopped spending more time with the novel object relative to the familiar object after the 4-hour interval, exceeding the performance of control males. This gender difference was consistent with the performance attained by controls in other laboratories, such as in the study by Soloman *et al.,* (2011). The ratio of time spent exploring the novel object relative to the familiar object by female control offspring after a 1, 2 and 4-hour interval appeared to be consistent with values obtained from other laboratories using Wistar rats and other strains (Ennaceur *et al.,* 2005; Soloman *et al.,* 2011).

Both male and female offspring, regardless of maternal diet, failed to habituate to the open field over 24-hours. Conventionally rats show significant reductions in parameters indicative of exploratory behaviour such as locomotion and rearing in an open field, with repeated exposure to a novel environment (Bronstein, 1971; Rusell & Williams, 1973; Tamasy *et al.,* 1973; Einon *et al.,* 1975; Leussis & Bolivar, 2006; Wilson & Linster, 2008). Pharmacological or physiological disturbances to the function of neurotransmitter systems, including 5-HT, DA, acetylcholine and glutamate, have been demonstrated to impair habituation to an open field (Leussis & Bolivar, 2006). For example Bidzinski *et al.,* (1998) demonstrated that acute 5-HT depletion using the synthesis inhibitor p-chlorophenylalanine is associated with a lack of intra-trial habituation. Wistar rats normally habituate to the open field after 24-hours exposure, even after invasive experimental

manipulations (Vianna *et al.,* 2001; Moojen *et al.,* 2006; Pedrazza *et al.,* 2007). It is difficult to explain why animals failed to habituate in this particular instance as conditions such as strain, age, light cycle and housing conditions in the experiments cited above were similar to the present study.

To the authors knowledge little research has been published examining the effect of a maternal hyperenergetic diet during critical periods of development upon behavioural measures of learning and memory in rodents. Despite this, multiple studies have been published demonstrating deficits on behavioural measures of learning and memory in many strains of rats fed hyperenergetic diet directly (Winocur & Greenwood, 1999; Molteni *et al.,* 2002; Goldbart *et al.,* 2006) including Wistars (McNeilly *et al.,* (2011). Although many studies have reported deficits induced by direct overnutrition on measures of spatial memory and rule learning memory, to date little research has looked at the effect of such dietary manipulations on recognition memory.

One such study by Jurdak and Kanarek (2009) demonstrated using an NOD test that male Long-Evans rats exposed to conditions of sucrose induced obesity exhibited a deficit in object recognition after a 1-hour interval compared to controls. Compared to the present findings, failing to spend more time with the novel object after a 1-hour interval represents a marked deficit in performance, suggesting the effects of direct exposure to overnutrition and obesity may be greater in magnitude than that induced by maternal dietary manipulations. Differences are unlikely to be attributable to

strain differences between Long-Evans and Wistar rats, due to the fact that the former have been repeatedly documented to outperform the latter on a number of rodent memory tests (Holahan *et al.,* 2006; Vales *et al.,* 2006; Platano *et al.,* 2008; Keeley *et al.,* 2010). In the study by Jurdak and Kanarek (2009), as obese rats could not discriminate the novel object from the familiar after 1-hour, testing ceased and the effect of other longer intervals was not analysed. Little research to date has been undertaken examining the possibility of a potential programming effect by maternal hyperenergetic diet during critical periods of development in offspring.

The fact that direct exposure to hyperenergetic diet can lead to memory impairments suggests it may be plausible that maternal exposure to overnutrition during early critical periods of development could programme impairments to recognition memory detectable in offspring. This potentially could explain the acceleration of the delay dependent decay observed in female offspring by maternal exposure to the CD, but fails to explain the enhancement of recognition memory in males. Studies analysing the metabolic consequences of maternal over- and undernutrition upon offspring have reported that both manipulations can have similar consequences (Langley-Evans *et al.,* 1996a; Wilson & Hughes, 1997; Samuelson *et al.,* 2008; Benkalfat *et al.,* 2011). The observation that in female offspring exposure to CD during lactation impaired memory appears to be consistent with reports that maternal exposure to undernutrition during lactation also impaired performance on a range of memory tests in offspring (Castro *et al.*, 1989; 1996; Bedi, 1991; Fukuda *et al.,* 2002; Cordoba *et al.,* 1994).

What is particularly salient is the differential effect of maternal diet on object discrimination between sexes. It is difficult to explain why maternal hyperenergetic diet had a facilitatory effect in males and a detrimental effect in females. There are many instances in the programming literature where the effect of maternal dietary manipulations has been influenced by gender, including adipose tissue distribution (Bellinger *et al.,* 2006; Erhuma *et al.,* 2007), regulation of systolic blood pressure (McMullen & Langley-Evans, 2005b), cardiovascular function (Elmes *et al.,* 2009) and appetite related behaviour (Bellinger *et al.,* 2004; Bellinger & Langley-Evans, 2005; Bellinger *et al.,* 2006). It is likely that the effect of maternal diet upon brain systems implicated in the mediation of learning and memory function, through whatever the mechanism, is specifically related to gender. Gender specificity has been demonstrated in response to maternal dietary manipulations to multiple systems through both the effects of glucocorticoid release (Langley-Evans, 1997; McMullen & Langley-Evans, 2005b) and direct or epigenetic changes to gene expression (McMullen *et al.,* 2004; McMullen & Langley-Evans, 2005c; Elmes *et al.,* 2009) in offspring.

Given the complexity of the neural circuitry underlying memory processes in rats and without any detailed measurement of brain physiology associated with memory processes, it is difficult to explain what may be the mechanism underlying the acceleration of the temporal decay in object recognition in adult female offspring on the NOD test. Neuroanatomical, neurogenetic and behavioural pharmacological research has ascertained that the mechanisms

underlying such a deficit could be extensive (see the comprehensive review by Dere *et al.,* 2007).

Mechanisms could include perturbations to biogenic amine, GABA, glutamate, cannabinoid, neuropeptide and hormones systems, as well as their associated intracellular transcription factors in brain regions demonstrated to be involved in the mediation object recognition such as the hippocampus, the perirhinal cortex, the nucleus accumbens, the cholinergic basal forebrain and perhaps more (Bartolini *et al.,* 1996; King *et al.,* 2004; Winters & Bussey, 2005; Paban *et al.,* 2005; Barker *et al.,* 2006; Dere *et al.,* 2007; Meneses, 2007; King *et al.,* 2008; Watson *et al.,* 2011; Millan *et al.,* 2010). Biogenic amine and cholinergic mechanisms may be of particular interest as they have been most extensively documented to be perturbed by nutritional programing manipulations during pregnancy and the neonatal period (Kehoe *et al.,* 2001; Barreto Medeiros *et al.,* 2002; Levin & Dunn-Meynell, 2002; Stevens *et al.,* 2008; Ryan *et al.,* 2008; Wong-Goodrich *et al.,* 2008; Vucetic *et al.,* 2010).

Interestingly, it is worth noting that in many instances glucose administration has been demonstrated to enhance memory performance in rats (Gold *et al.,* 1986; Stefani & Gold, 1998; McNay & Gold, 2002). Despite such observations, the facilitatory effect of glucose on memory in rats has been demonstrated to vary based upon several factors, including strain, age, the behavioural measure used and the nature of the manipulation (e.g. site of intracranial injection) (Gold, 2005; Canal *et al.,* 2005; Salinas & Gold, 2005). As in humans, studies investigating the consequences of glucose administration in rats have

demonstrated such manipulations can improve performance on multiple measures of memory, through reversal of pharmacological and age related deficits, in performance on alternation and spatial memory tasks (Gold, 1995; Korol & Gold, 1998; Gold, 2005). Intracranial microinjections of glucose into brain regions such as the hippocampus, the amygdala, the striatum and the medial septal nuclei have also been demonstrated to enhance memory performance under several different conditions (Ragozzino *et al.,* 1995). As in humans, in young rats glucose administration can improve memory performance under conditions when the task complexity is increased to require a high level of cognitive demand (McNay & Gold, 1998; McNay *et al.,* 2000). It is possible that the high sugar content of the maternal diet could have induced the facilitatiory effect in male offspring. Human studies reported enhanced memory performance in young males who were poor glucose regulators on a variety of tasks (Craft *et al.,* 1994; Curwin *et al.,* 1995).

However, as the macronutrient intake of rats fed CD does not just consist of enhanced consumption of sugar (also greater fat and salt and less protein, carbohydrate and fibre), it is unclear whether enhanced maternal consumption of glucose and/or permanent alterations to glucose metabolism in offspring is the reason for the attenuation of the delay dependent decay in male offspring in the present instance. It is worth acknowledging we have very little information about the exact macro- or micronutrient intake of the pups. The consequences of maternal dietary effects may also be confounded by the fact that toward the end of the lactation period (after post-natal day 14) pups may begin to consume the diet directly.

In summary, the present study demonstrates an additional nutritional programming effect by reporting that maternal exposure to hyperenergetic diet throughout the suckling period can alter performance upon behavioural measures of learning and memory in adult offspring. The behavioural effects of hyperenergetic diet during lactation, in addition to anxiety, exploration and ingestive behaviour, appear to extend to performances in behavioural tests of learning and memory. Such findings add further credence to the importance of maternal diet during the lactation period in programming offspring behaviour.

3.3.4 Brain neurotransmitters determination

The present study shows that maternal consumption of a hyperenergetic diet during lactation can programme hypothalamic 5-HT neurochemistry, as measured by increased 5-HT concentration and reduced turnover in adult offspring. Hypothalamic DA concentration was also increased in adult offspring. There was no effect of maternal CD on 5-HT and DA neurochemistry in the hippocampus and the frontal cortex in adult offspring.

The observed overall elevation in 5-HT and reduction in its metabolism could be indicative of a diminished release of the neurotransmitter, although changes in metabolite/neurotransmitter ratio are not always associated with changes in neurotransmitter release (Commissiong, 1985). In addition, changes in brain 5-HT concentration, even up to 60 %, do not necessarily alter the stimulated release (Hall *et al.,* 1999; Rex *et al.,* 2005). For example, Rex *et al.,* (2005) reported that microinjection of the selective 5-HT neurotoxin 5,7-

Dihydroxytryptamine (5,7-DHT) into the dorsal raphe nuclei reduced 5-HT levels within a key terminal projection area (the ventral hippocampus) by 60% compared to rats subjected to sham lesions. However, when both sets of animals were exposed to a mildly aversive situation in the form of the elevated plus maze, stimulated release measured by microdialysis did not differ between the 5-HT lesion animals and the sham lesion animals.

However, even relatively small changes in tonic release could possibly induce adaptive counter-regulations. For example, even reduction in 5-HT metabolism below 60 % led to postsynaptic receptor up-regulation (Voigt *et al.* 2008). As 5-HT metabolism was reduced by 42 % in males, and 44 % in females in the present study, it is more likely that tonic release rather than stimulated release would be affected. However, the situation might be further complicated by the fact that 5-HT transporters are subject to nutritional regulation (Zhou *et al.,* 1996, Huether *et al.,* 1997) and a programming effect on 5HT transporters cannot be excluded at this stage. Hypothalamic 5-HT neurochemistry has been demonstrated to be susceptible to maternal dietary manipulations in infant rats and is also a known target for programming by maternal protein restriction in adult offspring (Kehoe *et al.,* 2001; Lopes de Souza *et al.,* 2008).

A similar complexity could possibly be assumed for the DA system. Despite a significant increase in concentration in offspring of both sexes, DA metabolism was unaffected by maternal CD. As with 5-HT, the DA system has been demonstrated to be potentially sensitive to programming manipulations. DA

concentrations in multiple brain regions were attenuated in pups in rats subjected to protein deprivation throughout gestation (Hisatomi *et al.,* 1979). Hypothalamic DA neurochemistry in infant rats has been demonstrated to be susceptible to maternal dietary manipulations, however, unlike 5-HT such an effect was only detectable under conditions of acute stress (Kehoe *et al.,* 2001). Hypothalamic DA gene expression in adult offspring has been shown to be sensitive to programming manipulations, such as maternal overnutrition through exposure to a high fat diet (Vucetic *et al.,* 2010).

Combined with the findings of the present study, such observations demonstrate that aspects of monoamine neurochemistry may be susceptible to programming by maternal overnutrition during lactation. The present findings provide some indication, all be it limited, of a possible cause of the behavioural changes induced by maternal overnutrition.

3.3.5 Brain neurotransmitter determination and ingestive behaviour

The data suggest that some of the programming effects of early exposure to CD may relate to changes in dopamine metabolism and, in particular, to hypothalamic 5-HT function. It is clear that feeding a cafeteria diet during lactation has a long lasting effect on brain neurotransmitter metabolism in the adult rat. There seems to be a lack of information as to how behavioural parameters of the BSS relate to *in vivo* neurotransmitter release, which could be measured by microdialysis, for example. Measuring brain neurotransmitter content is a reasonable intermediate step to examine potential mechanisms through which programming of behaviour may be mediated. Both 5-HT and

DA are involved in the control of feeding behaviour and reward (Blundell, 1977; Di Chiara, 2002; Hayes and Greenshaw, 2011; Hoebel, 1985; Leibowitz and Rossakis, 1979; Meguid *et al.,* 2000; Wellman, 2005), and impact also on the microstructure of feeding behaviour (Clifton, 2000). However, the neurochemical data provided here cannot easily be related to the behavioural changes as the functional significance of the observed alterations in post-mortem brain neurochemistry needs to be confirmed in further *in vivo* studies.

Considering the satiating effect of 5-HT (Rodgers & Blundell, 1979), the observed decrease of hypothalamic 5-HT metabolism could relate to alterations to the BSS seen in CD fed offspring, although it would not explain the obvious differences seen between male and female offspring. The present study noted gender differences in DA metabolism. As multiple 5-HT–DA interactions exist (Ferre and Artigas, 1993; Fetissov *et al.,* 2000; Fink and Gothert, 2007; Thorre *et al.,* 1998), some of these could potentially contribute to the differences in the BSS pattern observed between CD exposed males and females. However without actual measures of both structural and functional elements of the 5-HT and DA systems, respectively, no definitive role for the observed alterations in the observed 5-HT and DA concentration and metabolism can be assigned to explain the observed differences in the BSS.

Other brain structures could possibly be involved and even in the hitherto analysed brain regions an analysis of substructures, for example in the

hypothalamus, would be required (Williams *et al.,* 2000, Berthoud, 2002). Our analysis, which examined the whole hypothalamus, could be considered too rudimentary considering the number of substructures within the hypothalamus which make differing contributions to behaviour. Furthermore, other systems could potentiality be altered as well, as postnatal responses to nutritional challenges demonstrate (Zippel *et al.,* 2003; Davidowa & Plagemann 2004; Franke *et al.,* 2005). Nutritional effects on the central nervous system as manifested by changes in behaviour have only been shown recently (Melhorn *et al.* 2010). Although using a somewhat different approach, Melhorn *et al.*, revealed that even acute exposure to a high-fat diet alters meal pattern. An acute exposure to such a diet changed daily pattern of food intake, leading to adiposity. This, along with the present study, highlights the fact that hyperenergetic diets can change feeding behaviour itself, thus potentially contributing to the development of obesity via behavioural modification.

The present study further emphasises that early periods in development are potentially sensitive to nutritional effects on feeding behaviour that last until adulthood. Our findings are essentially in line with earlier findings providing evidence for hypothalamic neuronal plasticity due to maternal overfeeding (Page *et al.,* 2009, Davidowa *et al.,* 2003) and altered responses to DA administered into the hypothalamus (Zippel *et al.,* 2003). The observed changes in BSS could possibly suggest an increased susceptibility to "nutritional challenges" with highly palatable food in later life, as was alluded to by Bayol *et al.,* (2007), who reported an enhanced propensity to consume

highly palatable human food items in adult offspring subjected to maternal CD during early life**.** The fact that we did not see severe obesity in our rats is not surprising therefore, as a high-fat-diet-attenuated DA turnover can be independent of the development of obesity (Davis *et al.,* 2008). The latter finding indicates that reward mechanisms could also be subject to early life programming.

3.3.7 Brain neurotransmitter determination and learning and memory

There was no effect of maternal CD during lactation on 5-HT or DA metabolism in the hippocampus and frontal cortex in adult offspring of either sex. Both are regions implicated in the mediation of learning and memory functions essential for performance in both novel object discrimination and open field habituation tasks (Dere *et al.,* 2007; Leusis & Bolivar, 2006). On the surface it may appear that in this instance, 5-HT and DA systems may not be responsible for alterations to novel object discrimination in offspring subjected to maternal hyperenergetic diet throughout lactation. However, multiple studies have demonstrated that drugs which perturb 5-HT and DA systems can alter performance on novel object discrimination tasks (Meneses, 2007; King *et al.,* 2008; Watson *et al.,* 2011; Millan *et al.,* 2010).

Behavioural pharmacology experiments have revealed that the contribution of the 5-HT system to performance in novel object discrimination tasks is exceedingly complex, involving a wide range of sub-receptors across a number of brain regions and interactions with a number of other neurotransmitters (King *et al.,* 2004; 2008). A similar complexity could be assumed for the DA

system. It is almost certainly the case that simple measurements of 5-HT and DA metabolism in whole brain regions may not be sensitive enough to detect alterations induced by maternal CD upon the neural circuitry known to mediate performance in object discrimination tasks.

Other candidates may be responsible for a programming effect upon recognition memory by maternal CD during lactation. Multiple studies have demonstrated programming effects induced by perturbations to maternal choline intake (the precursor to acetylcholine) during critical periods of development, inducing impairments to performance on behavioural procedures measuring rule learning and spatial memory in adult offspring (Stevens *et al.,* 2008; Ryan *et al.,* 2008; Wong-Goodrich *et al.,* 2008). This may be related to alterations to the epigenome as choline has been demonstrated to determine DNA methylation patterns (Mehedint *et al.,* 2010; Zeisel, 2011, Okabe *et al.,* 2011). Cholinergic neural circuitry within multiple brain regions including the hippocampus and frontal cortex has been demonstrated to contribute to performance upon one trial recognition tasks (Dere, *et al.,* 2007). Like biogenic amine neurotransmitters, acetylcholine synthesis is dependent upon the acquisition of its dietary precursor. Due to the fact that choline intake was not recorded, it is difficult to make assertions about associations between choline content of diet and deficits in performance on the NOD test.

As discussed earlier, in addition to those discussed above there are many other neurotransmitters implicated in mediating performance on one trial

object recognition tasks including, glutamate, GABA, cannabinoids, neuropeptides and hormones (Dere *et al.,* 2007; King *et al.,* 2004; Barker *et al.,* 2006). Other brain regions have also been demonstrated to contribute to performance on one trial object recognition tasks such as the rhinal cortices and other regions within the cholinergic basal forebrain (Bartolini *et al.,* 1996; Winters & Bussey, 2005; Paban *et al.,* 2005; Barker *et al.,* 2006; Dere *et al.,* 2007).

Despite alterations in performance in a novel object discrimination task, maternal CD feeding during lactation had no effect upon 5-HT and DA metabolism in the hippocampus and frontal cortex in adult offspring of both sexes. The current measures of 5-HT and DA neurochemistry were not designed to infer causation in relation to behaviour, but examine the possibility gross changes to monoamine metabolism. Changes to monoamine neurochemistry in this instance may have not been detected by the methods currently employed or may be attributable to other neurotransmitter systems and/or in alternative brain regions.

3.3.8 Conclusion

In summary, the data presented in this chapter demonstrated that maternal exposure to hyperenergetic diet during lactation programmed alterations to ingestive behaviour and recognition memory, as well as hypothalamic 5-HT and DA neurochemistry in adult offspring of both sexes. As in the previous chapter, the present findings reiterate the importance of the lactation period in programming behaviour in adult offspring. Our findings demonstrate

further the wide range of behaviours which are potentially susceptible to programming by maternal overnutrition in adult offspring of both sexes. Despite it being difficult to make any direct associations between brain neurochemistry at time of death and behaviour, our results also suggest a speculative role for changes to hypothalamic monoamine neurochemistry in the mediation of some of the programmed changes to behaviour.

4.0 Chapter 4 - General discussion

4.1 Introduction

Epidemiological statistics cited in *Chapter 1* report that, as with levels of obesity in the general UK population, there has been a substantial increase in the prevalence of obesity in women of childbearing age (16-44) (NHS, 2011). Literature investigating the effects of obesity and/or hyperenergetic diet on behaviour in children (Wiles *et al.,* 2009; Peacock *et al.,* 2011), the elderly (Paile-Hyvarinen *et al.,* 2009; Baker *et al.,* 2011; Bruehl *et al.,* 2010) and other vulnerable groups (Schmidt *et al.,* 1997; Stevenson, 2006; Emond *et al.,* 2010; Blunden *et al.,* 2011; Kim & Chang, 2011) appears to show that diet can influence behaviour but that the exact nature of the relationship is not well understood. In parallel, rodent studies have demonstrated that chronic overnutrition can impair performance in multiple measures of learning and memory (Winocur & Greenwood, 1999, Stranahan *et al.,* 2008; Jurdak *et al.,* 2008; Jurdak & Kanarek 2009), can increase the propensity to engage in behaviours associated with anxiety (Souza *et al.,* 2007), depression (Abildgaard *et al.,* 2011) and aggression (Hilakivi-Clarke *et al.,* 1996), and lead to alterations in ingestive behaviour (Sclafani and Springer, 1976; La Fleur *et al.,* 2007).

Independently, epidemiological literature has demonstrated numerous associations between nutritional constraints during pregnancy and metabolic abnormalities and disease experienced many years later by the adult offspring (Barker *et al.,* 1989; Hales, *et al.,* 1991; Barker *et al.,* 1991; Barker & Martyn, 1992; Barker & Bagby, 2005; Stanner & Yudkin, 2001; Li *et al.,* 2011 Roseboom

et al., 2011). In parallel, the findings of rodent experiments present a persuasive picture regarding associations between maternal under- or overnutrition during critical periods of early development and metabolic and physiological abnormalities in adult offspring (Plagemann, *et al.,* 1999; Davidowa & Plagemann, 2000; Davidowa *et al.,* 2002; McMillen & Robinson, 2005; Langley-Evans & McMullen, 2010). Few studies to date have documented the effects of maternal overnutrition upon offspring behaviour (Bayol *et al.* 2007; Spencer & Tilbrook, 2009). Data presented in *Chapters 2* and *3* provide strong evidence for a nutritional programming effect upon behaviour in adult offspring induced by exposure to an obesogenic maternal diet. Our findings contribute significantly to literature reporting associations between maternal overnutrition during early sensitive periods and alterations to behaviour in adult offspring and add further credence to the assertion that the effects of maternal dietary perturbations extend from not just physiology but to also behaviour.

4.2 *Maternal overnutrition and offspring adiposity*

When taken with the findings of the literature described in the introduction, the results presented in this thesis suggest that the effects of maternal consumption of an obesogenic diet extend from abnormalities indicative of the metabolic syndrome, diabetes, cardiovascular disease and other disorders to behaviour in adult offspring. However, in this particular instance the nutritional programming effect on behaviour in adult offspring appeared to be independent of any major increases to adiposity. Maternal exposure to CD

throughout all periods led only to limited increases in adipose tissue mass, primarily in rats maternally subjected to CD throughout the lactation period and/or pregnancy. Maternal consumption of CD during lactation increased whole carcass fat content in female offspring, with interactions demonstrating that all the maternal feeding periods contributed in some way.

In contrast to these observations that any effects of maternal CD upon offspring adiposity were subtle and that the timing of the effect was largely limited to the lactation period, other studies have reported strong effects of maternal obesity upon offspring adiposity. For example, using Long-Evans rats and a high fat feeding regimen, White *et al.,* (2009) reported that maternal obesity induced by a hyperenergetic diet prior to conception continued through pregnancy and lactation, had an independent effect compared to maternal exposure to dietary fat alone and direct obesogenic feeding in adulthood. Maternal adiposity was of primary importance in programming obesity and metabolic abnormalities in adult offspring (as effective as direct exposure to the high fat diet). Work from our own laboratory using Wistar rats (Akyol *et al.,* 2011), employed a similar design to that used in *Chapter 2,* but after weaning split groups again either feeding weaned animals the CD or the control chow creating sixteen different groups. It was observed that maternal obesity, as well as CD feeding during pregnancy and lactation all had a major contribution to the programming of adiposity and glucose tolerance in the offspring, but only when combined with direct exposure to CD post-lactation.

The current findings bear more similarity to those of Akyol *et al,.* (2011) as the effects on offspring adiposity overall were only minor but seemed to be increasing in magnitude across all three feeding periods. Such findings demonstrate the variability and complexity of dietary models employed in fetal programming experiments, with results ranging from strong salient effects directly observable in adult offspring (White *et al.,* 2009; Benkalfat *et al.,* 2011), to more subtle programmed phenotypic changes which may only surface in response to dietary conditions in the current environment (Akyol *et al.,* 2011). The matter is further complicated when considering variations to the constitutional susceptibility of mothers and offspring to the obesogenic properties of diet, which may alter the propensity by which adiposity and metabolic abnormalities may occur (Levin, 2000a; 2000b; 2006; Muhlhausler *et al.,* 2006; Muhlhausler & Ong, 2011). The prospect of programming occurring across more than one or even several generations is intriguing and a concept which is already being investigated by researchers (Torrens *et al.,* 2008; Harrison *et al.,* 2009).

4.3 Maternal overnutrition and behaviour

It was hypothesized in *Chapter 2* that maternal CD feeding would programme emotional behaviour in adult offspring. Whilst the hypothesis presented in *Chapter 2* can be accepted, the issue of the timing of the effect i.e. maternal obesity vs. hyperenergetic diet during the latter periods is slightly more complicated due to the number of interactions between effects of CD feeding at different developmental stages, and some direct effects. However, the

present findings consistently show that maternal overnutrition during the lactation period, compared to maternal obesity and gestational overfeeding, had the most profound effect on behaviour. Whilst there was almost certainly some interaction, it would appear that the effects of the hyperenergetic diet can be clearly discriminated from the effect of maternal obesity. The importance of the lactation period was confirmed further due to fact that the hypothesis presented in *Chapter 3* could also be accepted, as CD feeding during lactation in isolation programmed changes to both ingestive behaviour and performance on measures of learning and memory. The issue of the timing of the effect is a salient one as the current findings differ from those reported in previous studies examining metabolic abnormalities.

The observations of Spencer and Tillbrook (2009), who produced similar findings to our own by exposing pups to overnutrition via a reduction to litter size also showed that the lactation period is of key importance for the programming of behaviour. Earlier studies investigating the programming of emotional behaviour in rodents using protein restriction models, which like the current study show an anxiolytic effect, also point to lactation as being critical (Almeida *et al.,* 1991; 1993; 1996b; Francolin-Silva *et al.,* 2006). The fact that hyperenergetic diet during lactation reduced parameters related to anxiety in offspring (as did protein restriction), may on the surface look like a beneficial effect of the early diet. In reality this may not be the case. In the numerous papers published by Almeida and colleagues, increased time spent on the open arms of the plus maze or the open field has been purported to represent enhanced impulsivity and should not necessarily be seen as positive

(Almeida *et al.,* 1991; 1993; 1996b; Francolin-Silva *et al.,* 2006). Despite this assertion, little evidence is presented to confirm that the reduction in anxiety related parameters is actually attributable to impulsivity. Rodent measures of impulsivity described in the comprehensive review by Winstanley, (2011), such as the stop signal task, the continuous performance task and the delay discounting task could confirm that offspring subjected to maternal dietary perturbations actually exhibit greater impulsivity.

Studies investigating the effect of neonatal undernutrition, mainly through protein restriction, on performance on behavioural measures of learning and memory in adult offspring also demonstrate the importance of the lactation period. The effect of maternal protein restriction on measures of learning and memory almost universally consists of deficits in performance compared to controls (Castro *et al.,* 1989; Bedi, 1991; Fukuda *et al.,* 2002). It is worth noting that maternal protein restriction during pregnancy has similar effects to that of lactation upon measures of learning and memory in adult offspring (Jordan *et al.,* 1981; Cordoba *et al.,* 1994).

The majority of studies investigating the effect of maternal diet upon ingestive behaviour in adult offspring show that dietary perturbations during pregnancy can induce alterations to macronutrient selection and subtle changes to the structural integrity of feeding behaviour in adult offspring (Bellinger *et al.,* 2004; Bellinger & Langley-Evans, 2005; Bayol *et al.,* 2007; Orozco-Solis *et al.,* 2009). The findings presented in *Chapter 3* demonstrated that in addition to

pregnancy, the lactation period may also act as a window where nutritional manipulations can programme ingestive behaviour in adult offspring.

4.4 Why the lactation period?

4.4.1 Maternal milk

The observation that a hyperenergetic maternal diet during the lactation period can programme offspring behaviour and brain neurochemistry raises the question of what may be occurring during the lactation period that is inducing change. Two key questions are, what is the effect of CD feeding upon the content of maternal milk; and what is the effect of CD feeding upon maternal behaviour and mother-pup interaction? Del Prado *et al.,* (1997) demonstrated that lactating rats fed a high fat diet has a greater milk yield and secreted milk with a significantly higher lipid content than milk of dams fed a low fat diet. One early study reported that feeding lactating rats CD not only attenuated the protein content of milk, but increased the lipid content compared to that given by control dams (Rolls *et al.,* 1986). The milk of rats rendered obese by cafeteria diet feeding was hypercaloric compared to the milk of lean animals.

As milk has been reported to contain large amounts of leptin, it may be the case that maternal overnutrition induces alterations to the leptin content of milk and that this contributes to the programming of metabolic abnormalities and behaviour in adult offspring. Sanchez *et al.,* (2008) reported that Wistar rats which received oral doses of leptin during the lactation period, at fifteen months of age had lower bodyweights, lower scores on an insulin resistance

index, lower circulating levels of insulin across various feeding conditions, lower levels of plasma leptin, higher levels of insulin sensitivity, as well as the propensity to consume less calories. Pico *et al.,* (2007a) gave male Wistar rats a dose of leptin five times greater than the levels present in maternal milk during the lactation period and measured adiposity, leptin expression and hypothalamic NPY, POMC and leptin OB-R expression at six months of age. Animals who received leptin supplementation exhibited lower bodyweight and adiposity and consumed fewer calories compared to controls. The same rats exhibited attenuated hypothalamic NPY and POMC mRNA levels in response high fat feeding, post-lactation. It is worth acknowledging that the doses of leptin administered in the two studies mentioned above were significantly higher than the amounts of leptin present in maternal milk from dams exposed to CD or high fat diets however, they provide more extreme examples how neonatal perturbations to leptin supply can programme metabolic abnormalities in later life.

Further investigations of the leptin content and macronutrient composition of milk produced by dams fed CD during early critical periods of development would provide a greater insight to potential mechanisms underlying changes to behaviour and brain neurochemistry in adult offspring. Also notable, was the observation that towards the latter periods of the lactation period, infant rats had a propensity to consume the CD food items directly as their reliance on suckling milk from the mother faded. Such observations raise the possibility that alterations to behaviour and brain neurochemistry may be partially attributable to direct CD feeding at the end of the suckling period.

Withdrawing the diet during the last week of the lactation period could potentially control for this, although this would potentially reduce the magnitude of the treatment effect. It would be interesting to target feeding during each individual week of lactation and compare the effect on behaviour of adult offspring. Despite this, as we found many interactions and some direct effects of maternal obesity and maternal diet during pregnancy in *Chapter 2*, it can be confidently asserted that the majority of alterations to behaviour were not attributable the effects of direct consumption of the diet by offspring.

4.4.2 Maternal behaviour

An alternative explanation of the effect of CD during lactation upon offspring behaviour is that that changes to behaviour and brain neurochemistry are, in part, mediated by changes to maternal behaviour. Experimental manipulations known to alter maternal behaviour have also been demonstrated to programme behaviour and brain neurochemistry in adult offspring (Smythe, et al., 1996; Meaney, *et al.,* 1996; Champagne *et al.,* 2006 Plotsky *et al.,* 2005; Cameron *et al.,* 2005). Maternal separation or repeated handling of dams during the neonatal period has been reported to alter the propensity of mothers to engage in beneficial maternal behaviours, such as licking and grooming of the pups and 'arched back rearing' which allows the pups easy access to suckle milk (Caldji *et al.,* 1998; Anisman *et al.,* 1998; Francis & Meaney, 1999; Champagne *et al.,* 2003). Maternal separation has been reported to reduce the propensity of mothers to express such beneficial

maternal behaviours, whereas handling has the opposite effect (Francis & Meaney, 1999).

Such manipulations have been demonstrated to alter anxiety and fear related behaviour, the responsiveness of the stress system and brain biogenic amine brain neurochemistry in adult offspring, primarily through glucocorticoid related mechanisms (Meaney *et al.,* 1988; 1989; Plotsky & Meaney, 1993; Caldji *et al.,* 1998; Smythe *et al.,* 1994; Laplante *et al.*, 2002; Champagne *et al.,* 2003). The question of whether exposure to CD during any of the feeding periods examined, particularly the lactation period, can alter maternal behaviour remains open and would be interesting to investigate using measures such as the pup-retrieval test and/ or measures of licking and grooming and arched back nursing.

In the past decade the argument for the programming of multiple brain systems in offspring through alterations to maternal behaviour has taken on an extra dimension. It has been hypothesized that maternal care during early life can induce epigenetic modifications leading to semi-permanent and sometimes irreversible phenotypic changes in offspring (Weaver *et al.,* 2004a; 2004b; Bagot & Meaney, 2010; Kappeler & Meaney, 2010; Caldji *et al.,* 2011; McGowan *et al.,* 2011). It is asserted that demethylation states can continue throughout the early neonatal period and that signals derived from the quality of environment and maternal care can trigger alterations to intracellular signalling pathways which lead to the remodelling of DNA methylation in offspring (Caldji *et al.,* 2011). Weaver *et al.,* (2004a) reported that offspring of

mothers who were more likely to engage in beneficial maternal behaviours such as LG-ABN showed greater levels of DNA methylation compared to offspring of mothers who were less likely to perform such behaviours. DNA methylation in offspring occurred during the first week of life and could be ameliorated by cross-fostering to competent mothers, but without intervention, would persist into adulthood. Changes induced by the quality of mother-pup interaction included alterations to glucocorticoid receptor gene expression in the hypothalamus, the hippocampus and the amygdala, as well as alterations to stress response and changes to activation of 5-HT systems within multiple brain regions (Weaver *et al.,* 2004b; Zhang *et al.,* 2004; Szyf *et al.,* 2005). Offspring deprived of beneficial maternal behaviours experienced changes to gene expression and exhibited concomitant behavioural signs of anxiety and fearfulness as adults (Zhang *et al.,* 2004; Diorio & Meaney *et al.,* 2007).

Preliminary research undertaken in our own laboratory has demonstrated that dams fed CD from the moment of birth were more likely engage in nest building behaviour and beneficial maternal behaviours such as licking and grooming of the pups, despite no change between groups upon a pup retrieval test (Speight, unpublished). When tested at weaning, pups from the CD fed litters exhibited an anxiolytic behavioural profile in the open field compared to offspring from chow fed mothers. Further investigations into both maternal behaviour and composition of maternal milk could potentially provide important insights into the mechanisms underlying the programming of behaviour in offspring.

4.5 Brain neurochemistry data – meaning and limitations

The finding that hypothalamic monoamine metabolism in adult offspring is sensitive to nutritional programming manipulations is not new (Kehoe *et al.,* 2001; Lopes de Souza *et al.,* 2008), although the fact that maternal CD during lactation programmed 5-HT neurochemistry in the hypothalamus is novel. The 5-HIAA/5-HT calculation has been devised as a method measuring the turnover ratio and thus 5-HT metabolism. The fact that 5-HT turnover was reduced in the hypothalamus suggests a possible overall reduction in serotoninergic tone in that region. It is possible that maternal hyperenergetic diet during lactation could remodel tissue morphology, inducing cytoarchitectural changes to 5-HT neurons within the hypothalamus, or from serotonergic projections from the dorsal raphe nuclei to the hypothalamus.

Studies demonstrating the programming effect of alterations to maternal behaviour (Weaver *et al.,* 2004a; 2004b; Zhang *et al.,* 2004; Szyf *et al.,* 2005), as well the programming of metabolic abnormalities by maternal dietary perturbations (Langley-Evans, 1997; Gardner *et al.,* 1997) suggest the most obvious candidates for driving such morphological adaptations and tissue remodelling in offspring are excessive glucocorticoid release, or epigenetic processes during the lactation period. Studies discussed in *Chapter 1* describe how maternal protein restriction, or overnutrition induced by litter size reduction can induce changes suggestive of morphological adaptations or tissue remodelling in the neural circuitry involved in the mediation of energy homeostasis, as accompanied by epigenetic changes (Plagemann *et al.,* 2000a;

2000b; Davidowa & Plagemann, 2000; Davidowa *et al.,* 2002; Davidowa & Plagemann, 2003).

Researchers documenting the ontogeny of the 5-HT system in the rat report that 5-HT neurons begin to appear approximately halfway through the gestation period, during embryonic days eleven and twelve and continue to develop after birth through the suckling period (Lauder *et al.,* 1982; Lauder, 1990; Liu & Lauder, 1992). Lauder (1990) demonstrated that during embryonic development, 5-HT acts as an ontological signal which can induce morphological and biochemical differentiation of neurons through changes to synaptogenesis and neurite outgrowth in the dorsal raphe nuclei and in terminal projection areas.

In vivo and *in vitro* experiments have demonstrated interactions between monoamine neural development and 5-HT axons, as well as neuroepithelial cell and glioblast proliferation during embryonic and postnatal development. These effects involve multiple 5-HT receptors (Lauder et al., 1982). Autoradiographic studies have revealed that although 5-HT and DA receptors are present at birth, throughout the suckling period the density of receptors and the 5-HT transporter increases rapidly across multiple regions in striatum, limbic system and cortex (Murrin *et al.,* 1985). It would appear that the 5-HT system, particularly 5-HT receptor and transporter density is sensitive to multiple insults during the lactation period, including perturbations to amino acid availability (Mathura *et al.,* 1986;), neurotoxins (Pranzatelli & Martens, 1992; Slotkin & Seidler, 2005; Slotkin *et al.,* 2009), manipulations of maternal

behaviour (Stamatakis *et al.,* 2006; Vicentic *et al.,* 2006) and maternal dietary manipulations (Kehoe *et al.,* 2001). Although we can only speculate as to what the effect of hyperenergetic diet during lactation was on the developing serotonin system, morphological alterations including synaptogenesis, neurite outgrowth, changes to receptor density, numerical density of dendritic spines and even the number of neurons are the most likely possible candidates.

Despite being able to make some broad assertions regarding links between changes in hypothalamic 5-HT and DA neurochemistry in adult offspring and changes to appetite related behaviour in adult offspring, a more effective method of linking changes to behaviour would have been advantageous. However, although methods such as microdialysis enable real-time measurement of neurotransmitter function, it is not a viable procedure to use in combination with many measures of behaviour due to the invasive insertion of an intracerebral or intracerebroventricular cannula directly into the brain. Also the fact that the whole hypothalamus was analysed makes more specific assertions regarding hypothalamic monoamine satiety mechanisms difficult. For example, as the hypothalamus contains numerous sub-regions, each containing serotonergic nuclei with multiple 5-HT sub-receptors, each sub-region may have variable contributions to the expression of behaviours related to satiety; Halford *et al.,* 2004; 2007; 2010). However, despite the limitations of the present findings, the fact that the data indicated that hypothalamic monoamine neurochemistry can be programmed by maternal overnutrition opens the door for further more detailed investigations into the nature of the effect.

4.6 Possibilities for future research

Given the effects of maternal CD upon anxiety and exploratory behaviour, it would be interesting to analyse neurotransmitter turnover and/or receptor gene expression in brain regions associated with anxiety, such as the amygdala (Stein & Stahl, 2000; Canteras *et al.,* 2010), the dorsal and median raphe nuclei (Costall *et al.,* 1988; Carli *et al.,* 1989; Higgins *et al.,* 1992), the periaqueductal grey (Brandao *et al.,* 2008; Graeff*,* 2004) and locus coeruleus (Weiss et al., 1994; Curtis *et al.,* 1999). Obvious neurochemical candidates for investigation into a programming effect upon anxiety and exploratory behaviour in offspring would include GABA (Little, 1991; File, 1988; Shephard, 1986), 5-HT (Shephard, 1986; Handley *et al.,* 1993; Handley & McBlane, 1993; Graeff *et al.,* 1996) and NA (Morilak *et al.,* 2005) systems. It would be interesting to investigate whether changes to the behavioural profile of rats subjected to maternal CD on the plus maze and the open field could be ameliorated or exacerbated by anxiolytic or anxiogenic drugs with affinity to 5-HT and NA receptor sub-types, as well as manipulations which perturb GABA neurochemistry. Investigations into the effects of maternal CD on behavioural measures sensitive to the administration of anti-depressant drugs, such as the forced swim test, measures of social defeat or the differential reinforcement of the low rate 72 sec schedule (DRL-72-s) in maternal CD offspring would also be valuable.

A programme of work which examined 5-HT and DA neurochemistry within sub-regions of the hypothalamus such as the ARC, the PVN, the VMH, the

DMH and the LHA, would provide a much greater insight into the effects of maternal overnutrition during lactation upon hypothalamic biogenic amine metabolism in offspring. Additionally measurement of other neurotransmitters, neuropeptides and hormones related to energy homeostasis such as NPY, AGRP, α-MSH, CART, leptin, CCK, ghrelin and the orexins would also be usefull, as well as assays of endocannabinoid and endogenous opioid neurotransmitters and neuropeptides.

It would also be interesting to analyse the effects of the administration of serotonergic and dopaminergic drugs on macronutrient selection and the BSS in offspring exposed to maternal obesity and/or CD feeding during pregnancy and lactation. In order to elucidate the mechanisms potentially underlying alterations to behaviour, it would be of interest of see if alterations to the BSS induced by CD during lactation could be ameliorated or exacerbated by drugs known to act at 5-HT sub-receptors.

Brain regions such as the NAc (the core and shell regions), the VTA, the amygdala, the bed nulceus of the stria terminals (BNST), the hippocampus and the substructures of the prefrontal cortex have been demonstrated to mediate the hedonic, emotional, contextual and executive aspects of appetite related behaviour, in addition to the critical role played by the hypothalamus (Morrison & Berthoud, 2007; Berthoud & Morrison, 2008; Zheng *et al.* 2009; Berthoud *et al.,* 2011). Analysis of neurotransmitter, neuropeptide and endocannabinoid systems in the regions mentioned above in adult offspring

subjected to maternal CD would provide an insight into mechanisms potentially underlying programmed changes to behaviour.

Given the fact that the neural circuitry known to mediate the desire, or 'craving' to consume highly palatable food items is more or less the same circuitry known to mediate the rewarding and reinforcing properties of drugs of abuse (Berridge & Robinson, 2003; Wise, 2006; Morrison & Berthoud, 2007; Berthoud & Morrison, 2008; Berthoud *et al.,* 2011), the issue of whether offspring exposed to maternal CD during pregnancy/lactation could have an enhanced susceptibility to engage in addictive behaviours as adults is an intriguing question.

With this in mind it would also be interesting to measure behaviour in rats maternally subjected to obesogenic dietary conditions during early sensitive periods using rodent models of addiction. Such measures could include operant measures of drug self-administration involving various schedules of reinforcement, place preference procedures, drug discrimination procedures and intracranial self-stimulation procedures (Schaefer & Michael, 1992; Lu *et al.,* 2003; Lynch *et al.,* 2010; Koob & Volkow, 2010). It may be the case that animals subjected to maternal overnutrition may have an alternated propensity to consume drugs of abuse.

As discussed in the previous chapter, both 5-HT and DA (King *et al.,* 2004; 2009; Watson *et al.,* 2011), as well as acetylcholine and glutamate (Wooley *et al.,* 2003; King *et al.,* 2004; Kendall *et al.,* 2011), have been demonstrated to mediate performance on measures of one trial object recognition tasks,

involving their numerous respective receptor sub-types. The hippocampus and other regions such as the rhinal cortices and the cholinergic basal forebrain are other potential candidates for investigation into what may underlie alterations to performance on measures of object discrimination (Bartolini *et al.,* 1996; Winters & Bussey, 2005; Paban *et al.,* 2005; Barker *et al.,* 2006; Dere *et al.,* 2007).

4.7 Notes on age

It is worth acknowledging that the animals in the present instance were only tested during young adulthood (10-20 weeks of age). What we have not been able to document is the longitudinal effect of such manipulations upon behaviour across the lifetime of the offspring. There are many instances when the nature of the programming effect is dependent on age (Langley-Evans & Sculley, 2005; 2006; Erhuma et al., 2007). It may be the case that behavioural alterations induced by maternal overnutrition may normalize or increase throughout adulthood. Further research investigating the more long term effects of such manipulations would provide a more comprehensive insight into the effects of maternal overnutrition on offspring behaviour.

4.8 Strain comparisons

In this particular instance Wistar rats were used to investigate the consequences of maternal overnutrition for behaviour in adult offspring. Several studies conducting comparisons between strains of rat have demonstrated that other strains may be better suited for behavioural testing, principally the Lister Hooded strain (Broersen & Uylings, 1999; Commissaris *et*

al., 2000; Weiss *et al.,* 2000; Manahan-Vaughan, 2000; Ennaceur *et al.,* 2005; McDermott & Kelly, 2006). Lister Hooded rats have been demonstrated to out-perform Wistar rats on measures of recognition and spatial memory with females often out-performing males (Ennaceur *et al.,* 2005).

The Long-Evans strain has also been demonstrated to out-perform Wistar rats when tested on a wide range of measures of learning and memory (Holahan *et al.,* 2006; Vales *et al.,* 2006; Platano *et al.,* 2008; Keeley *et al.,* 2010). In addition to this it should be noted that there may be strain-specific differences in response to dietary change. It has recently been demonstrated that Lister Hooded rats are more vulnerable than Wistar rats to effects of iron deficiency during pregnancy (Cornock *et al*., unpublished data). For future investigations into the programming of behaviour in rodents, researchers may want to take strain comparisons into consideration when planning experiments in order to get the best results.

4.9 From rats to humans

In terms of the applicability of the current findings to humans, differences in the timing of CNS development between rats and humans make direct cross-species comparisons difficult. The phase of brain development occurring during the lactation period in rats is far from the same phase of brain development occurring during lactation in humans. Compared to humans, rats are a far more altricial species born with the CNS in an undeveloped state and entirely dependent upon the quality of maternal care provided by the mother to ensure adequate development (Francis & Meaney, 1999; Caldji *et*

al., 2000). During the early neonatal period in rats, layers of the cerebral cortex and crucial aspects of the visual system are still developing and continue to form throughout the lactation period, whilst the same would be occurring in humans early on in gestation (Bayer *et al.,* 1993; Clancy *et al.,* 2001; 2007).

Using data from numerous studies to construct a model capable of translating developmental time points of CNS ontogeny across mammalian species, Clancy *et al.,* (2001) reported that events such as cerebral cortex development occurring in the early neonatal period in rats occur during the 2nd and 3rd trimesters of pregnancy in humans. The developmental timing of 95 neural events preceding eye opening listed from previous studies were complete in humans by the end of the 2nd trimester of pregnancy with eye opening in rats occurring two thirds of the way through the lactation period (Clancy *et al.*, 2001). Such observations suggest that experimental manipulations effecting rat CNS development during the lactation period would equate to the 3rd trimester of pregnancy in humans. According to Clancy *et al.,* rats are born with their CNS approximately half as developed as humans.

In rats the hippocampus develops after 75-85 percent of the gestation period whereas this only occurs after approximately 19-23 percent in humans (Clancy *et al.,* 2001). In the rat, substructures of the hypothalamus including the PVN, the VMH, the DMH, the LHA and ARC have been demonstrated to form during embryonic days 13-15 out of the 21 day gestation period, compared to embryonic days 30 to 45 of the 161 day gestation period in the macaque

(Altman & Bayer, 1986; Swanson, 1992; van Eerdenburg & Rakic, 1994; Markakis 2002). In the rat, hypothalamic neural circuitry known to mediate appetite regulation continues to develop during the latter half of gestation with development continuing post-partum throughout the lactation period compared to humans and non-human primates where such structures develop relatively early on in gestation (Seress, 1985; Kagotani *et al*., 1989; Markakis, 2002; Singer *et al.,* 2000; Brogan *et al.,* 2000; Grove *et al.,* 2003; Grayson *et al.,* 2006). Despite this incongruence, what remains clear is that maternal exposure to obesogenic diet during early critical periods of development can programme behaviour in adulthood and this raises important questions.

Although it is not always easy is to equate changes to behaviour in animal studies with that in primates and humans, studies examining offspring subjected to maternal starvation in the womb during the Dutch winter famine showed increased prevalence of psychiatric disorders during adulthood including affective disorders (Brown *et al.,* 1995; Brown *et al.,* 2000; Stein *et al.,* 2009), schizophrenia (Hoek *et al.,* 1998; Hulshoff Pol *et al.,* 2000; Brown & Susser, 2008) and addiction (Franzek *et al.,* 2008). Similar to the present findings, Almeida and colleagues (1993; 1994) reported that maternal protein restriction led to reduced anxiety and enhanced exploratory behaviour, which they attributed to be a result of increased impulsivity. Increases on psychometric measures of impulsivity have been shown to be a characteristic feature of numerous psychiatric disorders, addiction to alcohol and drugs and the propensity to engage in dangerous and risky behaviour, as well as suicide

(Dervaux *et al.,* 2010a; 2010b; Nolan *et al.,* 2011; Dumais *et al.,* 2011). Although our controls represented a 'low anxiety' phenotype, the fact that anxiety was reduced even further is notable, but not necessarily indicative of psychopathology.

Researchers have speculated that maternal factors such as emotional problems during pregnancy, substance misuse, history of failed pregnancy, as well as lack of breastfeeding and fever or illness in early life are risk factors for psychopathology in offspring such as anxiety, depression, behavioural disorders and substance misuse (Allen *et al.,* 1998; Hill *et al.,* 2000; Linnet *et al.,* 2003; Alder *et al.,* 2007; Rice *et al.,* 2010; Martini *et al.,* 2010). In humans, shorter durations of breastfeeding have been reported to be associated with attention deficit hyperactivity disorder in childhood, as shown by impaired performance on psychometric measures of executive function, social competence and attention deficit hyperactivity symptom scores (Julvez *et al.,* 2007).

Although to date there are no viable animal models of eating disorders, as there are for measures of anxiety, fearfulness and depression, the fact that the structure of eating behaviour was programmed by the early diet may lead us to infer that similar disturbances to feeding related behaviour could be induced in humans. Anorexia nervosa, bulimia nervosa and binge eating disorder are disorders associated with abnormal patterns of eating behaviour (Van Binsbergen *et al.,* 1988; Walsh *et al.,* 1989a; 1989b; Elmore & de Castro, 1991; La Chaussee *et al.,* 1992; Boyle *et al.,* 2011). Although changes to the

BSS are unlikely to translate directly into feeding related psychopathology in humans it may be an increased risk factor to the development of such disorders.

4.10 Conclusion

Given the observations that direct exposure to obesogenic dietary conditions altered performance upon behavioural measures of emotional behaviour, learning and memory and appetite related behaviour in rats, we hypothesized that hyperenergetic diet during pregnancy and lactation, as well as maternal obesity would also alter performance on behavioural measures. The findings of the experiments presented in this thesis indicated that the effects of maternal obesity could be discriminated from the effects of maternal over-feeding (independent of obesity) and demonstrated that overnutrition during the lactation period was of primary importance in programming offspring behaviour. Lactational overnutrition also altered biogenic amine neurochemistry in adult offspring, most notably 5-HT concentrations and turnover in the hypothalamus. To date, despite the fact that many treatment strategies have been devised to curb obesity, in combination with campaigns to promote healthy eating and living habits and despite the considerable expense of obesity for society, the effectiveness of treatment overall is poor and levels of obesity continue to rise unabated. Although there have been several decades of intense scientific interest, to date little research has been undertaken investigating the behavioural effects overnutrition during early sensitive periods of life on the offspring. The findings outlined in the present

thesis demonstrate for the first time that maternal exposure to an obesogenic diet during early sensitive periods of development can programme a range of behaviours in adult offspring of both sexes.

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