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The Influence of Early Life and Positive Affect on Feeding Behaviour and Food Choice in the Rat

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Declaration

I, Amy Warnock, hereby declare that this thesis has been composed by myself, and that the work and data analysis carried out in this thesis is my own, unless clearly indicated throughout the thesis. Furthermore, this work has not been submitted for any other degree or professional qualification. All sources have been clearly acknowledged throughout the thesis.

Signed……………………………………………………………

Date………………………..

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Abstract

In recent years, worldwide obesity rates have risen dramatically, putting major strain on public health systems and the economy. Obesity is a multifaceted disease and its development can be influenced by a variety of factors including genetic, psychological and environmental influences. One area of current focus in obesity research is that of early life programming. It has been well-established that certain early life factors can impact the physiology and behaviour of the offspring. Because of this, early life programming has become increasingly well studied in order to develop a deeper understanding of how early life can influence obesity development. Another area of interest lies in positive mood. While there has been much research into the effects of negative states such as stress and anxiety on feeding behaviour, there is still very little known about how positive states can influence food choice. Using rat models of prenatal stress, neonatal overnutrition and positive affect, this thesis aimed to investigate the effects of early life and mood factors on feeding behaviour and food choice.

Prenatal stress has been extensively studied and is characterised by an enhanced stress response in the offspring. Using two rat models of prenatal stress-social and restraint stress, the effects of prenatal stress on feeding behaviour and food choice in the offspring were examined. In both models, no effects of prenatal stress on either food intake or food choice were observed. However, in both cases the expected alterations to the offspring’s stress responses when exposed to an acute stressor were not replicated. This may suggest that models of prenatal stress are not as robust as often cited in the literature.

As well as the prenatal environment, the early postnatal environment is also able to influence physiology and behaviour. In terms of obesity, a well-studied model is that of small litter size. Rats from small litters are over-nourished as neonates and because of this illustrate an increased body weight that persists throughout life. While this increase in weight gain has been well-established, there is no evidence examining the impact of neonatal overnutrition on long-term food choice. Therefore, food intake and food choice were measured in small and control litter rats over a 10-week period. When placed on an ad lib diet of bland chow, sucrose and lard, small litter rats consumed significantly more chow than control litter rats, whilst maintaining similar consumption of lard and sucrose. However, when offered a high-fat high-sugar (HFHS) pellet for two hours a day alongside ad
lib chow, small litter rats illustrated increased consumption of the HFHS pellet compared to controls. This suggests that small litter rats may be programmed to adjust their food choices to enable them to maintain their increased body weight in comparison to controls.

To examine the effects of positive affect on feeding behaviour, ultrasonic vocalisations (USVs, specifically those at 50 kHz) were used as a measure of positive affect in rats. In order to examine whether access to a food reward could induce a positive affect (as measured by an increase in 50 kHz USVs), rats were schedule-fed sweetened condensed milk and USVs measured before, during and after consumption. No differences in 50 kHz USVs were observed suggesting that a palatable food, whilst rewarding, does not alter affective state in the rat. Using heterospecific social contact (a tickling interaction simulating rough and tumble play) to induce positive affect, rats were presented with an hour-long sucrose preference test following social contact in order to examine the impact of positive affect on food choice. While no differences in sucrose consumption were found, a reduced sucrose preference was observed in rats receiving social contact compared to controls, suggesting that positive affect may play a role in mediating food choice. Finally, the effects of fasting (a negative stimulus thought to reduce 50 kHz USVs) and a food reward on motivation for social contact were examined. Both fasting and access to a food reward resulted in no differences in conditioned place preference to receive social interaction.

Overall, the results obtained in this thesis implicate both neonatal overnutrition and, for the first time, positive affect as possible mediators of food choice, although further studies are required to fully establish these effects. Importantly, these results also raise questions regarding the reproducibility of some early life models, such as prenatal stress, and highlights the importance of sharing precise experimental protocols across laboratories. Through further investigation of the effects of early life and affective states on food consumption and choice, and the mechanisms behind these, this may enable the development of therapeutic interventions and preventative measures that can help slow, or even reverse, the global obesity epidemic.
Lay Abstract

Obesity is a worldwide health issue that is reaching higher levels than ever before. Although the causes of obesity may seem simple: overeating and reduced exercise, the reality is that obesity development can be influenced by many different factors. One area that has been recently gaining interest is the effect of early life, such as how changes during pregnancy, or eating habits during early life can lead to differences in what and how much we eat. Mood is also an important factor when it comes to feeding behaviour. There has been much research into how negative moods, such as stress and anxiety, can change our eating patterns but there is still very little evidence on how positive mood can alter food choices. Therefore, this thesis set out to investigate the effects of early life and mood on feeding, using rat models of stress during pregnancy, early life over-eating and positive mood.

It is well-established that stress can lead to changes in what and how much we eat. Both humans and rats born of mothers experiencing stress during pregnancy often show higher sensitivity to stress, releasing higher levels of stress hormone when exposed to stress. Because of these changes to the stress system in offspring of stressed mothers, it was investigated whether experiencing stress during pregnancy would lead to any changes in food consumption or food choice. This was achieved using rat models: exposing rats to stress during pregnancy and then measuring feeding behaviour in their offspring. Both the total amount of food consumed, and food choices did not differ in rats who had been exposed to stress during pregnancy or control rats who had experienced no stress. However, when testing the levels of stress hormone in these rats, the expected increase in stress hormone was not seen. This may suggest that the models of stress during pregnancy used in these experiments did not work as expected, which raises questions about the use of rats to reliably model the effects of early life factors, such as stress during pregnancy.

Using rat models, it has been well-established that overeating in early life leads to increased weight gain and obesity development. This model is often achieved through reducing a rat’s litter size shortly after birth, as this leads to the pups having access to more milk and therefore becoming over-nourished. Although it is well-established that this leads to obesity, there is still very little evidence to show how overeating in early life can lead to changes in food choice. Therefore, this was investigated by placing rats from small litters on a diet of standard rat food, fat and sugar. It was found that small litter rats ate similar
amounts of sugar and fat compared to normal rats but surprisingly ate more of the standard, less palatable, food. These rats were then offered a high-fat high-sugar food alongside the standard food which led to the small litter rats eating more of the high-fat high-sugar food. This suggests that rats that have been over-nourished in early life develop changes in their food choice that enable them to maintain their increased body weight.

It is well known that negative moods such as stress and anxiety can lead to changes in what and how much is eaten, however there has still been very little research done on how positive mood can alter feeding. When studying mood in rats a tool known as ‘ultrasonic vocalisations’ (USVs) can be used. These are sounds made by rats outside the range of human hearing which can be recorded using specialised equipment. Sounds at a frequency of 50 kHz have been associated with positive experiences such as play and sex and are therefore assumed to indicate a positive mood in rats. Through measuring these sounds it was investigated whether giving rats a rewarding food would lead to changes in mood, however no differences in USVs were observed suggesting that a food reward does not induce a positive mood. It is possible to induce a positive mood in rats by tickling them, as this mimics their natural play behaviour. Therefore, food choice between a sugar solution and water was measured in rats after receiving a tickling interaction to investigate whether positive mood could alter food choice. No differences in the amount of sugar solution consumed were seen between tickled rats and controls, but when measuring preference between the sugar solution and water, a lower preference for the sugar solution was found in the tickled rats, suggesting that positive mood might be able to alter food choices.

Finally, it was examined whether access to a food reward or fasting would lead to any changes in the rat’s motivation to receive tickling, but no differences were found.

Overall these results suggest that both early life overfeeding and positive mood could have important influences on food choice. It is important to continue studying the effects of early life and mood on eating behaviour as by developing a deeper understanding into these issues, it may be possible to develop ways to prevent these factors influencing eating behaviour, and therefore possibly reducing obesity levels.
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List of Abbreviations

11β-HSD2 - 11-β-hydroxysteroid dehydrogenase 2
α-MSH – alpha melanocyte stimulating hormone
ACTH - adrenocorticotropic hormone
AgRP - agouti-related peptide
ARC - arcuate nucleus
AUC – area under curve
BMI – body mass index
Cort - Corticosterone
CPP – conditioned place preference
CRF - corticotrophin releasing factor
CV – coefficient of variability
DMH - dorsomedial hypothalamic nucleus
FM – frequency modulated
GR – glucocorticoid receptor
HFHS – high-fat high-sugar
HPA - hypothalamic-pituitary-adrenal
ICV – intracerebroventricular
LH - lateral hypothalamus
MR – mineralocorticoid receptor
NAc – nucleus accumbens
NSB – non-specific binding
NPY - neuropeptide Y
POMC - pro-opiomelanocortin
PNS – prenatal stress
PVN - paraventricular nucleus
RIA – radioimmunoassay
SCM – sweetened condensed milk
SD – standard deviation
SEM – standard error of mean
SON - supraoptic nucleus
USV – ultrasonic vocalisations
VMH - ventromedial hypothalamus
VTA - ventral tegmental area
Chapter 1: Introduction

1.1 Obesity

In recent years, worldwide obesity prevalence has dramatically increased, with at least 2.8 million deaths each year occurring as a result of excess weight (WHO, 2017). Although initially only a health issue associated with high income countries, obesity is becoming increasingly prevalent in low and middle income countries (Bhurosy and Jeewon, 2014). While recently some developed countries have observed a plateau of obesity rates, nowhere has observed a decline in obesity prevalence (Wabitsch et al., 2014). Obesity is defined as an excessive accumulation of fat that may impair health and is often diagnosed by measurement of a patient’s body mass index (BMI), calculated by dividing body weight (in kg) by height$^2$ (in meters). A BMI between 25-30 is considered overweight, whereas a BMI over 30 is classified as obese (Williams et al., 2015). In 2016, it was estimated that around 13% of the global adult population was obese, a figure that has nearly tripled since 1975 (WHO, 2017). Alarmingly, recently childhood levels of obesity have also risen dramatically, which is problematic as weight gain in childhood is a risk factor for adulthood obesity (Kumar and Kelly, 2017).

Obesity is associated with an increased risk of a multitude of health problems. One of the most prevalent is an increased likelihood of cardiovascular diseases, such as heart disease and stroke. Obesity is also associated with development of type 2 diabetes, some forms of cancer and musculoskeletal problems. Furthermore, there are also associations between obesity and psychosocial problems such as depression, anxiety and low self-esteem due to the stigma often associated with increased weight (Williams et al., 2015).

1.1.1 Causes

Fundamentally, the cause of obesity is an imbalance in calorie intake and expenditure, leading to accumulation of excess fat. The rise in obesity rates is often attributed to recent changes in our environment; processed foods high in fat and sugar are not only readily and cheaply available, but portion sizes of such foods have also increased (Young and Nestle, 2002). Furthermore, advances in technology, whilst increasing productivity, may result in decreased activity levels further contributing to caloric imbalance (Ladabaum et al., 2014).
While these arguments are often presented as the primary cause of increased obesity prevalence, evidence supporting these links is lacking, mainly due to the extreme difficulties in accurately measuring energy intake and expenditure in large populations. In reality the aetiology of obesity is much more complex and can involve a range of factors including genetic, psychological, physiological, environmental and economic influences (Wright and Aronne, 2012).

1.1.2 Treatments
Whilst in theory body weight can be reduced by reducing calorie intake and increasing calorie expenditure, the reality is that obesity is very difficult to treat. Traditional methods such as dieting and exercise, whilst effective in the short term, often result in patients gaining back the excess weight within several years (Mann et al., 2007, Ochner et al., 2015). In the past there has been much effort to combat obesity through therapeutic treatment, however whilst several drugs have been trialled that do lead to weight loss, these are often associated with other unwanted side effects, for example combined treatment of d,l-fenfluramine and phentermine (commonly known as Fen/Phen) while successful in reducing body weight was associated with increased prevalence of valvular heart disease (Connolly et al., 1997). Currently, the only effective treatment for obesity is bariatric surgery: surgical reduction of the stomachs capacity through either bypass, band, or removal of part of the stomach. However, although this method has been shown to be effective in the long term, there are many risks associated with surgical procedures, it is expensive, and it also results in significant dietary change for life (Eldar et al., 2011).

Because of the difficulties in treating obesity, much research is now focusing on establishing the many causes associated with increased weight gain, in the hope that this will lead to the development of more effective preventative measures.

1.1.3 Food Choice
When considering the increased calorie intake and reduced energy expenditure that can lead to weight gain, it is also important to consider food choice. Particular food choices, such as increased consumption of foods high in fat and sugar could lead to increased calorie intake and therefore weight gain. In obese individuals there is some evidence for an increased preference for fatty foods (Cox et al., 2016), however this is not always the case.

The formation of dietary preferences is complex and can be influenced by many factors. Firstly, biological determinants such as physiological state, as well as the palatability and
sensory aspects of food can influence food choice (Saper et al., 2002). Economic factors can also contribute to dietary choice, and it has been illustrated that lower income groups are more likely to consume fewer fruits and vegetables and could therefore be considered to have unhealthier diets (Irala-Estévez et al., 2000). Other social factors such as cultural influences can also lead to differences in food preferences (Larson and Story, 2009) and psychological states such as stress can contribute to food choice alterations (Zellner et al., 2006). Finally early life experiences such as flavour learning in utero and repeated early exposure to certain foods can also lead to the development of long-term taste preferences (Ventura and Worobey, 2013).

Although many of the determinants of food choice are known, there is still a need for a greater understanding of how dietary choices develop and the mechanisms behind the development of certain food preferences. Furthermore, there is also the need for further investigation into how changes in food choice could potentially lead to weight gain and vice versa. Crucially, these mechanisms need to be understood to allow for the development of successful interventions to modify food choice. Therefore, this thesis will examine how certain factors, specifically early life programming and positive affect can influence feeding behaviour and food choice.

1.2 Control of Feeding

Key to determining the causes of obesity is understanding the control of feeding. While this is a complex system involving many hormones and pathways, feeding can be arbitrarily divided into two main drives: homeostatic – the consumption of food to maintain physiological needs, and hedonic – the motivated consumption of food for pleasure. The control of feeding is a multifaceted process involving both homeostatic and hedonic drives.

1.2.1 Homeostatic Mechanisms

To ensure the consumption of nutrients necessary for survival, homeostatic mechanisms in the brain monitor and, via influencing behaviours, regulate various nutrients in the blood and body stores and promote feeding accordingly. Areas regulating the homeostatic control of feeding behaviour are mainly located in the hypothalamus and include the paraventricular nucleus (PVN), lateral hypothalamus (LH), arcuate nucleus (ARC) and ventromedial hypothalamus (VMH) (Sohn et al., 2013).

The ARC is now perhaps the best studied brain region for the control of appetite, and its main function is to integrate inputs signalling energy balance and then relay these to other...
brain areas (Benarroch, 2010). The ARC contains two neuronal populations: pro-opiomelanocortin (POMC) neurons and neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons, responsible for inhibiting and promoting food intake respectively. POMC neurons produce α-melanocyte stimulating hormone (α-MSH) which acts as an agonist at anorectic melanocortin receptors (MC3R and MC4R) distributed in other brain areas associated with the control of feeding such as the PVN, VMH and LH (Benarroch, 2010). Both humans and mice deficient in POMC and MC3R/MC4R develop hyperphagia and obesity (Yaswen et al., 1999, Krude and Grüters, 2000, Sutton et al., 2006, Martinelli et al., 2011). Furthermore, optogenetic stimulation of POMC neurons results in a reduction in food intake in mice when stimulated over a 24 hour period, although no differences were observed when stimulated for 2 hours (Aponte et al., 2011).

AgRP acts as an inverse agonist at MC3R/MC4R receptors, inhibiting the action of α-MSH, whilst NPY acts independently of melanocortin receptors at G-protein-coupled NPY receptors to inhibit anorexigenic neurons (Sohn et al., 2013). Whilst developmental loss of NPY/AgRP neurons has no influence on food intake, when ablated in adults the loss of these neurons results in reduced food intake and lower body weight suggesting that compensatory mechanisms may counteract the loss of NPY/AgRP during development (Luquet et al., 2005). Optogenetic simulation of NPY/AgRP neurons results in an acute increase in food consumption in mice over a one hour period (Aponte et al., 2011).

Many of these ARC neurons express receptors for leptin and ghrelin (see sections 1.2.1.1 and 1.2.1.2), as well as insulin which has an inhibitory effect on the activity of both POMC and AgRP neurons (Varela and Horvath, 2012), and serotonin (5HT) which stimulates POMC neurons thereby reducing food intake (Romanova et al., 2018) (see Figure 1.1).

1.2.1.1 Leptin

The hormone leptin is produced by white adipose tissue and acts as a satiety signal. Leptin was identified through the study of two naturally occurring obese mouse models: the ob/ob mouse and the db/db mouse (Coll et al., 2007). In 1994, researchers found that ob/ob mice were deficient in a satiety factor, resulting in the discovery of a loss of function mutation in the gene encoding leptin (Zhang et al., 1994). Subsequently, it was found that leptin administration to obese, leptin deficient animals reversed their hyperphagia (Pelleymounter et al., 1995). Similarly, humans lacking leptin also illustrate hyperphagia and obesity, and respond well to leptin treatment (Farooqi et al., 1999). However, while leptin
administration is effective in leptin deficient, as well as normal rodents, it is relatively ineffective when administered to mice with diet-induced obesity, as in the majority of obese humans, suggesting that leptin resistance may be responsible for failure to regulate energy observed in most forms of obesity (Coll et al., 2007). Although leptin does have peripheral targets (Muoio and Lynis Dohm, 2002), direct administration of leptin into the central nervous system without altering peripheral concentrations reverses the metabolic phenotype of ob/ob mice (Friedman, 1999), illustrating the importance of central leptin receptors in the control of feeding behaviour. Leptin can cross the blood brain barrier through receptor mediated transport and has receptors in several brain areas, although the highest expression is in those areas involved in the control of food intake: the ARC, VMH, PVN and dorsomedial hypothalamic nucleus (DMH) (Pénicaud et al., 2012). Activation of leptin receptors in the ARC promotes expression of α-MSH and inhibits expression of AgRP and NPY (Benarroch, 2010).

As well as involvement in the control of feeding, leptin also appears to have a developmental role. Electrophysiological evidence from ob/ob mice illustrates that leptin deficiency alters synaptic inputs to the ARC, increasing excitatory inputs on NPY/AgRP neurons but decreasing excitatory inputs to POMC neurons. However, leptin administration was able to reverse these effects within hours (Pinto et al., 2004). Furthermore, there is also evidence to show that leptin acts as a neurotrophic growth factor during hypothalamic development, as ob/ob mice have disruptions in ARC innervations to the PVN, LH and DMH. Treatment of neonates with leptin reversed these effects, although this was only effective when applied in a critical window corresponding to a period of elevated leptin secretion in wild type rodents (Bouret et al., 2004).

1.2.1.2 Ghrelin

Ghrelin is produced and secreted by cells in the stomach and is currently the only known orexigenic peripheral hormone. Secretion of ghrelin is increased by weight loss and fasting, and peripheral administration of ghrelin leads to increased food intake (Coll et al., 2007). Ghrelin receptors are found in both the VMH and ARC. The binding of ghrelin to its receptors results in stimulation of NPY/AgRP neurons, therefore promoting food intake (Benaroch, 2010). The importance of ARC neurons in the initiation of ghrelin’s effects has been demonstrated by experiments using NPY antagonists, which blunt the actions of ghrelin (Nakazato et al., 2001). While deletion of ghrelin or its receptor lead to no changes...
in feeding behaviour or body composition, there is evidence to show that these models are able to resist diet-induced obesity (Wortley et al., 2005).

Human studies have also illustrated a possible role for the importance of ghrelin in motivation for food consumption. Patients who had lost weight via dieting (which generally results in weight regain) had significantly increased ghrelin levels with exaggerated peaks in pre-meal ghrelin secretion compared to obese controls, whereas patients who had undergone bariatric surgery (and are generally more successful at maintaining weight loss) illustrated reduced ghrelin levels (Cummings et al., 2002).

Recent evidence has also suggested that ghrelin may play a role in mediating food choice. Schéle et al. (2016) found that when rats were placed on a food choice paradigm consisting of bland lab chow, sucrose pellets and lard, intracerebroventricular (ICV) injection of ghrelin led to an increased chow consumption when measured at 3, 6 and 24 hours after injection. An increase in lard consumption was also observed, although this was only present when food intake was measured at 3 and 6 hours. Fasting (which raises ghrelin levels) illustrated similar effects and these changes in food choice were prevented by administration of a ghrelin receptor antagonist. Moreover, Bake, Hellgren and Dickson (2017) found that ICV ghrelin also increased chow consumption during a 2 hour binge-style feeding paradigm, where rats had 2 hours of daily access to a high-fat diet in addition to ad lib chow. These studies illustrate that as well as promoting food consumption, ghrelin may play an important role in mediating food preference.
Two distinct sets of ARC neurons represent the core of this system: those expressing POMC and neurons that express AgRP and NPY. POMC neurons suppress feeding and promote energy expenditure, while AgRP/NPY neurons stimulate appetite and reduce energy usage. Many of these neurons contain receptors for leptin (LepRb), insulin (InsR), and ghrelin (GHSR), as well as 5HT (5HT2cR).
1.2.2 Hedonic Feeding

Feeding, whilst necessary for survival, is also rewarding. Hedonic feeding can be broadly defined as the consumption of food for pleasure in the absence of an energy deficit. Almost all mammals will consume calories beyond their homeostatic need when presented with a highly palatable food (Saper et al., 2002). In an environment where palatable foods are readily and cheaply available, it seems reasonable that the hedonic control of food intake may be contributing to the obesity epidemic and driving food consumption outside of homeostatic energy requirements.

The hedonic system is partially mediated by palatability cues. Food deprived animals will avoid bitter and sour tastes as these are often associated with poisonous substances. On the other hand, salty and sweet foods will be consumed beyond homeostatic need as these tastes indicate the presence of important nutrients. Animals also develop taste aversions to those foods that have made them sick in the past, illustrating the importance of taste in modulating feeding behaviour (Saper et al., 2002).

Food reward can be separated into two components. Firstly the ‘liking’ aspect of food is associated with the subjective pleasure experienced with consumption of certain foods and is mainly generated by the release of endogenous opioids. Opioids are a key regulator for both reward and food intake and administration of an opioid receptor antagonist will decrease feeding through blocking the effects of AgRP (Hagan et al., 2001). While administration of opioids stimulates all food intake, its effects are particularly powerful for palatable food, highlighting their importance in the regulation of reward and ‘liking’ of foods (Nogueiras et al., 2012).

On the other hand, ‘wanting’ is the motivational component of food consumption and is mediated by the dopamine system (Finlayson and Dalton, 2012). Dopamine is a key neurotransmitter in the regulation of reward mainly through its projections from the ventral tegmental area (VTA) into the nucleus accumbens (NAc) (Volkow et al., 2011). There is evidence to illustrate the importance of dopamine signalling in feeding. Dopamine deficient mice are hypophagic and die of starvation, however they resume feeding after introduction of the tyrosine hydroxylase gene (involved in generating L-dopa, a precursor to dopamine) into the caudate putamen (Szczypka et al., 2001). Furthermore, presentation of highly palatable foods leads to the release of dopamine in the NAc (Lutter and Nestler, 2009). However, upon repeated presentation of a food reward the dopamine response
gradually habituates, and instead becomes associated with cues linked with the food reward, highlighting the role of dopamine in the development of incentive salience (Volkow et al., 2011). Through implementing a short hairpin RNA approach to selectively knockdown D2 receptors (which have an inhibitory influence on dopamine activity) in the VTA of adult rats, de Jong et al. (2015) found an increased motivation for sucrose self-administration on a progressive ratio lever pressing task. This is further supported by evidence illustrating that chemogenetic activation of dopamine neurons in the VTA of rats also increases motivation for sucrose when using the same progressive ratio schedule task (Boekhoudt et al., 2018).

There is some evidence to suggest that reward pathways related with feeding are altered in those with obesity. There is evidence from both human and animal studies that dopamine D2 receptors are down-regulated in obesity (reviewed in: Baik, 2013). Rothemund et al. (2007) found that obese women illustrated increased activation of the dorsal striatum when viewing high calorie foods in comparison to lean controls. Furthermore, it was reported that obese individuals are willing to work harder to obtain high calorie snack food, even though there was no difference in ‘liking’ of the snack foods between obese participants and lean controls (Giesen et al., 2010). This evidence suggests that obesity may be associated with an increased ‘wanting’ of foods.

1.2.3 Interactions Between Homeostatic and Hedonic Systems

The rewarding nature of food is modulated by the current satiety state of an animal: for example, a food that may have been rewarding when hungry could lose its reward value once in a satiated state (Saper et al., 2002). There are many connections between brain areas controlling hedonic and homeostatic feeding (see: Rossi and Stuber, 2018). Furthermore, there is evidence that peripheral signals affect not only those brain areas associated with homeostatic feeding such as the ARC, but also areas associated with reward such as the VTA and NAc (Benarroch, 2010). For example, leptin has been shown to inhibit dopamine activity in the VTA, with direct administration of leptin to the VTA resulting in decreased food intake (Hommel et al., 2006). Furthermore, there is evidence that, while food restriction increased the reward value of sucrose, administration of leptin decreases its reward value, likely through modulation of the dopamine system (Hebebrand et al., 2014, Domingos et al., 2011). Ghrelin has also been shown to be important in the motivation for food rewards. Ghrelin receptors are expressed in the VMH, and ghrelin
increases lever pressing for sucrose, an effect which also appears to be modulated by the dopamine system (Skibicka et al., 2013). This evidence illustrates that there are clear links between the homeostatic and hedonic feeding systems, and that they work together to promote or inhibit food intake.

1.2.4 Oxytocin and Feeding
Oxytocin is a peptide that has been classically implicated in the control of reproductive behaviours, such as mediating the milk-ejection reflex (Nishimori et al., 1996), sexual behaviours (Veening et al., 2015) and maternal behaviours (Pedersen et al., 1982). Early efforts in oxytocin research focused on social bonding, building on observations that monogamous prairie voles and non-monogamous montane voles illustrated contrasting patterns of oxytocin receptor expression in the brain, with prairie voles showing high oxytocin receptor density in areas such as the prelimbic cortex, nucleus accumbens and the lateral amygdala, which showed little binding in the montane vole (Insel and Shapiro, 1992). Furthermore, it was shown that ICV oxytocin administration in female prairie voles increased social contact and facilitated the development of partner preferences (Williams et al., 1992). Further research has illustrated that oxytocin administration in reward related brain areas such as the NAc and VTA has been associated with social reward. Hamsters injected with oxytocin in the VTA increase their time spent in a chamber associated with social interaction in comparison to a neutral chamber, whereas administration of an oxytocin receptor antagonist prevents these effects and leads to no chamber preference (Song et al., 2016). Furthermore, oxytocin receptor antagonist administration to the NAc in male mice also prevents a conditioned place preference for social interaction (Dölen et al., 2013). These data suggest a role for oxytocin in mediating some of the rewarding properties of social interaction.

As well as its role in social and reproductive behaviours, there is also increasing evidence that oxytocin may be involved in the control of feeding. Oxytocin is produced by magnocellular neurons in the supraoptic nucleus (SON) and PVN of the hypothalamus which project to the posterior pituitary gland. These magnocellular neurons also release oxytocin from their dendrites (Sabatier et al., 2007). The PVN also contains parvocellular neurons that project within the brain (Sabatier et al., 2013). Oxytocin receptors are expressed in various brain regions, including those involved in the control of feeding such as the VTA, NAc and VMH (Olszewski et al., 2016).
Studies injecting ICV oxytocin have illustrated the anorexigenic effects of this peptide, showing a dose-dependent inhibition of food intake, which was reversed upon administration of an oxytocin receptor antagonist (Arletti et al., 1990). However, whilst oxytocin has been implicated in food consumption, its role is not essential. Oxytocin knockout mice show similar feeding patterns to wild type mice, although they are prone to the development of late-onset obesity (Camerino, 2009). However, while oxytocin knockout mice show few alterations in their basal food intake, there is evidence for alterations in food choice. Oxytocin knockout mice illustrate an increased consumption of sucrose solution (Amico et al., 2005) as well as a saccharin solution (Billings et al., 2006), however no effects were observed on consumption of a high-fat solution (Miedlar et al., 2007). Further implicating oxytocin in food choice is evidence showing that peripheral injection of a blood-brain barrier penetrant oxytocin receptor antagonist increases sucrose intake but has no effect on lipid intake (Olszewski et al., 2010). Furthermore, it has been illustrated that gavage of a high-sugar, but not high-fat food, increases firing of oxytocin neurons in the SON (Hume et al., 2017). Overall, these data suggest that oxytocin may be have a specific involvement in the consumption of sweet-tasting carbohydrates. These effects may be mediated by the reward system as oxytocin injection in the VTA suppresses the intake of sucrose solution (Mullis et al., 2013) and oxytocin infusions into the NAc core also decrease sucrose consumption, as well as decreasing chow intake in fasted rats (Herisson et al., 2016).

1.3 Stress
Stress is defined as ‘a state of real or perceived threat to homeostasis’ (Smith and Vale, 2006). Exposure to a stressor leads to the activation of the stress response which initiates a variety of adaptive physiological and behavioural changes designed to enhance the individual’s survival, often known as ‘fight or flight’. Behavioural adaptations include increased awareness and improved cognition while physiological changes can include increased heart and respiratory rate, as well as inhibition of many ‘background’ functions such as digestion and reproduction (Adam and Epel, 2007). Although these behavioural and endocrine alterations may have been adaptive when confronted with stressful situations in our hunter-gatherer past, in the modern world where stress is an inevitable part of daily life, the stress response can be maladaptive, and particularly experiencing chronic stress can lead to development of disease (de Kloet et al., 2005, Cerqueira et al., 2007).
1.3.1 The HPA Axis
An acute stressor activates the hypothalamic-pituitary-adrenal (HPA) axis. The structures mediating the stress response are found in both the central and peripheral nervous systems and are comprised mainly of the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal gland. Corticotrophin releasing factor (CRF) is the fundamental regulator of the HPA axis and is secreted from the median eminence from axon terminals with their cell bodies in the PVN. CRF binding to its receptor on the anterior pituitary leads to the release of adrenocorticotrophic hormone (ACTH) into the systemic circulation, which targets the adrenal cortex where it stimulates the synthesis and secretion of glucocorticoids. These glucocorticoids, cortisol in humans and corticosterone in rodents, initiate the behavioural and physiological changes associated with the stress response (Smith and Vale, 2006).

Glucocorticoids have many functions in the body including regulating glucose, fat and protein metabolism, anti-inflammatory actions, and can also affect mood and cognitive functions. Under basal conditions the HPA axis is modulated by both circadian and ultradian rhythms, however when exposed to a stressor HPA activity increases (Spiga et al., 2014). Glucocorticoids also play a role in regulating the HPA axis. Following stress, circulating glucocorticoids act through negative feedback at the hypothalamus and pituitary to inhibit HPA axis activity (Smith and Vale, 2006). Glucocorticoids also act at higher brain areas such as the hippocampus where they exert a tonic inhibitory influence on PVN activity, as well as mediating some of the behavioural responses to stress (Reul et al., 2015). The prefrontal cortex (PFC) has also been implicated in the negative feedback of the HPA axis with the medial PFC likely having an inhibitory influence on the HPA axis, while the infralimbic PFC has been implicated in the initiation of HPA responses (Ulrich-Lai and Herman, 2009). Additionally, the amygdala has also been suggested to have a feed-forward role on the stress response (Xu et al., 1999).

1.3.2 Effects of Stress on Feeding
The effects of stress on feeding behaviour are both complex and varied. Both human and rodent models have illustrated that stress can impact feeding behaviour, although these effects can vary with the duration, intensity and timing of the stressor (Adam and Epel, 2007). A selection of experimental evidence from humans and rodent studies is summarised below.
1.3.2.1 Human Evidence

It has been well established that stress can lead to changes in feeding behaviour in humans, although it seems to vary between individuals, with some eating more when confronted with stress whilst others eat less (reviewed in: Torres and Nowson, 2007). Administration of glucocorticoids has been shown to increase food intake in humans (Tataranni et al., 1996) and in some studies stress has been associated with an increased drive to eat (Groesz et al., 2012). Peripheral injection of CRF also leads to increased food intake, with food consumption directly correlated with the cortisol response to the injection (George et al., 2010).

The effects of stress on feeding behaviour seem to be moderated by a wide variety of factors. Some have suggested that stressor severity may play a role in how feeding is mediated, where mild stressors may induce hyperphagia whilst more severe stressors may lead to hypophagia (Stone and Brownell, 1994). Stress can also have differing effects on food intake in restrained and unrestrained eaters, with unrestrained eaters more likely to decrease their food intake when stressed, while restrained eaters who consciously restrict their diet to maintain or lose weight are more likely to increase their food intake when stressed (Lowe and Kral, 2006). Furthermore, it appears that the effects of stress in humans can be dependent on the cortisol reactivity of the individual, with studies showing that high cortisol reactivity to stress is a predictor for greater food intake (Newman et al., 2007).

1.3.2.2 Rodent Studies

Similarly to humans, the literature on how stress effects food intake in rodents is varied. While it is often asserted that exposure to an acute stressor leads to a reduction in food intake, this appears to be highly dependent on the type of stressor, and the time of day at which food measurements are taken. Some forms of stressor have been shown to reduce food intake, for example 2 hours of daily immobilisation stress over 9 days leads to a lower body weight gain in stressed animals, as well as reduced food intake (Ricart-Jané et al., 2002). Calvez et al. (2011) found that three hours of restraint stress led to a reduction in 24-hour food consumption, however rats exposed to a 10-minute forced swim test, only illustrated reduced food intake for 1 hour following the stressor. Consequently, only rats exposed to the restraint stress illustrated a reduced body weight gain.
Harris et al. (2002) found that when exposed to 3 hours of restraint stress for 3 consecutive days, rats that were fed *ad libitum* or fed during the dark phase experienced reduced weight gain as well as reduced food intake. However, rats that were fed only during the light phase illustrated no changes in body weight or food consumption compared to controls, illustrating how timing of feeding can be an important factor in how stress influences food consumption.

A repeated social defeat stressor lead to a decreased weight gain, however unexpectedly an increased food intake was observed. Whereas a single application of this social defeat paradigm resulted in decreased food intake (Bhatnagar et al., 2006). Furthermore, 3 consecutive days of a forced swim test, whilst resulting in a decreased body weight gain for both males and females, illustrated no differences in total 24 hour energy intake, although a decrease was observed during the first 3 hours following the stressor (Diane et al., 2008).

These studies highlight the huge variation in feeding behaviour in response to stress.

### 1.3.2.3 Stress and Food Choice

As well as having an impact on food intake, stress can also impact on food choice. Stress has been associated with an increased preference for palatable foods high in fat and sugar. Pecoraro et al. (2004) illustrated that when offered sucrose and lard as well as chow, 5 consecutive days of restraint stress resulted in an increased proportion of total calories being obtained from the palatable foods in comparison to unstressed controls. Human studies have illustrated that high cortisol reactors (here defined as an increased cortisol secretion during stress) consumed more sweet foods (Epel et al., 2001), and Zellner et al. (2006) found that when offered the choice between M&Ms and grapes, stressed women consumed a larger quantity of M&Ms and fewer grapes in comparison to unstressed controls. Furthermore, Oliver, Wardle and Gibson (2000) found that stressed emotional eaters consumed more sweet and high-fat foods in comparison to unstressed non-emotional eaters.

There is some evidence to suggest that consumption of foods high in fat and sugar, often referred to as ‘comfort foods’ may help to ameliorate some of the effects of a stressor. La Fleur et al. (2005) found that rats given access to lard as well as chow had reduced ACTH and corticosterone responses on exposure to a 30-minute restraint stress. In addition to this, Ulrich-Lai et al. (2010) found that access to a sucrose solution not only dampened the HPA responses to a restraint stressor, but also led to reduced stress induced tachycardia.
and reduced anxiety responses in comparison to controls. Furthermore, it was found that highly stressed women reported higher levels of stress eating, and consequently had lower HPA responses to an acute stressor (Tomiyama et al., 2011). This evidence suggests that stress can lead to a preference for palatable foods as this may help ameliorate some of the endocrine and behavioural responses associated with exposure to a stressor.

1.3.3 Links Between Stress and Feeding Systems

These changes in feeding behaviour in response to stress are likely due to the many links between both the stress and feeding systems. The PVN is the site of convergence of many neural circuits involved in the control of energy intake, however it also contains the cells bodies of CRF neurons (Maniam and Morris, 2012). A subset of these CRF neurons express MC4Rs (Lu et al., 2003). These receptors appear to play a role in HPA axis regulation, as ICV administration of a melanocortin agonist resulted in an increase in plasma corticosterone levels (Lu et al., 2003). CRF is anorexigenic and central administration has been shown to reduce food intake (Carr, 2002). Conversely, glucocorticoids are orexigenic and therefore peripheral administration of CRF increases glucocorticoids which generally stimulates feeding behaviour (Keen-Rhinehart et al., 2013). Specifically glucocorticoids appear to promote palatable feeding, as adrenalectomized rats will not consume saccharin, but saccharin consumption increases with corticosterone replacement in a dose dependent manner (Bhatnagar et al., 2000).

Furthermore, glucocorticoids are able to alter the expression of peptides involved in feeding. Adrenalectomy is associated with reduced feeding and body weight gain (la Fleur, 2006), and also decreased levels of POMC and AgRP in the hypothalamus, an effect which can be reversed through the administration of glucocorticoids (Savontaus et al., 2002). Administration of dexamethasone (a synthetic glucocorticoid) in both obese and lean individuals increases plasma leptin (Dagogo-Jack et al., 1997), and 3 day ICV treatment of dexamethasone in rats resulted in increased levels of leptin, along with increased food intake and body weight in comparison to controls (Zakrzewska et al., 1999). These data may suggest a role for glucocorticoids in reducing the sensitivity of leptin, although in rats the route of administration of glucocorticoids appears to play an important role in how its effects are mediated, as while intraperitoneal injection of glucocorticoids does increase plasma leptin in rats (although not to the extent of ICV injection), it also leads to a reduction in food intake and in body weight (Zakrzewska et al., 1999).
There is also evidence for the involvement of ghrelin in the stress response. Ghrelin knockout mice illustrate lower corticosterone and ACTH in response to acute stress in comparison to wild type controls. Furthermore, ghrelin knockouts show heightened anxiety after acute restraint stress implicating a possible role for ghrelin in the modulation of the HPA axis and anxiety behaviours (Spencer et al., 2012).

Genetic models of obesity, such as the obese Zucker rat, have been associated with elevated levels of glucocorticoids (Livingstone et al., 2000) and human obesity has also been associated with alterations in HPA axis reactivity although these changes appear variable. High central adiposity has been associated with higher cortisol secretion (Epel et al., 2000). Rosmond et al. (1998) found that salivary cortisol was correlated with BMI and plasma ACTH has also been positively associated with BMI (Veldhuis et al., 2009). Others have observed lower plasma cortisol levels in obese individuals in comparison to non-obese controls (Vgontzas et al., 2007). Furthermore there have also been links established between some stress related disorders and obesity, for example posttraumatic stress disorder has been established as a significant risk factor for obesity (Perkonigg et al., 2009). These many and complex links between the HPA axis and neuroendocrine systems controlling feeding could explain the varying effects that different stressors have on feeding behaviour.

1.4 Early Life Programming
It was originally the epidemiologist Barker who established that experiences in utero may influence the physiology and behaviour of the offspring later in life when he established a link between maternal malnutrition and cardiovascular disease in the offspring (Barker and Fall, 1993). Since this pivotal study, there has been much research into the area of early life programming, investigating the effects of both prenatal and early postnatal influences on the offspring. In the early stages of life neural plasticity (how readily the brain responds and is influenced by a stimulus) is much higher than in adulthood and as a consequence at this stage of life the brain is extremely sensitive to environmental changes which can influence its development (Parlee and MacDougald, 2014). This may lead to adaptive changes to suit changing environmental conditions, however occasionally these changes may be maladaptive which can lead to the development of disease. This theory that a mismatch of prenatal and postnatal environments can lead to disease development later in life has been termed the ‘thrifty phenotype hypothesis’ (Hales and Barker, 2001).
1.4.1 Maternal Nutrition

In terms of obesity research, it has already been established that certain early life influences can increase the risk of obesity development later in life, for example maternal nutrition during pregnancy. As an example of the thrifty phenotype hypothesis, it has been illustrated that maternal malnutrition can lead to a higher incidence of obesity in offspring (Jones et al., 1984, Vickers et al., 2000). This can be rationalised through the fact that if a foetus develops in a food deprived environment, it may undergo adaptations that lead to altered feeding behaviour and food preferences to maximise caloric intake. However, if then born into a food rich environment, these adaptations will become maladaptive and therefore lead to an increased likelihood of obesity development.

Research into prenatal programming in humans has its flaws, such as the inability to carefully control experimental conditions, as well as ethical problems concerning subjecting pregnant women to interventions that may potentially harm them or their foetuses. Therefore, much human evidence for the effects of prenatal programming comes from case studies, whereby researchers have studied the effects of naturally occurring situations on the offspring later in life.

For example, the Dutch hunger famine is often used when examining the effects of maternal malnutrition on programming effects on the offspring. This occurred during the winter of 1944 when Nazi blockades prevented the transportation of food shipments from farm areas to the densely populated western provinces of the Netherlands, and as a consequence calorie consumption in these areas dropped to below 1000 Kcal a day. This makes a relevant case study as it has a very fast onset and resolution unlike many other cases, and furthermore there are very detailed birth records of the time. What this data has shown is that those who were prenatally exposed to the famine during the first and second trimester of pregnancy have a much higher incidence of obesity as adults (Roseboom et al., 2006).

However, it is not possible to use case studies for all cases of early life programming, particularly if specific hormonal or neural evidence needs to be collected. Therefore the development of rodent models of early life programming has become increasingly common over the years and has enabled the development of much knowledge in the area. In terms of maternal nutrition, it is possible to perform more detailed studies where, as well as altering the amount of food consumed, it is also possible to restrict specific nutrients.
consumed during pregnancy. Bieswal et al. (2006) illustrated that when dams were fed a protein restricted diet (8% protein compared to 20% of the control diet, although diets were isocaloric) or restricted to 50% caloric intake of control dams during pregnancy, this resulted in pups with a reduced birth weight. When these restrictions were continued during lactation this reduction in body weight persisted, however if during lactation pups were overfed (induced by reducing litter sizes to 4 pups) this led to fast catch up growth and resulted in protein restricted and calorie restricted rats becoming permanently heavier than controls, regardless of whether they were fed a control or a high calorie diet supplemented with sucrose and corn oil after weaning. The type of early life restriction was also important in determining adult body weight, with those submitted to calorie restriction during gestation showing significantly higher body weights than those who had been protein restricted.

As well as influencing weight gain, maternal diet during pregnancy can also influence food choice of the offspring. Rats born of dams fed a ‘junk food’ style diet high in fat and sugar during pregnancy and lactation showed a higher preference for foods high in fat, sugar and salt at the expense of protein compared to control rats (Bayol et al., 2007). It was also found that dams who were protein restricted during pregnancy had offspring who were more likely to choose foods high in fat when given a free choice of foods high in fat, protein or carbohydrates. (Bellinger et al., 2004).

There is some evidence that these changes in food preferences in the offspring could be caused by alterations in the reward system as a consequence of perinatal changes to the environment. Naef et al (2011) found that offspring of mothers fed a high-fat diet during pregnancy and lactation illustrated reduced release of dopamine in the NAc in response to amphetamine when measured by microdialysis. Additionally, in adulthood there was reduced expression of the D2 receptor in the VTA in offspring of high-fat fed dams. Furthermore, when tested on different operant responding paradigms using both fixed ratio and progressive ratio reinforcement, it was found that while there were no differences in operant responses for high-sugar pellets between control rats and offspring of high-fat fed dams, the high-fat group did illustrate an increased response rate for high-fat pellets in comparison to controls. These data suggest that changes to maternal diet during pregnancy can lead to alterations in dopamine signalling and dopamine mediated behaviours in their offspring.
1.4.2 Prenatal Programming

1.4.2.1 Prenatal Stress

The effects of stress on the body have been well established, however as stress is incredibly prevalent in modern society and due to expansion in the field of early life programming, research has recently been focusing on the effects of prenatal stress on the developing foetus.

Animal models of prenatal stress (PNS) illustrate an impairment of HPA axis activity, generally resulting in an enhanced and/or prolonged ACTH and corticosterone response when subjected to an acute stressor (reviewed in: Weinstock, 2017). This HPA axis hyper-reactivity is also apparent from human case studies. One example is that of the Chernobyl disaster, after which there was very little information available in Finland and fear spread quickly across the country questioning the safety of food and water. When examining a cohort of children, some of whom were prenatally exposed to stress related to the Chernobyl disaster, it was found that those who were exposed to maternal stress from the second trimester onwards had higher cortisol levels than control groups. However, no differences in cortisol were observed in those subjected to stress from the first or third trimester of pregnancy onwards (Huizink et al., 2008).

As well as hyperactivity of the HPA axis, rodent PNS offspring also demonstrate increased anxiety-like behaviour, illustrated by the elevated plus maze, open field test and light-dark box (Grundwald and Brunton, 2015, Vallee et al., 1997). PNS rats often also illustrate increased depressive like behaviours (Morley-Fletcher et al., 2003), and PNS has also been shown to be associated with deficits in learning and memory, although this appears to be dependent on the type, timing and duration of the stressor (reviewed in: Weinstock, 2008).

Many of the behavioural and neurochemical effects of prenatal stress appear to be sex dependent. For example, heightened anxiety behaviour is typically only demonstrated by male offspring (Brunton and Russell, 2010). Furthermore, female offspring appear more resistant to the effects of prenatal stress on alterations to learning and memory (Zuena et al., 2008), while female rats appear to indicate more depressive-like behaviour in the forced swim test (Sickmann et al., 2015). Although more resistant to some of the behavioural aspects of prenatal stress, female PNS offspring tend to illustrate a more pronounced HPA axis response to prenatal stress (Weinstock et al., 1992).
The HPA axis is under negative feedback control by glucocorticoid (GR) and mineralocorticoid (MR) receptors. The prolonged corticosterone responses observed in PNS offspring are associated with reduced levels of GR and MR in the hippocampus. Furthermore, the expression of MR mRNA is also lower in the hippocampus of adult PNS offspring. This suggests impaired glucocorticoid negative feedback control of the HPA axis in PNS offspring (Maccari et al., 2014).

1.4.2.1.1 Rodent Models of Prenatal Stress

Whilst case studies are useful for examining the effects of prenatal stress in a natural environment, animal models are required to enable researchers to fully examine the mechanistic effects of prenatal stress on physiology and behaviour. Rodent models of prenatal stress can be generated in several ways using either a psychological stressor (e.g. restraint, Van den Hove et al. 2014), a social stressor (social defeat, Brunton and Russell 2010), or variable stress using a variety of stressors (Sickmann et al., 2015). Stressors are generally applied in the final week of pregnancy, as at this point in development GR mRNA is present in the hypothalamus and pituitary of the neonatal brain (Kapoor et al., 2006). The implementation of prenatal stress models varies widely across different laboratories and as a result the effects of prenatal stress are also variable (Table 1.1). Whilst an elevated or prolonged corticosterone response is often hailed as the most well-established feature of prenatally stressed offspring, this can vary greatly depending on the model in use, as can be observed in Table 1.1. Some of these differences may be explained by the differences in acute stressors used in the offspring, as while the majority have used a 20 or 30 minute restraint stressor, some have also used foot-shock (Cannizzaro et al., 2006) or forced swimming (Szuran et al., 2000). Furthermore, this wide variety of models have also observed differences in pup body weight, anxiety behaviour and spatial learning (reviewed in Weinstock, 2008; Weinstock, 2017).
### Table 1.1: Rat Models of Prenatal Stress and Their Resultant Effects on Corticosterone Responses in Offspring

A selection of publications on prenatal stress, the maternal stressors (and days during gestation which they were applied) and the offspring’s corticosterone (cort) responses to an acute stressor in comparison to unstressed control offspring. M = males, F = females

<table>
<thead>
<tr>
<th>Reference</th>
<th>Laboratory</th>
<th>Dam Stressor type</th>
<th>Days in gestation</th>
<th>Offspring acute stressor</th>
<th>Cort responses in comparison to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Henry et al., 1994)</td>
<td>Maccari</td>
<td>45 min restraint 3 x a day</td>
<td>14 -21</td>
<td>Exposure to novelty</td>
<td>Elevated peak cort (M, F not tested)</td>
</tr>
<tr>
<td>(Vallee et al., 1997)</td>
<td>Maccari</td>
<td>45 min restraint 3 x a day</td>
<td>15-21</td>
<td>30 min restraint</td>
<td>Prolonged duration of cort (M, F not tested)</td>
</tr>
<tr>
<td>(Szuran et al., 2000)</td>
<td>Welzl</td>
<td>30 min restraint 3 x a day</td>
<td>15-19</td>
<td>Forced Swim</td>
<td>Prolonged duration of cort (in F not M)</td>
</tr>
<tr>
<td>(Maccari et al., 2003)</td>
<td>Maccari</td>
<td>45 min restraint 3 x a day</td>
<td>14-21</td>
<td>30 min restraint</td>
<td>Prolonged duration of cort (M, F not tested)</td>
</tr>
<tr>
<td>(Van den Hove et al., 2005)</td>
<td>Steinbusch</td>
<td>45 min restraint 3 x a day</td>
<td>14-21</td>
<td>20 min restraint</td>
<td>Reduced peak cort (M, F not tested)</td>
</tr>
<tr>
<td>(Koenig et al., 2005)</td>
<td>Koenig</td>
<td>Variable stress</td>
<td>14-21</td>
<td>30 min restraint</td>
<td>Prolonged duration of cort (M, F not tested)</td>
</tr>
<tr>
<td>(Richardson et al., 2006)</td>
<td>Rivier</td>
<td>45 min restraint 3 x a day</td>
<td>14-21</td>
<td>Footshock</td>
<td>Lower peak cort (M) Prolonged duration of cort (F)</td>
</tr>
<tr>
<td>(Cannizzaro et al., 2006)</td>
<td>Cannizzaro</td>
<td>Variable stress</td>
<td>14-21</td>
<td>Footshock</td>
<td>No change (M and F)</td>
</tr>
<tr>
<td>(Brunton and Russell, 2010)</td>
<td>Brunton</td>
<td>120 min immobility</td>
<td>16</td>
<td>Footshock</td>
<td>Reduced peak cort (M, F not tested)</td>
</tr>
<tr>
<td>(García-Cáceres et al., 2010)</td>
<td>Chowden</td>
<td>45 min restraint 3 x a day</td>
<td>14-21</td>
<td>45 min restraint</td>
<td>No change (M and F)</td>
</tr>
<tr>
<td>(Brunton et al., 2013)</td>
<td>Brunton</td>
<td>10 mins daily social defeat</td>
<td>16-20</td>
<td>Glucose Tolerance Test</td>
<td>No change (M and F)</td>
</tr>
</tbody>
</table>
1.4.2.1.2 Mechanisms of Prenatal Stress

The exact mechanisms causing the effects of prenatal stress in the offspring remain unclear. Foetus exposure to excessive levels of maternal glucocorticoids has been implicated as one mechanism by which prenatal stress may exert its effects. Maternal treatment with synthetic glucocorticoids (such as dexamethasone) during pregnancy recapitulates some of the effects of PNS in the offspring such as reduced birthweight and increased anxiety like behaviour (Welberg et al., 2001). However, while maternal adrenalectomy (therefore preventing stress induced increases in corticosterone) has been shown to ameliorate some of the effects of prenatal stress, treatment with corticosterone in adrenalectomized dams is not able to fully recapitulate all of the effects of PNS (Salomon et al., 2011). Furthermore, the hormone 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which is released by the placenta during pregnancy and metabolises corticosterone into inactive 11-dehydrocorticosterone, acts to protect the foetus against maternal glucocorticoid exposure (Jensen Peña et al., 2012). Additionally, maternal HPA responses are reduced during late pregnancy (Brunton et al., 2008) suggesting that other factors may also be implicated in generating the effects observed in PNS offspring.

Others have speculated that maternal care may influence the PNS phenotype, as some models have illustrated reduced maternal behaviours in dams stressed during pregnancy (Smith et al., 2004). Adoption has been shown to reverse the stress hyperactivity often observed in PNS offspring, regardless of whether the adoptive mothers had experienced stress or not, as adoption appeared to increase maternal behaviours such as licking (Maccari et al., 1995). Further studies have indicated that adoption is also able to reduce some of the anxiety-like behaviours observed in PNS offspring, however this is only successful when the stressed pups are adopted by an unstressed dam and is not reversed when rats are adopted to a stressed dam (Barros et al., 2006). However, some have reported no differences in maternal behaviour of stressed dams to controls (Brunton, 2013), and some have illustrated that stressed dams spend more time nursing pups compared to unstressed dams (Muir et al., 1985). Therefore, the exact effects of maternal behaviour on prenatal stress are still contested.

1.4.3 Early Postnatal Programming

As well as the prenatal period, the early postnatal period is also an important stage of development, and environmental influences can lead to long term changes in physiology.
and behaviour. In a large number of mammalian species, organ development is not complete at birth, and many structures involved in metabolism, such as connections between the brain feeding centres such as the ARC, PVN and LH, are still developing in the early postnatal period (reviewed in: Spencer, 2013). Therefore, alterations to these systems at this stage of development can lead to changes in metabolism and feeding behaviour that may persist throughout life. The development of connectivity between these structures is partially dependent on a surge in circulating leptin which occurs between P4 and P16 in rodents (Bouret and Simerly, 2004). This leptin surge can be modified by both maternal and postnatal diet, as well as by early life stress which can lead to disruption in the development of these pathways (Spencer, 2013).

1.4.3.1 Early Life Nutrition and Litter Size

With childhood obesity on the rise, and the high likelihood of obese children carrying their excess weight into adulthood, early life nutrition has become an area of increasing interest. In rodent models it is well established that neonatal overfeeding results in increased weight gain which is persistent throughout life (Boullu-Ciocca et al., 2005, Stefanidis and Spencer, 2012, Hou et al., 2011), which suggests that changes in neonatal over-nutrition could be a risk factor for obesity development.

One of the most well-established models of early life nutrition in rodents is achieved through adjusting litter size. By adjusting to a small litter, pups will then be over-nourished due to increased access to the dam’s milk (Moreira et al., 2009). In the converse, rats raised in large litters will have reduced access to milk, and will therefore be under-nourished in comparison to control offspring (Remmers et al., 2008). In order to generate varying litter sizes, litters can be adjusted shortly after birth and allowed to remain with their dams (Portella et al., 2015, Noschang et al., 2014), or cross fostered to generate the required litter sizes (Sominsky et al., 2017b, Spencer and Tilbrook, 2009). Some studies have used naturally occurring small or large litters (Dimitsantos et al., 2007), although this can create difficulties when large numbers are needed for experimental purposes as naturally occurring small/large litters are uncommon.

1.4.3.1.1 Small Litter Rats

The most prominent effect of small litter rearing on rats is an increased body weight in comparison to control rats which generally becomes apparent early in the pre-weaning
stage and often persists throughout life (Boullu-Ciocca et al., 2005, Xiao et al., 2007). This increased body weight is associated with larger fat mass and increased lean mass in small litter rats (Mozes et al., 2014, Stefanidis and Spencer, 2012), as well as elevated plasma leptin (Stefanidis and Spencer, 2012) and hyperinsulinemia (Plagemann et al., 1999). Furthermore, there is also evidence to suggest alterations to the HPA axis, with small litter rats illustrating increased circulating corticosterone as adults (Hou et al., 2011) and prolonged corticosterone secretion following a mild psychological stressor (Boullu-Ciocca et al., 2005).

As well as small litter rats consuming a larger amount of milk in comparison to control rats (Cunha et al., 2009, Moreira et al., 2009), there is evidence to show that milk from small litter dams has higher fat levels than that of control dams (Mozes et al., 2014). Furthermore, in the early stages of life, rodents receive much of their leptin from their mother’s milk, and therefore rats from small litters may consume more leptin than control litter rats. This could lead to changes in the leptin surge and therefore alter the development of hypothalamic feeding centres (Spencer, 2013), resulting in alterations to feeding behaviour that could persist throughout life.

Although there is some evidence that the increased body weight observed in small litter offspring is associated with increased food intake (Spencer and Tilbrook, 2009, Rodrigues et al., 2009), others have found no differences in food intake between control and small litter rats (Noschang et al., 2014, Portella et al., 2015, Mozes et al., 2014). Therefore, it is important to uncover the physiological and behavioural changes that lead to increased weight gain in neonatally over-nourished rats.

1.4.3.1.2 Large Litter Rats

Large litter rats show a reduced body weight which persists throughout life (Bulfin et al., 2011). This is associated with reduced plasma leptin and glucose-stimulated insulin secretion (López-Soldado et al., 2006). Large litter rearing is also associated with changes in stress reactivity, with corticosterone levels returning to basal levels significantly faster in large litter rats compared to controls after exposure to a stressor (Bulfin et al., 2011).

In humans, multiple births may be considered a comparable model of neonatal undernutrition. Studies have shown that twins often have a lower BMI compared to singletons both in childhood (Estourgie-van Burk et al., 2010) and adulthood (Silventoinen
et al., 2013, Andrew et al., 2001). Some studies have reported a lower incidence of obesity and lower fasting blood glucose in twins compared to singletons (Jayaweera et al., 2018), but there have also been reports of higher obesity and type 2 diabetes prevalence in elderly twins (Poulsen et al., 2009). However, often twin studies have small sample sizes and may not take into account differences between monozygotic and dizygotic twin pairs (Rumball et al., 2008). Furthermore, whilst twin studies may be thought to be comparable to rats reared in a large litter, this may not be an appropriate model of neonatal undernutrition as twins often have a lower birthweight in comparison to singletons (Muhlhausler et al., 2011) and therefore may be considered to have undergone intrauterine growth restriction. In contrast, many of the protocols used for rat models implement cross-fostering after birth to ensure that the effects are only due to postnatal nutrition and not due to intrauterine growth restriction.

1.5 Mood and Feeding
There have been many links established between mood and feeding behaviour, particularly the impact of negative states such as stress and anxiety on food consumption (reviewed in: Maniam and Morris, 2012). Mood disorders such as depression, anxiety and post-traumatic stress disorder are all risk factors for obesity development (Wurtman and Wurtman, 2017), which could be due to associations between the drugs used to treat these disorders and weight gain (Fava et al., 2000). Furthermore, many drug treatments that have been used in the past to treat obesity have often been withdrawn due to mood related side effects, such as Rimonabant which while effectively producing weight loss also led to increased incidence of depressive disorders and suicidality (Burch et al., 2009).

The majority of research into mood and feeding is associated with negative states, and therefore an area which has been significantly neglected is the effect of positive mood on feeding. It is often postulated that negative emotions lead to an increased food consumption and unhealthier food choices, and therefore the converse may be assumed for positive states. However, the evidence is still conflicting as to whether this is the case.

In agreement with this idea, Gardner et al. (2014) found that participants in a positive mood were more likely to evaluate healthier foods more favourably (for example giving higher ratings for tastiness) in comparison to those in a neutral mood. Furthermore, participants in a positive mood consumed a greater proportion of ‘healthier’ foods in comparison to those in a negative mood when measuring the consumption of raisins and
M&Ms. It was inferred that participants in a happy state put more emphasis on long-term health and therefore made choices accordingly, whereas participants in a negative mood were more focused on immediate reward. Similarly, Turner et al. (2010) found that participants exposed to a positive mood condition consumed fewer chocolate chip cookies than those in a neutral condition.

However, evidence from Evers et al. (2013) suggests that positive mood may lead to an increase in food intake, as participants in a positive mood had an increased calorie intake during a 10-minute taste test in comparison to control participants. It was also found that, when measuring self-reported snack intake over a week, a larger percentage of unhealthy snacking was preceded by positive emotions in comparison to negative emotions. Furthermore, experiments conducted by Macht, Roth and Ellgring (2002) found that motivation to eat is often higher during ‘joy’ in comparison to ‘sadness’, and that chocolate is rated as more pleasurable when in a positive mood. Bongers et al. (2013) found that those subjects who classified themselves as emotional eaters ate more in a positive mood in comparison to a negative mood, whereas non-emotional eaters ate a similar amount in each condition.

In human studies when examining positive affect, it is important to also account for other influencing factors such as social context. Positive mood is often associated with a social context and socialising and food consumption are also often tightly associated (Evers et al., 2013). Patel and Schlundt (2001) found that meals eaten in a positive mood were larger than those eaten in a neutral mood, however when meals were consumed in a positive mood and social context these meals were even larger. Furthermore, social context appeared to alter food choices, with more fat and fewer carbohydrates being consumed in the social context condition compared to when eating alone. Therefore, when examining the effects of mood on feeding behaviour it is important to account for other influencing factors.

A problem associated with mood research in humans is that it is hard to accurately and reliably induce and measure mood states without interference from previous and current experiences of the participants (Hammersley et al., 2014). Furthermore, in many human studies concerning feeding behaviour, dietary restraint - the tendency to consciously restrict food intake - can be a confounding factor (Johnson et al., 2012). Therefore, it is
important to develop a model whereby a positive state can be accurately and reliably produced whilst also allowing control of external factors such as dietary restraint.

1.5.1 Mood and Affect
It is important to establish the differences between mood and affect as they are often used interchangeably. Affect refers to the immediate expression of an emotion and is generally generated from a specific stimulus or event. Affect is instantaneous, instinctual and generally short term. On the other hand, mood is a long term emotional state, and occurs without a specific stimulus or reason (Manjunatha et al., 2009). Although mood states are complex and varied, basic affective states can be broadly subdivided into two categories, positive and negative affective states which are common to all animals (Brudzynski, 2013).

1.5.2 Ultrasonic Vocalisations
Whilst there is clear evidence that animals can express affective states (Panksepp, 2011), the largest problem facing research in this area is the ability to measure subjective emotional state. In recent years, scientists have discovered that rodents emit ultrasonic vocalisations (USVs). These are generally in the range of 20-100 kHz and are thought to serve a communicative function (Schwarting and Wöhr, 2012). These vocalisations can be recorded via specialised microphones and then quantified through analysis of a spectrogram.

Between rats the production of the number and types of USVs can vary greatly, however within an individual they appear to remain stable over time (Mallo et al., 2007). In rats there are three classes of USVs, each thought to reflect differing affective states. 40 kHz USVs are emitted by infant rats, whereas adolescent and adult rats have two classes of USVs: 50 kHz and 22 kHz.

1.5.2.1 40 kHz USVs
Vocalisations at around 30-65 kHz (referred to as 40 kHz USVs) are emitted by infant rats in response to distressing situations such as maternal separation, isolation from the nest and unfamiliar odours (Brudzynski et al., 1999). These calls are generally around 80-150 ms in duration are thought to serve an adaptive function to elicit a maternal response towards endangered pups (Schwarting and Wöhr, 2012). These calls are generally emitted at the highest level between postnatal days 5-10 and are no longer expressed after weaning (Simola, 2015).
USVs that range between 20-30 kHz, termed 22 kHz USVs, are long in duration (generally in the range of 500 and 3000 ms) and monotonous (flat when viewed on a sonogram) (Brudzynski, 2013). These calls are associated with negative experiences such as predator exposure (Litvin et al., 2007), aggression (Burgdorf et al., 2008), and the withdrawal of some drugs, including opiates and cocaine (Barker et al., 2015) and are therefore suggested to reflect a negative state. Further supporting this idea, Wöhr, Borta and Schwarting (2005) found that both the number and amplitude of 22 kHz USVs increased with increasing foot shock intensities. It appears that individual differences in anxiety levels also contribute to 22 kHz USV emission. Rats that were classified as highly anxious using the elevated plus maze, demonstrated increased levels of 22 kHz USVs in comparison to those who illustrated fewer anxiety related behaviours (Borta et al., 2006).

It is thought that 22 kHz USVs serve a communicative function, specifically as a warning call to others. Kim et al. (2010) illustrated that in pair-housed rats, when 22 kHz were emitted by a rat that had undergone fear conditioning, their conspecific would also emit 22 kHz USVs and show fear-like behaviour such as freezing, however this was only the case in those conspecific rats that had previously experienced an aversive event and did not occur in naïve partners. Furthermore, playback of 22 kHz USVs leads to avoidance behaviour (Burgdorf et al., 2008), further evidence to suggest that these vocalisations signal an aversive situation.

Nevertheless, not all studies have found a strong behavioural response to playback of 22 kHz USVs. Endres et al. (2007) found an increase in time spent freezing in response to 22 kHz USV playback, but this was only significant when a number of stimuli including artificial 22 kHz sine tones were grouped together and compared to ‘other stimuli’ which included 50 kHz USVs, silence and modified 22 kHz USV tones. Moreover, some have found no behavioural responses with playback of 22 kHz USVs (Parsana et al., 2012, Sadananda et al., 2008). However, although naïve rats may not illustrate an aversion to 22 kHz USVs, there is evidence to suggest that rats may be primed to quickly learn to associate aversive events with 22 kHz calls. When undergoing a Pavlovian fear conditioning programme, rats appear to learn the significance of 22 kHz alarm calls faster and retain this information longer in memory, in comparison to other stimuli (Endres et al., 2007). Furthermore, rats receiving footshocks that elicited 22 kHz USVs, froze in response to novel 22 kHz USVs but did not
freeze in response to novel 50 kHz USVs, whereas control rats who hadn’t received footshocks or vocalised did not freeze in response to either stimulus, which suggests learning through auto-conditioning (Parsana et al., 2012).

1.5.2.3 50 kHz USVs

Vocalisations termed 50 kHz are generally of a much shorter duration than those at 22 kHz, generally between 10-150 ms in duration, and although classified as 50 kHz, appear across a range of frequencies from 35-80 kHz (Brudzynski, 2013). 50 kHz USVs are associated with positive experiences such as play (Knutson et al., 1998), during sexual encounters (McGinnis and Vakulenko, 2003) and in anticipation of administration of rewarding drugs (Knutson et al., 1999), and are reduced by negative stimuli such as exposure to predator odour (Knutson et al., 2002) or anticipation of foot shock (Burgdorf et al., 2000). 50 kHz vocalisations can be separated into two categories, flat and frequency modulated (FM). Frequency modulated 50 kHz USVs can be further subdivided into categories based on their spectrographic shapes such as trill, step and step trill (see Figure 2.3) (Brudzynski, 2013). Whilst there has still been little research to illustrate the exact function of the different subtypes of 50 kHz USVs, current evidence suggests that frequency modulated vocalisations are more associated with positive experiences such as rough and tumble play (Burgdorf et al., 2008) and administration of amphetamines (Wright et al., 2010). On the other hand flat 50 kHz USVs are associated with more adverse situations, such as separation from a cage mate (Wöhr et al., 2008) and aggression (Burgdorf et al., 2008). This is further illustrated by the fact that rats will self-administer playback of frequency modulated 50 kHz USVs, but not flat 50 kHz vocalisations (Burgdorf et al., 2008), suggesting that FM 50 kHz USVs are more indicative of positive affect whilst flat 50 kHz USVs may serve a communicative function associated with social coordination (Wöhr et al., 2008).

As with 22 kHz USVs, there is evidence to suggest that 50 kHz USVs serve an important communicative function. Adolescent rats seek the company of rats who emit high levels of 50 kHz USVs more than those who emit fewer vocalisations (Panksepp et al., 2002). Furthermore, playback of 50 kHz USVs induces approach behaviour in juvenile rats (Wohr and Schwarting, 2007).

It appears that vocalisation levels may have a genetic component as rats can be selectively bred to produce high or low levels of 50 kHz vocalisations, and those rats emitting higher numbers of USVs show higher levels of play behaviour, and also find tickling stimulation less
aversive than wild type rats (Panksepp and Burgdorf, 2000). Overall, lines bred for high levels of 50 kHz USVs appear to be more stress resilient whereas rats bred for low levels of USVs are more anxiety prone (reviewed in: LaFollette et al., 2017).

### 1.5.3 Heterospecific Social Play

In 1999, Burgdorf and Panksepp found that they were able to replicate the increase in 50 kHz USVs observed during play by imitating the rats’ natural rough and tumble play behaviour through a ‘tickling’-like interaction with the experimenter. This interaction involves vigorous finger movements over the dorsal surface of the rat, as well as repeated pinning (Panksepp and Burgdorf, 1999). This interaction, often referred to as ‘heterospecific social play’ or ‘tickling’, induces a large increase in 50 kHz USVs suggesting the induction of positive affect (Burgdorf and Panksepp, 2001, Cloutier et al., 2013, Hori et al., 2013b). Furthermore, this interaction has been considered as rewarding to rats as it has been well established that approach latency to the hand of the experimenter following tickling interaction is shorter in rats who have been tickled in contrast to light touch controls (Hori et al., 2009, Yamamuro et al., 2010). Furthermore, when exposed to an experimenter’s hand, tickled rats made more hand contacts and produced more 50 kHz USVs in comparison to rats who had experienced passive contact (Cloutier et al., 2012) and illustrated a preference for the experimenters’ hand over an anaesthetised conspecific (Burgdorf and Panksepp, 2001).

It has been well established that positive emotional states in humans can modify resilience to fear and anxiety (Ong et al., 2006, Tugade and Fredrickson, 2004), and this has also been shown using tickling as a model of positive affect in rats. Tickling treatment prior to a conditioned fear response reduced fear-induced freezing in rats. Additionally, tickled rats showed lower levels of plasma adrenaline and noradrenaline, although no differences in corticosterone were observed (Hori et al., 2013b). Tickling has also been shown to reduce the detrimental effects on fear responsiveness and spatial learning induced by isolated rearing during adolescence (Hori et al., 2014). Cloutier et al. (2013) found that rats who had regularly experienced heterospecific social play with an experimenter illustrated reduced anxiety in an open field test. Furthermore, exposure to rough and tumble play behaviour has been shown to be able to reverse the effects of chronic unpredictable stress, in the forced swim test, sucrose preference and in novelty induced hypophagia (Burgdorf et al., 2017). These data together suggest that this model induces a positive affect as illustrated
by increased numbers of 50 kHz USVs and reduced approach latency to the hand of the experimenter, as well as the fact that tickling is able to induce stress resilience in rats.

Since the implementation of this model, many laboratories have used this paradigm to investigate neural pathways and behaviours associated with positive affect. Tickling increases dopamine release in the NAc and tickling-induced 50 kHz USVs were blocked by administration of D1 and D2 receptor antagonists into the NAc (Hori et al., 2013a). Dopamine receptor antagonists have also been shown to reduce amphetamine induced 50 kHz USVs (Wright et al., 2013). Tickling has also been shown to increase neurogenesis in the dentate gyrus of the hippocampus, an area which is thought to mediate memory formation (Yamamuro et al., 2010), as well as increase hippocampus cell proliferation, although this only occurred in high-calling rats (Wöhr et al., 2009). Furthermore, Hori et al. (2009) found an increase in expression of genes regulating feeding behaviour in adolescent rats who had been exposed to repeated tickling stimulation, including genes encoding POMC, AgRP and NPY. However, the use of this model to examine the effects of positive affect on feeding behaviour and food choice remains unstudied.

In addition to uncovering neural substrates of positive affect, this model has also been used in the study of animal welfare in a laboratory setting in an attempt to reduce stress associated with many routine procedures such as injections (Cloutier et al., 2015, Cloutier et al., 2014) and improve welfare of laboratory rats (Cloutier et al., 2012, Cloutier et al., 2013).
1.6 Thesis Aims and Hypotheses

This thesis is made up of three results chapters focusing on early life and mood factors and how these may affect food intake and food choice, and therefore possibly contribute to the development of obesity.

Firstly, due to the known effects of prenatal stress on the stress response of the offspring, and the effects of stress on appetite related behaviours, the impact of prenatal stress on feeding behaviour and food choice was examined. As there is some evidence to suggest that stress can lead to an increased palatable food consumption in rats, which may be able to attenuate the stress response (Pecoraro et al., 2004, Ulrich-Lai et al., 2010), the following hypotheses were tested:

- Prenatal stress will lead to an increased calorie intake in comparison to control rats when offered a palatable diet.
- Prenatal stress will lead to an increased preference for more energy dense food.

Secondly, while small litter rats are a well-established model of obesity, there is still little evidence on how neonatal overnutrition can influence food choice in later life. Therefore, small litter rats were used as a model of neonatal overnutrition to examine the effects of early life overfeeding on food choice in adulthood. Evidence from Conceição et al. (2016) and Noschang et al. (2014) suggests that small litter rats may illustrate an increased preference for foods high in fat and sugar, therefore the following hypothesis was tested:

- When given a food choice, small litter rats will show a preference for high energy foods such as lard and sucrose over bland chow.

It is well-established that consumption of foods, particularly those high in fat and sugar, can be rewarding (Kenny, 2011). The concept of comfort eating suggests that consumption of these foods can help to reduce some of the behavioural and endocrine responses to stress (Ulrich-Lai et al., 2010), however there is still little evidence to suggest whether consumption of these foods can lead to an induction of positive affect. Therefore, using ultrasonic vocalisations to measure affect in rats, the effects of food reward on USVs were examined. The hypothesis tested was:

- 50 kHz USVs will be increased before and after access to sweetened condensed milk, as both the anticipation and consumption of a food reward will induce a positive affect.
Furthermore, as human evidence on whether positive affect can influence food choice is conflicting, heterospecific social interaction was used as a model of positive affect in the rat to establish the impact of positive affect on food preference, and the following hypothesis was tested:

- Positive affect will lead to a decreased consumption of sucrose, and therefore a reduced sucrose preference, in comparison to controls.
2 General Methods

2.1 Animals
For experiments using Sprague Dawley and Long Evans rats, rats were bred in-house (unless otherwise stated). Wistar rats for experiments using ultrasonic vocalisations were sourced from Charles River UK.

2.1.1 Housing
Rats were housed in a temperature-controlled room (20 ± 2°C) and on a 12-hour light/dark cycle (lights on at 7:00). Rats were housed in plastic tub cages with a wire lid containing a food hopper and a water bottle. Cages for group housing were of dimensions: 585 x 405mm (height 200mm) and for single housing dimensions were: 400 x 255mm (height 190mm). Rat bedding consisted of aspen chips and shredded tissue. Unless otherwise specified, rats received *ad lib* access to a standard lab chow (Rat Mouse 1 – Special Diet Services - SDS) and water. All experimental procedures took place in the light period.

2.1.2 Timed Matings
For experiments requiring timed matings, nulliparous females were mated overnight with a sexually experienced male in a cage with mesh flooring. Presence of a semen plug was used as a confirmation of pregnancy and determined as day 1 of pregnancy. Shortly before birth, female rats were transferred into single housing and provided with extra bedding for nest building. Pups remained with the dam for 21 days before being weaned. Dams were maintained on a breeding diet (Rat Mouse 3 – SDS) throughout pregnancy and weaning.

2.1.3 Cross Fostering
Cross fostering was performed on postnatal days 1 or 2 (with P0 as day of parturition). Pups were briefly removed from dams and reassigned to new dams, taking care to ensure that none of the dams received her own pups, and ensuring each dam received a mix of pups from at least 2 different litters. Before placing with the new dam, pups were gently rolled in the bedding of the new dam to reduce the chance of rejection. Excess pups were culled by an appropriate schedule 1 method.
2.1.4 Ethics
All experiments and procedures were approved by a local committee and performed under UK Home Office Regulations by trained individuals holding a personal licence.

2.2 Diets
Rat body weight and food and water consumption were measured manually using digital scales with a precision of 0.01g. The weight of food and water was subtracted from the previous day’s/week’s measurement in order to obtain a value for daily/weekly consumption. Care was taken when measuring food to ensure that there was no food remaining in the cage. Water dripping and evaporation was controlled for by daily measurement of a control water bottle which was inverted in an empty cage under the same housing conditions as the rats.

2.2.1 Lab Chow (RM1)
The lab chow (hereafter referred to as ‘bland chow’) consisted of a nutritionally complete lab chow (RM1 – Special Diet Services) designed for long-term maintenance of rodents. Pellets were held in the food hopper on the cage lid. For nutritional data on bland chow see Table 2.1.

2.2.2 Breeding Diet (RM3)
The breeding diet is similar to the bland chow in composition, however it is slightly higher in protein and fat. This is given to dams during pregnancy and lactation due to the higher requirements for protein and fat during this period (NRC, 1995). RM3 was sourced from Special Diet Services. For nutritional data see Table 2.1.

2.2.3 High-Fat High-Sugar Pellet
The high-fat high-sugar diet (RM AFE45%FAT 16%SUC - referred to as the HFHS diet) was sourced from Special Diet Services. It consisted of a soft pellet, high in fat and sugar. During feeding these pellets were held in the food hopper on the cage. For nutritional data see Table 2.1.

2.2.4 Sucrose Solution
Sucrose solution was either prepared at 5% or 10% w/v. Water was warmed in a microwave and sucrose (Fischer Scientific) dissolved using a stirrer. Sucrose solution was presented using standard water bottles. For experiments using sucrose solution, fresh solution was given to rats every two days.
2.2.4.1 Sucrose Preference Tests

For sucrose preference tests both sucrose solution and water consumption were measured over a specified period. Sucrose preference was then calculated as the percentage of the volume of sucrose intake of the total volume of fluid intake.

2.2.5 Sucrose Pellets

Sucrose pellets were obtained from Sanddown Scientific UK (TestLab). Pellets weighed approximately 1g each in weight and were held in the food hopper for *ad lib* access.

2.2.6 Lard

Lard (obtained from Tesco, UK) was placed into cages via a stainless-steel bowl (diameter: 80 mm, depth: 42 mm), bolted to the feeding hopper of the cage. Lard was changed every 2 days.

2.2.7 Sweetened Condensed Milk

Sweetened condensed milk (SCM, Carnation, Nestle, UK) was placed into the cage using a small glass bowl (external diameter: 75mm, depth: 25mm). SCM was diluted 1:1 using tap water and frozen in aliquots at -20°C. Before use SCM was thawed at room temperature.

<table>
<thead>
<tr>
<th>Table 2.1: Diet Nutritional Information</th>
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<tbody>
<tr>
<td>Diet</td>
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<td></td>
</tr>
<tr>
<td>RM1</td>
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<td>RM3</td>
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<tr>
<td>HFHS</td>
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<tr>
<td>Sucrose pellet</td>
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<tr>
<td>Lard</td>
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<tr>
<td>50% SCM</td>
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</table>

2.3 Blood Sampling

2.3.1 Tail Nick

Prior to blood sampling, rats had been regularly handled by the same experimenters undertaking the blood sampling in order to reduce stress from the procedure. For PNS experiment 2 (see section 3.3), rats were gently restrained in towel for 2 minutes a day for
a week prior to blood sampling. For blood collection, rats were gently restrained in a towel and their tails placed in warm water to encourage vasodilation. A small, horizontal incision was made around half way down the tail using a scalpel blade and the tail massaged to encourage blood flow. Blood (approximately 300 µl per sample) was collected into chilled EDTA tubes with capillary action (Microvette® CB 300 K2E, Sarstedt) and stored on ice until processing. When completing serial sampling, after warming the tail in water, a paper towel was swiftly wiped over the incision to remove the clot and to enable further blood collection. When obtaining serial samples over a short period (less than 24 hours), the total amount of blood collected did not exceed 10% of total blood volume. The blood volume for adult animals is estimated as 55 to 70 ml/kg body weight (Parasuraman et al., 2010), therefore blood volume was calculated based on the lower limit of 55 ml/kg body weight to ensure the limits were not exceeded.

2.3.2 Plasma Preparation
Blood samples were centrifuged in the EDTA tubes at 14,000 rpm for 10 minutes at room temperature to enable plasma collection. After centrifugation plasma was separated from the pellet using a pipette and aliquots of plasma were stored in 500 µl Eppendorf tubes and flash frozen on dry ice. Samples were then stored at -80°C before analysis.

2.3.3 Blood Glucose
Blood glucose was measured directly from whole blood using a glucose meter and test strips (Aviva Accuchek, UK).

2.4 Stressors
2.4.1 Restraint Stress
For restraint stress, rats were placed into a transparent tube with a diameter of 63mm (554-BSRR, PLAS-LABS) with adjustable length and breathing holes. Rats were closely monitored throughout the restraint period.

2.4.2 Prenatal Restraint Stress
For rats undergoing the prenatal restraint stress procedure, dams were subjected to a restraint stressor for 30 minutes, 3 times a day from days 16-20 of pregnancy. Dams received the stressor at different times each day in order to prevent habituation. Tubes were cleaned between each rat using warm water and soap.
2.4.3 Prenatal Social Stress
The prenatal social stress paradigm was based on that used by Brunton and Russell (2010). Pregnant rats were subjected to a social defeat stressor from days 16-20 of pregnancy. They were placed into a cage with an unfamiliar lactating ‘resident’ female (days 2-8 of lactation) for 10 minutes a day, with each intruding rat experiencing a different lactating resident each day. Behaviour was video-recorded and aggression scored using the following scale: punching = 1; single bite = 2; bite with pin down = 2; prolonged biting = 3; tumble attack and pin down = 3 (based on the scale used by Brunton and Russell, 2010).

2.5 Assays
2.5.1 Corticosterone Assay
Corticosterone measurement was performed by radioimmunoassay (MP Biomedical). In this assay, a limited amount of specific antibody is reacted with $^3$H labelled corticosterone. Therefore, on addition of increasing amounts of corticosterone, a correspondingly decreasing fraction of the $^3$H added is bound to the antibody. After the separation of the bound from free $^3$H by precipitation, the amount of bound radioactivity is counted using a gamma counter and this data used to construct a standard curve, from which the unknown samples are interpolated. Minimum detectable plasma concentration of this assay is 7.7 ng/ml. All samples were measured in duplicate.

2.5.2 Leptin Assay
Plasma leptin was measured by ELISA (DRG Diagnostics). This assay is based on the sandwich principle - microtiter wells are coated with a monoclonal antibody directed towards a unique antigentic site on a leptin molecule. Plasma is incubated in the well with a specific biotinylated monoclonal anti-leptin antibody. A sandwich complex is formed and following incubation the unbound material is washed off and a Steptavidin Preoxidase Enzyme Complex is added for detection of the bound leptin. The intensity of colour developed is proportional to the concentration of leptin in the sample. Using a plate reader to determine absorbance, a standard curve is plotted and used to determine the concentration of the unknown samples. All samples were measured in duplicate. Assay sensitivity was 1 ng/ml.

2.5.3 Inter-assay Coefficient of Variability
For some assays where there were a large number of samples, it was necessary to perform more than one assay in order to analyse all of the samples. The inter-assay coefficient of
variability (CV) monitors the precision of results between assays. Inter-assay variability is calculated from the standard deviations (SD) of the means of duplicates over the grand mean of the duplicates. Inter-assay CV was calculated from the raw data counts for the standards used in each assay using the equation:

\[
\frac{SD \text{ of means of duplicates}}{Grand \text{ mean of the duplicates}} \times 100
\]

A value of under 10% is satisfactory and indicates good accuracy between assays.

2.5.4 Intra-assay Coefficient of Variability
The intra-assay CV measures the precision of results within the assay by measuring the replicates in the assay. The intra-assay CV is calculated by the mean of the standard deviation of the duplicates over the grand mean of the duplicates. Intra-assay CV was calculated from the raw data counts for the standards used in the assay by the following equation:

\[
\frac{Mean \ of \ SD \ of \ the \ duplicates}{Grand \ mean \ of \ the \ duplicates} \times 100
\]

A value of under 10% is satisfactory and indicates good accuracy between duplicates within an assay.

2.5.5 Assay Data Analysis
Assay data was analysed using Graphpad Prism 6. An average of the counts of the two duplicates was taken before subtracting the blank/non-specific binding (NSB) counts. Percentage binding was then calculated by dividing the corrected values by the corrected B0 (total binding) value. These values were then used in Graphpad to construct a non-linear standard curve from the standards and then interpolating unknown concentrations. This was done using a sigmoidal equation.

2.6 USVs
2.6.1 Recording Set Up
Recordings took place in a ‘recording arena’ of dimensions 585 x 405 mm (height 200mm). The microphone was suspended on a tripod approximately 350 mm above the base of the recording arena. Recording was carried out using an Avisoft-UltraSoundGate 116H recorder connected to a laptop and recorded using Avisoft RECORDER USGH (see Figure 2.1). Recordings took place in the rats’ home room. The recording arena was cleaned with a
disinfectant between each rat in order to prevent distraction by olfactory cues left by the previous rats.

For those rats receiving social contact in their home cages, rats were individually housed in cages of the same dimensions as the recording arena to ensure sufficient space for heterospecific social contact. Recording set up was otherwise the same as illustrated in Figure 2.1.

2.6.2 Baseline Recordings
There is a degree of inter-individual variability in rat vocalisations, however vocalisations in individual rats remain stable over time (Mallo et al., 2007). Therefore, before each experiment a 5-minute baseline recording was made from each animal and rats were grouped based on these recordings, ensuring an equal spread of USV characteristics across groups.

2.6.3 Heterospecific Social Play – ‘Tickling’
The simulated social contact procedure was similar to that used by Burgdorf and Panksepp (2001). For pilot experiments a 5-minute protocol was used, whereby rats were placed in the recording arena before receiving 30 seconds of passive contact (experimenters hand resting passively in the cage) followed by 30 seconds of heterospecific social play from the experimenter. This pattern was repeated until a total recording time of 5 minutes was reached. For experiments following the pilot experiments, a 2-minute protocol was used (as described by Burgdorf and Panksepp, 2001). This was similar to the 5-minute protocol but involved 15 second intervals of passive contact/social contact until a total recording time of 2 minutes was reached. The social contact from the experimenter mimicked the natural rough and tumble play behaviour of the rat and involved vigorous, whole body stimulation of the rat, including repeated pinning (see Figure 2.2 for social contact examples). During social contact a white cotton glove was used (one per rat in order to avoid olfactory cues) in order to reduce friction on the fur. Control rats received passive contact for the full 5/2-minute recording period.
Rats received social contact in the recording arena. The microphone was suspended approximately 350 mm above the floor of the arena at a slight angle to ensure efficient recording of USVs. The microphone was connected to the Avisoft recorder which was connected to a laptop.
Figure 2.2: Examples of different types of hetero-specific social play

2.6.4 USV Quantification

USVs were manually counted from spectrograms generated using Avisoft SASLab Lite. For each vocalisation duration, maximum amplitude, maximum frequency and shape were recorded. Shapes were scored based on the categories described by Brudzynski (2013). Vocalisations were split into two broad categories: flat and frequency modulated (FM). FM USVs were then further categorised as trill, step, step trill or other FM (see Figure 2.3 for spectrogram examples). USVs that were under 0.003 s in duration, or those whose shape was unable to be scored (~1-2% of total vocalisations) were not counted.

When quantifying, total number of USVs were divided by the total recording time in order to provide a value for USVs/minute.

2.6.5 Contact Latency

Contact latency is used as a measure of the rewarding nature of heterospecific social play, as rats that find the stimulation rewarding will show a higher motivation for contact to the hand of the experimenter. Contact latency was measured in the same arena used for simulated social contact. The experimenter placed their hand in the opposite corner of the recording arena to the rat and the amount of time it took for the rat to make contact with the hand, either nose or front paws, was recorded. If no contact was made after 180 seconds recording was stopped.
Figure 2.3: Spectrogram Examples

A: Examples of flat 50 kHz USV vocalisations. B: Trill vocalisations. C: Step vocalisations. D: Step trill vocalisations. E: Other frequency modulated vocalisations. Any USVs which did not fit the characteristics for the other categories were categories as other FM. F: 22 kHz USVs – associated with negative experiences.
2.7 Place Preference Test

2.7.1 Place Preference Apparatus
The place preference apparatus was constructed from a wooden base surrounded by an adjustable stainless-steel frame (Figure 2.4). Acrylic sheeting was slotted into the frame to give final dimensions of 910 mm x 430 mm. For one side of the box, electrical tape was used to create a cross pattern on the acrylic sides and Lego sheeting was taped to the floor. The other side had taped vertical stripes and a floor covered with linoleum. This was to allow the rat to easily distinguish the two sides of the box.

2.7.2 Place Preference Test
Rats were placed into the place preference box (on the checked side) and allowed to habituate to the box for 15 minutes. The conditioned place preference (CPP) test then took place the following day at the same time as the habituation. Again, the rat was placed in the place preference box for 15 minutes and their behaviour video recorded. The videos were later manually scored and total amount of time on one side of the box was calculated, as well as total entries to that side of the box counted. A rat was counted as entering a certain side of the box when its head and front paws crossed the boundary between the two boxes.

The box was cleaned between each rat to prevent olfactory cues from previous rats influencing the results.
Figure 2.4: Conditioned Place Preference Box

A: Checked side of the CPP box with a Lego textured flooring and black divider. Checks were approximately 40 x 50 mm. B: Striped side of CPP box, stripes were approximately 40 mm apart. Flooring was made of linoleum sheet. C: View of full box. Total box dimension was approximately 910 x 430 mm (height 400 mm). Door opening between the two chambers measured 125 x 150 mm.
2.8 Statistics
Statistical analyses were performed using Graphpad Prism 6.01. All graphs are plotted as mean ± standard error of mean (SEM) unless otherwise stated. For all statistical testing, p < 0.05 indicates a statistically significant result.

2.8.1 Normality Testing
Data were tested for normality using a Shapiro-Wilk normality test. Data that passed this test (p > 0.05) were assumed to fit a normal distribution pattern and were therefore analysed using parametric analysis. Data failing this test were assumed not to fit a normal distribution and non-parametric analysis was performed.

2.8.2 Parametric Testing
When comparing normally distributed data between two groups, an unpaired two-tailed t test was used. In the case where multiple t tests were used to analyse data, the Holm-Sidak method was used to correct for multiple comparisons and determine statistical significance.

For comparisons of 3 or more groups, one-way ANOVA analysis was implemented. If a significant difference was observed, Tukey’s multiple comparisons test was used post-hoc to determine which data illustrated a significant difference.

For comparison of data with two factors, such as group and diet, a two-way ANOVA was used to measure any significant differences in the data. If a significant p value was observed, Tukey’s multiple comparisons test was used post-hoc to illustrate which data showed significance.

For comparison of two or more groups of data over time, 2-way repeated measures ANOVAs were used. If a significant difference was detected (p < 0.05) the Sidak’s multiple comparisons test was used post-hoc to determine the location of the significant difference.

When comparing 3 factors (e.g. stress condition, sex and diet), 3-way ANOVAs were used. If a significant main effect was detected (p < 0.05), Tukeys or Dunnets multiple comparison tests were used post hoc to determine significant differences.

Significance is denoted on figures as * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

2.8.3 Non-Parametric Testing
For comparison of two groups that had failed normality testing a two-tailed Mann Witney U test was implemented to indicate statistical significance.
In the case of comparison of three or more groups, Kruskal-Wallis testing was implemented.

For data presented over time that was not normally distributed, area under the curve (AUC) analysis was performed (see section 2.8.4) in order to represent the data as a single value. This data was then tested for normality and analysed with parametric or non-parametric testing as appropriate.

Significance is denoted on figures as * = \( p < 0.05 \); ** = \( p < 0.01 \); *** = \( p < 0.001 \).

2.8.4 Area Under the Curve Analysis
In order to represent data over time as a single value, Area under the curve (AUC) analysis was used. AUC was calculated for each individual animal and then averaged to establish one value for each animal per timepoint (total AUC/(total number of time points – 1)). These data could then be tested for normality and analysed with parametric or non-parametric testing as appropriate.

2.8.5 Linear Regression Analysis
Linear regression analysis was implemented to determine whether there was a relationship between two sets of independent data. A value of \( p < 0.05 \) signifies a significant difference compared to a line with no correlation (slope = 0) and therefore indicates a linear relationship between the two variables. \( R^2 \) is a statistical measure of how well the data are fitted to the linear regression line, with a value of 1 indicating a perfect fit of the data to the linear regression line.

2.8.6 Power Analysis
For experiments for which pilot data had been collected, retrospective power analysis was performed in order to estimate the sample size required in subsequent experiments to detect a statistically significant difference (provided there was a difference) with a power of 0.8 (an 80% probability of correctly rejecting the null hypothesis when false).

An online tool was used for power calculations:

http://www.statisticalsolutions.net/pssZtest_calc.php
3 The Effects of Prenatal Stress on Feeding Behaviour and Food Choice.

3.1 Chapter Background

Stress during pregnancy is a common occurrence and has been shown to cause many programming effects to the offspring in both rodent models and humans (reviewed in: Weinstock, 2008). One of the most well characterised effects of prenatal stress (PNS) is an impairment of HPA axis activity, generally resulting in enhanced and/or prolonged corticosterone secretion in response to an acute stressor (Henry et al., 1994, Koenig et al., 2005, Brunton and Russell, 2010). As there is much evidence suggesting that elevated glucocorticoids due to stress can lead to changes in food intake and choice (see section 1.3.2), it could be speculated that the hyperactivity of the stress axis observed in prenatally stressed offspring could result in altered feeding behaviour. Therefore, the aim of the experiments carried out in this chapter was to use a rat model of prenatal stress to establish the effects of PNS on feeding behaviour and food choice.

There is already some evidence suggesting that PNS can lead to changes in certain metabolic parameters. Prenatal stress (or glucocorticoid exposure during pregnancy) is associated with development of type 2 diabetes mellitus, hyperglycaemia, and insulin resistance (Lindsay et al., 1996, Nyirenda et al., 1998, Lesage et al., 2004, Balasubramanian et al., 2015). Some studies have also suggested that prenatal stress can lead to an increase in body weight. Tamashiro et al. (2009) found that when PNS pups were weaned onto a high-fat diet, both male and female offspring illustrated an increased body weight, which was associated with heavier subcutaneous and retroperitoneal fat pads, as well as increased plasma leptin. While in this study these differences were not apparent when rats were weaned onto a chow diet, Schulz et al. (2011) found an increase in body weight in PNS male rats at 12, 24 and 40 weeks of age on a standard chow diet, although there were no differences in the body weight of control and PNS females.
Paternain et al. (2013) illustrated that when rats were placed onto a high-fat high-sugar (HFHS) diet at 8 weeks of age, PNS rats gained more body fat mass than control rats, although this was statistically significant only in females. Interestingly, this increase in fat mass was not mirrored by a significant increase in body weight. However, both male and female rats that had experienced prenatal stress had a greater food intake than the control groups on both bland chow and HFHS diets. Lesage et al. (2004) also found changes in food consumption, illustrating that in aged rats (24 months) prenatal stress resulted in an increased food intake after a period of fasting, although no effects of PNS on body weight were observed. Interestingly, studies in mice have found that whilst PNS mice demonstrate hyperphagia on both a bland and HFHS diet, they illustrate reduced body weights compared to control male mice on both bland and HF diets (Pankevich et al., 2009).

Whilst these studies do suggest that prenatal stress could lead to an obesity predisposition, evidence is lacking as to whether any increase in weight gain observed in PNS offspring can be attributed to an increased calorie intake in comparison to controls. Furthermore, these studies have mainly been carried out in aged rats, and therefore there is little evidence for body weight differences, or differences in food intake in younger rats. Finally, these studies give no indication on whether there are any effects of prenatal stress on food choice.

As there is some evidence that stress can lead to an increased palatable food consumption in rats which may then be able to blunt the elevated stress response (Pecoraro et al., 2004, Maniam and Morris, 2010), the following two hypotheses were tested:

1 – Prenatal stress will lead to an increased calorie intake in comparison to control rats when offered a palatable diet.

2 – Prenatal stress will lead to an increased preference for more energy dense food.

As a secondary outcome it was also hypothesised that prenatal stress would lead to changes in body weight, specifically that PNS rats, particularly those on a HFHS diet, would show an increase in body weight gain in comparison to controls.
3.2 Experiment 1: Prenatal Restraint Stress

3.2.1 Background

For the following experiment the aim was to establish whether prenatal stress could lead to changes in food intake for rats on a standard chow diet or on a palatable HFHS diet. Furthermore, it was examined whether prenatal stress could lead to alterations in food choice when given an ad lib free choice between a standard chow, a HFHS pellet, and a sucrose solution. For this experiment, PNS offspring were generated using a model of prenatal restraint stress, and shortly after weaning rats were placed onto one of three diets. Rats body weight was measured once a week and weekly food intake was also calculated.

Prenatal restraint stress is a well-established model, although models of prenatal restraint have varied greatly in terms of the duration of the restraint stress, the number of restraint stress sessions per day, and the number of days of application of the stressor (reviewed in Weinstock, 2017). Using restraint as a stressor as opposed to other methods has its advantages as its duration can be easily and accurately controlled, and it is not susceptible to variations in rodent behaviour during the stressor. The model of restraint stress used in this experiment consisted of delivering 3, 30-minute restraint stressors per day on days 16 – 20 of gestation. In order to prevent adaptation to repeated use of the same stressor, the timings of the restraint sessions were altered each day as this has been shown to minimise stressor adaptation (Weinstock et al., 1988).

As rats are social animals, single housing can act as a stressor (Weintraub et al., 2010). Therefore, to prevent the stress effects of single housing confounding the results, all offspring were group housed and the cage taken as the experimental unit.

Many prenatal stress studies are carried out solely on male rats. As there have been many reported sex differences in PNS offspring, including differences in certain metabolic and body weight parameters (Brunton et al., 2013, Paternain et al., 2013, Schulz et al., 2011) both male and female offspring were tested in this study.
3.2.2 Methods

3.2.2.1 Animals

38 nulliparous female Sprague-Dawley rats (bred in house) were mated with a sexually-experienced male. The day at which a plug was found was designated day 1 of pregnancy. Rats were housed under standard conditions (see section 2.1.1). Rats were maintained on a standard ad lib breeding diet (RM3) and water throughout pregnancy and weaning. Rats were singly housed from day 14/15 of pregnancy onwards. Body weight and food and water consumption were measured in pregnant rats from day 16 to term and thereafter on postnatal day 14. Females were randomly assigned to a PNS (n=17) or control (non-stressed) group (n=20).

3.2.2.2 Prenatal Restraint Stress Procedure

On days 16-20 of pregnancy, 17 of the pregnant rats (PNS group) were subjected to 3 bouts of 30-minute restraint stress. The stress was carried out using a clear Plexiglas tube (63 mm) with breathing holes and adjustable length. The times of the stressor were changed each day in order to prevent habituation, however they always took place in the light period, between 7am and 7pm in the rats housing room. Control dams were housed under the same conditions as stressed dams and remained undisturbed in their cages, apart from body weight and food intake measurements. Pups were weighed and sexed on P1/2 and then further body weight measurements were taken at P7, P14 and at weaning (P21). No effort was made to record maternal behaviour during the pre-weaning period. One control dam rejected her litter and was therefore removed from the experiment. On weaning, male rats were housed in groups of 3 and females were pair-housed with same sex litter mates and each cage was taken as an experimental unit.

3.2.2.3 Experimental Groups

Cages were randomly assigned to one of three diets. A ‘bland diet’ consisting of standard lab chow (RM1) and water; a ‘palatable diet’ consisting of a high-fat high-sugar (HFHS) food pellet (RM AFE 45%FAT 16%SUC) and a 10% sucrose solution; or a ‘choice diet’ where rats were offered a free choice of the bland chow, HFHS pellet, water and sucrose solution. This resulted in 12 groups in total (Table 3.1). Only one cage per litter was assigned to each group in order to prevent intra-litter effects confounding the results.
Table 3.1: N Numbers for Each of the 12 Experimental Groups

<table>
<thead>
<tr>
<th>DRAIN</th>
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<td>n = 8</td>
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Pups were maintained on ad lib standard RM1 chow and water for 2 weeks after weaning prior to being placed on the experimental diets. Following placement onto their diets, weekly measurements were made for body weight and food and fluid consumption for 7 weeks. These were taken as whole cage measurements (i.e. all rats in the cage were weighed simultaneously) and then averaged per rat for analysis.

3 cages were removed from the analysis – one male was culled due to evidence of malocclusion (PNS bland), one female died unexpectedly during the experiment (PNS Bland) and a further cage was removed due to incorrect sexing of pups at weaning (Control Palatable).

Diet group abbreviations: Control bland (Control-B), PNS bland (PNS-B), Control palatable (Control-P), PNS palatable (PNS-P), Control choice (Control-C), PNS choice (PNS-C).

3.2.2.4 Blood Sampling

On week 7 of diet, blood samples were taken from one rat in each cage before, during and after an acute 30-minute restraint stress (sampled at -30min, -1min, +15min, +30min and +240min with t=0 being the onset of the restraint stress) via tail nick. Samples were processed to collect plasma and stored at -80°C until measurement of corticosterone (see Section 2.3 for full procedural details).

Plasma corticosterone concentrations were measured in three separate radioimmunoassays (RIAs, MP biomedical) from the samples taken before, during and after the restraint stressor. All samples were assayed in duplicate. Intra-assay variability was under 4% for all three RIA’s and inter-assay variability was 2%.

At the end of the experiment animals were euthanized using an appropriate schedule one method.
3.2.2.5 Data Analysis

Statistical analyses were carried out using GraphPad Prism 6 and Minitab 18. All figures were plotted using GraphPad Prism 6 and data are presented as mean ± SEM. Data for the whole cage was divided by the number of rats per cage to obtain average data values per rat. Weekly food intake values were divided by 7 to give an average value per day. Data were tested for normality using the Shapiro-Wilk test. Normally distributed data were analysed using repeated measures, 2-way analysis of variance (ANOVA), with Sidak’s post hoc test; or unpaired student t test. For multiple t tests the Holm-Sidak method was used to correct for multiple comparisons and determine statistical significance. For data not fitting a normal distribution, Mann Whitney U tests were used. In cases where data presented over time were not normally distributed, area under the curve (AUC) analysis was employed and then tested using parametric or non-parametric tests as appropriate.

Data for Kcal intake was analysed by calculating the area under the curve for total daily Kcal consumption (averaged per rat) and then divided by 6 (total number of weeks on diet– 1) to give an average value for daily Kcal consumption.

For food choice analysis, daily Kcal data (averaged per rat) for each of the three foods available to rats on the choice diet was analysed using AUC analysis, and then divided to give an average value per day for each food.

Sucrose preference data was analysed by calculating weekly sucrose preference (see section 2.2.4.1) and then analysing area under the curve and dividing to give an average sucrose preference.
3.2.3 Results

3.2.3.1 Reproductive Parameters

Figure 3.1 illustrates dam parameters during pregnancy. There were no significant differences in body weight between control and stressed dams from days 16-20 (Figure 3.1A, 2-way RM ANOVA, F(1, 21) = 2.848, p = 0.106). Similarly there were no significant differences between control and stressed dams in percentage body weight increase from day 16-20 (Figure 3.1B, 2-way RM ANOVA, F(1, 21) = 0.310, p = 0.584).

No significant differences were observed between control and stressed dams in total Kcal consumption from days 17-21 (Figure 3.1C, 2-way RM ANOVA, F(1, 21) = 0.078, p = 0.783),

There were also no significant differences in water intake between the control or stressed dams (Figure 3.1D, 2-way RM ANOVA, F(1, 21) = 0.002, p = 0.963).

Average gestation period for stressed dams was 21.7 ± 0.143 days and 22.2 ± 0.202 days for control dams. There were no significant differences for gestation time between groups (data not shown, Mann Whitney U test; U = 104.0, p = 0.118).

Figure 3.2 shows litter data for the control and PNS offspring. There were no significant differences in total number of pups born to control and PNS litters (Figure 3.2A, t(33) = 1.878, p = 0.069). Figure 3.2B shows the average number of male and female pups per litter. A 2-way ANOVA, whilst showing no significant effect of prenatal stress (F(1, 66) = 3.932, p = 0.0515), illustrated a significant effect of sex (F(1, 66) = 14.56, p = 0.0003) with fewer female pups being born to PNS litters. Body weight was not significantly different between groups on postnatal day 1 for either males (Figure 3.2C; multiple t tests; t(23) = 0.855, p = 0.076) or females (Figure 3.2C; t(22) = 1.571, p = 0.131). Body weight at weaning (postnatal day 21, Figure 3.2D) did not differ significantly between control and PNS males (multiple t tests; t(26) = 0.268, p = 0.791) or females (t(26) = 0.163, p = 0.872).

There were also no significant differences in body weight observed on postnatal days 7 and 14 between control and PNS males (data not shown, multiple t tests with Holm Sidak method to determine statistical significance; P7: t(31) = 0.522, p = 0.606, P14: t(33) = 2.082, p = 0.045) or females (P7: t(28) = 0.370, p = 0.714, P14: t(32) = 1.650, p = 0.109).
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**Figure 3.1: Dam Gestation Parameters**

All statistical tests performed were 2-way RM ANOVAs.

**A:** Dam absolute body weight from days 16 – 20 of gestation. No significant differences were observed between stressed (n = 17) and control dams (n = 6, 14 rats were unable to be analysed as data collection was incomplete due to administrative issues) (p = 0.106).

**B:** Dam percentage body weight increase from gestation day 16 to day 20. No significant differences were observed between the two groups (p = 0.584; n: control = 6, stressed = 17).

**C:** Dams total Kcal consumption from days 17-21 of gestation. No significant differences were observed between total Kcal consumption between control (n=6) and stressed (n=17) dams (p = 0.783).

**D:** Water consumption for stressed and control dams from days 17 – 21 of gestation. No differences were observed between the two groups (p = 0.963, n: control = 11, stressed = 12).
Figure 3.2 - Reproductive Parameters for Pups from Stressed (PNS) and Control Litters

A: Total number of pups born to control (n = 19) and stressed (PNS, n = 16) litters. No significant differences were observed between the two groups (unpaired t test: p = 0.069).

B: Number of male and female pups per litter. 2-way ANOVA analysis showed a significant difference of sex (p = 0.0003) and post hoc analysis illustrated that this difference was between the PNS females and all other groups (control males and females n = 19; PNS males and females n = 16). C: Pup body weights on postnatal day 1. Multiple t tests found no significant differences between control and PNS groups for either males (p = 0.076) or females (p = 0.131) (males n: control = 10, PNS = 15; females n: control = 10, PNS = 14). D: Pup body weight at weaning (P21). No significant differences were observed between control and PNS rats in males (multiple t tests, p = 0.791) or females (p = 0.872, n = 14 for all groups).
3.2.3.2 Total Kcal Intake

Figure 3.3 shows AUC analysis for average daily Kcal intake for rats on the bland, palatable and choice diets. There were no significant differences in total Kcal consumption per week between control and PNS male (Figure 3.3A, t(13) = 0.330, p = 0.747) or female rats on the bland diet (Figure 3.3B, t(13) = 0.235, p = 0.818). There were also no significant differences in total Kcal intake for male (Figure 3.3C, t(14) = 0.810, p = 0.432) or female rats (Figure 3.3D, t(13) = 0.297, p = 0.772) on the palatable diet. Finally, no significant differences were observed between PNS and control rats for males (Figure 3.3E, t(14) = 1.060, p = 0.307) or females (Figure 3.3F, U = 27, p = 0.629) for daily Kcal intake on the choice diet.

A 3-way ANOVA was performed on the AUC data from Figure 3.3 with sex, diet and stress condition as the factors. A significant effect of diet was observed (F (2, 72) = 31.24, p < 0.0001) and post hoc testing illustrated that rats on both the palatable and choice diets consumed more Kcal than those on the bland diet. A significant effect of sex was also observed (F (1, 72) = 154.2, p < 0.0001) with post hoc testing showing that male rats consumed more Kcal than female rats. No significant main effect of stress condition was found (F (1, 72) = 2.009, p = 0.166).

3.2.3.3 Food Choice

Figure 3.4 shows AUC data for average daily total Kcal intake for the three different calorie sources available to rats on the choice diet over the 7-week experiment. For males on the choice diet (Figure 3.4A), multiple t tests showed no differences between PNS and control groups for Kcal intake from the bland chow (t(14) = 0.099, p = 0.922), the HFHS diet (t(14) = 0.532, p = 0.603) or the sucrose solution (t(14) = 0.529, p = 0.605).

Similarly, female rats on the choice diet (Figure 3.4C) also showed no significant differences in Kcal consumption between control and PNS groups for the chow (t(14) = 0.618, p =0.547), the HFHS pellet (t(14) = 0.559, p =0.584), or the sucrose solution (t(14) = 1.187, p = 0.255).

There were also no differences observed in average sucrose preference for male rats on the choice diet (Figure 3.4B, t(14) = 0.154, p = 0.880) or female rats on the choice diet (Figure 3.4D, U = 26, p = 0.555).
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Figure 3.3 - Total Kcal Intake for Male and Female Rats on the Bland, Palatable and Choice Diets

Statistical tests are unpaired t tests unless otherwise stated. For n numbers see Table 3.1.

A: AUC analysis of average Kcal per day for male rats on the bland diet. No significant differences were observed between control and PNS offspring (p = 0.747).
B: AUC analysis for female rats on the bland diet. Again, there were no significant differences between control and PNS groups (p = 0.818).
C: AUC Kcal per day for male rats on the palatable diet. There were no significant differences in Kcal intake between control and PNS offspring (p = 0.432).
D: AUC Kcal per day for female rats on the palatable diet. There were no significant differences between control or PNS groups (p = 0.772).
E: AUC Kcal intake for male control and PNS rats on the choice diet. No significant differences were observed between groups (p = 0.307).
F: AUC total Kcal intake for female rats on the choice diet. Mann Whitney U analysis illustrated no significant differences between control or PNS groups (p = 0.629).
FIGURE 3.4: Kcal Consumption and Sucrose Preference for Male and Female Rats on the Choice Diet

N = 8 for all groups.

A: AUC average daily Kcal intake for control and PNS male rats on the choice diet. Multiple t tests showed no significant differences for calorie intake between control and PNS groups for chow (p = 0.922), HFHS diet (p = 0.603) or sucrose solution (p = 0.605).

B: AUC analysis of sucrose preference for male control and PNS rats on the choice diet. There were no significant differences in sucrose preference between control or PNS offspring (unpaired t test, p = 0.880).

C: AUC average total daily Kcal intake for female rats on the choice diet. Multiple t tests illustrated no significant differences between control and PNS groups for the chow (p = 0.547), HFHS pellet (p = 0.584) or the sucrose (p = 0.255).

D: AUC average of sucrose preference for females on the choice diet. There were no significant differences between control or PNS groups (Mann Whitney U test, p = 0.555).
3.2.3.4 Body Weight

3.2.3.4.1 Males

Figure 3.5 illustrates body weight data for male rats over the 7 weeks of the experiment. Figure 3.5A shows body weight per rat for male control and PNS rats on the bland diet. Over the 7 week period, 2-way repeated measures ANOVA analysis showed no significant differences between control or PNS offspring (F(1, 13) = 0.509, p = 0.488). There were also no significant differences between male control or PNS rats on the palatable diet (Figure 3.5B, 2-way RM ANOVA; F(1, 14) = 1.372, p = 0.261). Male rats on the choice diet also showed no significant differences in body weight for control and PNS rats (Figure 3.5C, 2-way RM ANOVA; F(1, 14) = 0.295, p = 0.595).

Figure 3.5D shows AUC data (averaged per week) for male control and PNS rats in the three different diet groups. Two-way ANOVA analysis illustrated no significant differences between control or PNS groups (F(1, 41) = 0.588, p = 0.448), although a significant effect of diet was observed (F(2, 41) = 15.45, p < 0.0001). Post hoc analysis showed that PNS palatable, control choice and PNS choice groups were all significantly different compared to the control bland group.

3.2.3.4.2 Females

Figure 3.6 illustrates body weight data for female control and PNS rats over the course of the experiment. Panel A shows bodyweight data for female control and PNS rats on the bland diet. No significant differences were observed between groups (2-way RM ANOVA; F(1, 13) = 1.159, p = 0.301). There were also no significant differences in body weight between control and PNS female rats on the palatable diet (Figure 3.6B; 2-way RM ANOVA; F(1, 13) = 0.089, p = 0.770). For female rats on the choice diet there were also no significant differences between control or PNS offspring (Figure 3.6C; 2-way, RM ANOVA; F(1, 14) = 0.052, p = 0.823).

2-way ANOVA analysis of averaged AUC body weight data between female rats in all three diet groups showed no effect of PNS (Figure 3.6D, F(1, 40) = 0.099, p = 0.755) although as with males there was a significant effect of diet (F(2, 40) = 7.623, p = 0.002). Post hoc testing found this difference to lie between PNS rats on the bland diet, and PNS rats on the choice diet.
A 3-way ANOVA on the AUC data from Figure 3.5D and Figure 3.6D with the factors as stress condition, sex and diet, found no significant effect of stress condition (F (1, 72) = 0.019, p = 0.888) although a significant effect of sex (F (1, 72) = 402.5, p < 0.0001) and diet (F (2, 72) = 19.37, p < 0.0001) was found, with females having lower body weights than males.

### 3.2.3.5 Plasma Corticosterone

Figure 3.7 illustrates plasma corticosterone data for male and female rats on the bland and palatable diets before, during and after an acute 30-minute restraint stressor. There were no significant differences in corticosterone responses between control and PNS male rats on the bland diet (Figure 3.7A; 2-way RM ANOVA; F(1,10) = 0.07, p = 0.791). For males on the palatable diet there appears to be an increase in plasma corticosterone in the PNS group at t(-1), however 2-way RM ANOVA analysis found no significant differences between groups (Figure 3.7B; F(1, 9) = 3.238, p = 0.106).

For female rats on the bland diet, there were no significant differences observed between control and PNS offspring (Figure 3.7C; 2-way, RM ANOVA, F(1, 9) = 0.1, p = 0.758). Similarly for female rats on the palatable diet there were no significant differences between control and PNS groups (Figure 3.7D; 2-way, RM ANOVA; F(1, 9) = 0.53, p = 0.485).

These results show that the rats in this experiment did not illustrate the expected hyperactivity of the stress axis usually observed in PNS offspring. This may suggest that the protocol implemented in this experiment was insufficient to programme the offspring.

When peak corticosterone values (t(30)) for male and female control rats on the bland diet were compared, unpaired t test analysis showed a significant difference between males and females (t(8) = 4.437, p = 0.002), with females illustrating higher peak corticosterone than males.
Figure 3.5: Body Weight Data for Male Control and PNS Rats on the Bland, Palatable and Choice Diets

For n numbers see Table 3.1. All statistical tests were 2-way repeated measures ANOVA unless otherwise stated.

A: Body weight data for male rats on the bland diet. There were no significant differences between control and PNS rats over the 7-week testing period (p = 0.488). 

B: Body weight data for male rats on the palatable diet. There were no significant differences between control and PNS rats over the 7 weeks of testing (p = 0.261). 

C: Body weight data for male rats on the choice diet. Over the 7-week period, no significant differences between the control or PNS offspring were observed (p = 0.595). 

D: AUC average body weight for male control and PNS rats on all three diets. 2-way ANOVA analysis illustrated no significant differences between control or PNS groups on each diet (p = 0.448) however a difference of diet was observed (p < 0.0001). Post hoc testing found that the PNS palatable as well as PNS and control choice groups were all significantly different from the control bland group (* indicates significance compared to control bland group).
FIGURE 3.6: BODY WEIGHT DATA FOR FEMALE CONTROL AND PNS RATS ON THE BLAND, PALATABLE AND CHOICE DIETS

For n numbers see TABLE 3.1. All statistical tests were 2-way repeated measures ANOVA unless otherwise stated.

A: Body weight data for female rats on the bland diet. There were no significant differences between control and PNS rats over the 7-week testing period (p = 0.301).

B: Body weight data for female rats on the palatable diet. There were no significant differences between control and PNS rats over the 7 weeks of testing (p = 0.770).

C: Body weight data for female rats on the choice diet. Over the 7-week period, no significant differences between the control or PNS offspring were observed (p = 0.823).

D: AUC average body weight for female control and PNS rats on all three diets. 2-way ANOVA analysis illustrated no significant differences between control or PNS groups on each diet (p = 0.755) but a difference of diet was observed (p = 0.002) and this difference was found to be between PNS-B and PNS-C rats.
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Figure 3.7: Corticosterone Profiles During an Acute Restraint Stressor (Shaded Area) for Male and Female Rats on the Bland and Palatable Diets

All statistical tests are 2-way repeated measures ANOVA

A: Plasma corticosterone concentration for control and PNS male rats on the bland diet during an acute 30-minute restraint stressor. No significant differences were observed between groups (p = 0.791, n = 6 for both groups; 4 missing data points). B: Plasma corticosterone profile for control and PNS rats on the palatable diet. There was no significant effect of group on plasma corticosterone concentration (p = 0.106, n: control = 6, PNS = 5). C: Plasma corticosterone concentrations for female rats on the bland diet. No significant differences were observed between control and PNS offspring (p = 0.758, n: control = 6, PNS = 5; 6 missing data points). D: Plasma corticosterone during an acute stressor for female rats on the palatable diet. No significant differences were observed between control and PNS offspring (p = 0.485, n: control = 5, PNS = 6; 6 missing data points).
3.2.4 Discussion

3.2.4.1 Reproductive Parameters

In this study, no divergence in body weight between control and stressed dams over the 5 days of restraint stress were observed. A reduction in percentage body weight gain may be expected as stress, particularly repeated restraint stress, has been associated with a reduction in food intake (Harris, 2015). However, lower body weight gain in stressed dams, whilst illustrated by some prenatal restraint stress studies (Govindaraj et al., 2017, Palacios-Garcia et al., 2015, Van den Hove et al., 2014), is not always present (Van den Hove et al., 2005, Zohar et al., 2015, Abe et al., 2007), and in those cases in which it does occur, it appears dependent on the duration of the stressor (reviewed in: Weinstock, 2017). During pregnancy hypothalamic systems regulating appetite are altered to drive increased energy intake and storage for the high metabolic demands of pregnancy and lactation, for example both humans and rats experience a pregnancy-induced leptin resistance to facilitate food intake (Ladyman et al., 2010). It may be that the stressor applied during the PNS protocol in this study was not sufficient to overcome these changes, therefore resulting in no body weight changes.

Whilst no differences were observed between control and stressed dams in the total number of pups per litter, a lower number of female pups born to stressed litters was observed. Generally, in prenatal stress studies there are few alterations in the number of male and female pups per litter, or male to female ratio (Tamashiro et al., 2009, Zohar et al., 2016). Pregnant hamsters exposed to social subordination in early pregnancy produce smaller, female biased litters, an effect which appears to be mediated by progesterone (Pratt and Lisk, 1991). In PNS rats when differences are observed in litter ratio, litters are generally female biased, unlike the present study (Sobrian et al., 1997, Lane and Hyde, 1973). The result seen in this study is unusual and it could be that alterations in male to female ratio may be due to selective reabsorption of pups in stressed dams, or due to selective infanticide shortly after birth.

As with maternal body weight, the birth weight of prenatally stressed pups can vary between models. Many illustrate a reduced birth weight in both male and female offspring (Van den Hove et al., 2014, Wilson and Terry, 2013), whilst in some studies no differences in birth weight are observed (Zohar et al., 2016, Sickmann et al., 2015). In some cases, mainly models using prenatal social stress, a lower birth weight is observed only in female
offspring (Brunton and Russell, 2010, Brunton et al., 2013). However, in those cases in which a lower birth weight is observed, fast catch up growth generally leads to body weight normalisation before weaning. Interestingly, Tamashiro et al (2009) found an increase in birth weight in both male and female PNS pups. In this study no birth weight differences were observed between PNS and control offspring. Considering the large variability in birth weight in studies of prenatal stress, this data provides little information on the success of the restraint model implemented, although it does suggest that the pups did not experience intrauterine growth restriction.

3.2.4.2 Total Kcal Consumption

In this study no significant differences in total daily Kcal consumption between control and PNS rats for any of the three diets were observed, for both male and female offspring. This suggests that there is no effect of prenatal stress on total calorie consumption when rats are placed on a standard laboratory diet (bland diet) or diets consisting of high-fat high-sugar foods (palatable and choice diet).

Rats on the palatable and choice diets consumed a higher number of Kcal per day in comparison to rats on the bland diet, however this was expected as when Sprague Dawley rats have access to a diet high in fat, they tend to consume a higher number of calories in comparison to rats on a standard chow diet (Marques et al., 2016). Increased food intake was also expected on the choice diet as there is evidence that when rats are offered a choice of foods, food consumption will increase (Rolls et al., 1983), and particularly diets offering sucrose in solution have been shown to dramatically increase calorie intake (Sclafani, 1989).

Females consumed a lower number of total Kcal in comparison to males, however this difference is also expected due to females’ lower body mass (NRC, 1995).

3.2.4.3 Food Choice

In terms of food choice, there were no significant differences observed in the daily Kcal consumption from the three different calorie sources available to rats on the choice diet (bland chow, HFHS pellet and sucrose solution). This suggests that there are no effects of prenatal stress on food choice in the rat.
There is some evidence that PNS rats show increased depressive-like behaviour through the forced swim test (Morley-Fletcher et al., 2003, Sickmann et al., 2015) and the sucrose preference test (Sun et al., 2013, Głombik et al., 2015). In this experiment no differences were observed in sucrose preference for male or female rats on the choice diet. However, the sucrose preference paradigm used in this experiment differs from those normally implemented in that it was calculated from the total weekly consumption of sucrose and water, rather than a shorter period. Furthermore, the concentration of the sucrose solution provided (10%) was higher than the standard concentration used in sucrose preference tests (Mateus-Pinheiro et al., 2014) which may have influenced consumption.

3.2.4.4 Body Weight

There were no significant differences in average body weight per rat between control and PNS offspring on either the bland, palatable or choice diets for either male or female rats. Previous studies have illustrated an increase in body weight or body fat in prenatally stressed rats, on both bland (Schulz et al., 2011) and HFHS (Tamashiro et al., 2009, Paternain et al., 2013) diets. However, in the case of some of these studies, the rats tested were considerably older than the rats used in this study. It could therefore be the case that the effects of prenatal stress on body weight are only apparent in aged rats.

3.2.4.5 Plasma Corticosterone

Corticosterone sampling at the end of the study illustrated that this cohort of rats did not display the expected stress hyperactivity generally observed in PNS offspring. This experiment involved imposing a 30-minute stressor, 3 times daily, from gestation days 16-21. However, some previous studies using prenatal restraint stress have used longer periods of stress (45 minutes), often starting earlier in pregnancy at gestation days 11 or 14 (reviewed in Weinstock, 2017). This suggests that the prenatal restraint paradigm used in this study may not have been robust enough to produce programmed prenatally stressed offspring.

There is also the possibility that the dams may have habituated to the restraint stress procedure. Habituation to restraint stress, and therefore a reduction in plasma corticosterone in response to the stressor, has been observed in several studies (Girotti et al., 2006, Barnum et al., 2007). Although in this study the timings of the stressor were altered each day in an attempt to minimise habituation, this may not have been sufficient...
to prevent adaptation to the restraint stressor procedure. Plasma corticosterone was not measured in the pregnant dams during the stressor, however previous studies have shown an increase in plasma corticosterone in dams subjected to both restraint (Williams et al., 1999) and a social stressor (Brunton and Russell, 2010), although the stress response of pregnant dams is reduced in comparison to non-pregnant females in order to minimise glucocorticoid exposure to the foetus (Brunton et al., 2008).

Another factor involved could be the blood sampling technique used in this study. In order to collect blood samples, rats were gently restrained in a towel whilst blood was taken via a tail nick. As an increase in plasma corticosterone can be observed for all rats between both baseline samples (t(-30) to t(-1)) it is clear that the blood sampling procedure itself caused some stress. This could have led to a maximal stress response being reached which can mask the elevated and prolonged corticosterone response generally observed in PNS offspring (Weinstock, 2008). The time of day at which the samples were taken did not appear to have an impact on the results, however it may be that this was masked by the stress of the blood sampling procedure.

There was an unexpected increase in plasma corticosterone for the male PNS palatable group at the second baseline sample timepoint (t(-1)), although this did not reach statistical significance. Whilst all groups illustrated an increase from the first to the second baseline sample, this increase was much higher in the male PNS palatable group which may suggest an increased sensitivity to stress in this group. However it is surprising that this increased response should only appear in male rats on the palatable diet as firstly, female PNS rats tend to more clearly illustrate the expected stress hyperactivity (Weinstock et al., 1992, McCormick et al., 1995) and secondly, it may be expected that the palatable diet could ameliorate the elevated stress response due to the reported effects of palatable food consumption blunting the corticosterone response (Pecoraro et al., 2004). Therefore, it is hard to conclude whether this result is due to the prenatal stress protocol, or whether it could be due to other factors such as the diet or blood sampling protocol.

There was a significant difference observed between the corticosterone responses of male and female rats, with females illustrating an enhanced plasma corticosterone response in comparison to males. In rodents it is well established that females illustrate higher corticosterone levels under both basal and stressed conditions (Aoki et al., 2010, Kant et al., 1983, Rivier, 1993). These sex differences can be attributed (in part) to sex steroids: in
females corticosterone secretion in response to stress is stimulated by ovarian estradiol whereas in males it is inhibited by testosterone (Kalil et al., 2013). When exposed to foot shock, females illustrated higher ACTH responses compared to males, however this difference was removed by ovariectomy (Rivier, 1999). Sex differences in corticosterone responses may be mediated by type of stressor as male and female had comparable corticosterone responses when exposed to a foot shock, but females illustrated higher corticosterone responses than males when exposed to a physical stressor (Rivier, 1999). However, there is evidence to suggest that ultradian and circadian rhythms of free corticosterone (the biologically active fraction of corticosterone) are similar in both male and female rats. Furthermore, peak levels of free corticosterone in male and female rats are similar after both a mild psychological stress (exposure to a novel environment) and forced swimming, although the free corticosterone response to forced swimming was significantly faster in female rats (Droste et al., 2009).

3.2.4.6 Experimental Limitations

3.2.4.6.1 Prenatal Restraint Stress Model

Restraint is the most commonly used form of stressor in rodent studies (Buynitsky and Mostofsky, 2009). While there are many advantages associated with restraint, such as the fact that it can be easily applied without causing any physical injuries to the subject and that the timing can be precisely controlled, there are also several limitations associated with its use as a stressor. Firstly, although psychological stress is common in society, restraint is not a particularly ecologically valid model for this. However, the most notable disadvantage to restraint is its susceptibility to adaption. In models using prenatal restraint stress, adaption to a stressor (generally measured by maternal corticosterone) is rarely reported or measured. For those studies that do measure maternal corticosterone during prenatal stress, generally a reduction in corticosterone response is observed with each successive stressor if applied at the same time each day (reviewed in: Weinstock, 2017). This may therefore suggest that prenatal restraint stress models are not the most robust in terms of generating prenatally stressed offspring.

Studies using restraint stress on pregnant dams to produce PNS offspring vary both in application of the stressor, and physiological and behavioural outcomes in the offspring. These models range from a daily application of a 20 minute restraint stressor to a 60
minute stressor applied three times a day (reviewed in: Weinstock, 2008). As there are such large variations in the models of prenatal restraint stress used, it can be difficult to directly establish the characteristics expected of prenatally stressed offspring, and which alterations are specifically due to the restraint stress rather than other parameters. This can be a particular problem in studies where prenatal restraint stress is associated with a reduction in body weight gain in dams, as this can contribute to intrauterine growth restriction which can have dramatic impacts on offspring development (Salam et al., 2014).
3.3 Experiment 2: Prenatal Social Stress

3.3.1 Background

Following issues in generating prenatally stressed offspring using a restraint stress paradigm, the study was repeated using a prenatal social stress model. Whilst using a social stressor can be more variable than with restraint stress due to the fact it relies on animal behaviour, this approach has been shown to be more successful at preventing habituation (Brunton and Russell, 2010). In addition this model of prenatal stress is more ecologically valid as social stress is the most common form of stressor experienced by pregnant women (Björkqvist, 2001).

In this study the number of experimental groups was reduced, eliminating the palatable diet group and only testing rats on a bland and choice diet. This was in order to increase the power of the experiment and prevent false positives from multiple comparisons. Whilst a larger effect of the stress hyperactivity is generally observed in female PNS offspring (Weinstock et al., 1992, McCormick et al., 1995), many of the behavioural and physiological changes due to prenatal stress are generally more pronounced in males (Brunton, 2013). Therefore, in this study only male offspring were tested.

In the choice diet the sucrose concentration was reduced from 10% to 5%. As in the previous study rats were consuming a large amount of their calories (around a third) from the sucrose solution it may be that this was masking any differences in food choice, therefore it was hoped that by reducing the sucrose concentration this would be prevented. Furthermore, this concentration of sucrose is more in line with concentrations often implemented in sucrose preference tests (Mateus-Pinheiro et al., 2014).

As exposing the rats on the diet experiment to an acute restraint stress when measuring corticosterone levels could lead to changes in food intake, a separate group of control and PNS male rats were group housed under standard laboratory conditions in order to allow for blood sampling prior to the end of the diet experiment. This not only meant that corticosterone measurements could be carried out earlier in the experiment in order to establish whether the PNS rats illustrated changes to their corticosterone profiles, but also ensured that the rats on the diet experiment were not exposed to a stressor which could affect their feeding behaviour. Furthermore, as the enhanced corticosterone responses often observed in PNS offspring are generally more pronounced in females than males.
a subset of control and PNS female rats were also group housed under the same conditions, allowing for comparison of male and female stress responses. Some studies of PNS rats employ single housing which can act as a stressor (Weintraub et al., 2010) and could therefore conceivably enhance the elevated stress response observed in PNS offspring. Therefore, a further group of rats was added to the study consisting of individually housed PNS males in order to examine the effects of single vs group housing on corticosterone responses during an acute stressor. As it appeared that the blood sampling method used during the acute restraint stressor caused some stress effects itself, the rats were habituated to being gently restrained in a towel every day for a week prior to the blood sampling procedure, in an effort to reduce stress associated with the blood sampling procedure.
3.3.2 Methods

3.3.2.1 Animals

45 nulliparous female Sprague-Dawley rats (bred in house) were mated and the day at which a plug was found was designated day 1 of pregnancy. Rats were maintained under standard housing conditions (see section: 2.1.1). Rats were maintained on an ad lib RM3 breeding diet and water throughout pregnancy and lactation. Rats were singly housed from day 14 of pregnancy onwards. Body weight was measured on day 1, 8 and 16-20 of pregnancy. Females were assigned to one of three groups: residents, intruders (PNS) and control. All procedures were carried out in the light phase between 9:00 and 13:00.

3.3.2.2 Prenatal Social Stress Procedure

In order to generate prenatally stressed offspring, 14 of the pregnant females (intruder group) were subjected to a social defeat stressor from days 16-20 of pregnancy. They were placed into a cage with an unfamiliar lactating ‘resident’ female (at days 2-8 of lactation) for 10 minutes a day, with each intruder interacting with a different lactating resident each day. Behaviour was video recorded and aggression scored using the following scale (based on that used by Brunton and Russell, 2010): punching = 1; single bite = 2; bite with pin down = 2; prolonged biting = 3; tumble attack and pin down = 3. Residents and intruders were housed in separate rooms and the social defeat sessions took place in the residents housing room.

Control dams remained undisturbed throughout pregnancy and weaning, other than food/water consumption and body weight measurements on postnatal days 7, 14 and 21. Shortly after birth (P3-4) the number of pups per litter was standardised to 9-10 in order to prevent litter size effects impacting on food intake or body weight (Chahoud and Paumgartten, 2009). Pups were weaned on postnatal day 21 and assigned to an experimental group, ensuring that no more than 1 cage per litter was in each group to prevent intra-litter effects confounding the results.

A subset of rats for blood sampling for corticosterone measurements were housed under standard conditions whilst the remaining rats were used in the ‘diet experiment’.
3.3.2.3 Corticosterone Sampling Groups

Male control and PNS rats were housed in pairs with same sex litter mates from weaning and provided with ad lib access to bland chow and water. Females were housed in groups of 5 or 6 in groups of either PNS or control rats. Furthermore 8 male PNS rats were individually housed in order to investigate the effects of single housing on the corticosterone profile of PNS rats.

In order to minimise the stress effects of the sampling procedure, rats were gently restrained in a towel for 2 minutes a day, for a full week prior to blood sampling.

At approximately 10 weeks of age rats were blood sampled by tail nick before, during and after an acute 30-minute restraint stress (see section 2.3 for full procedural details). Males were sampled 6 times at: -30, -1, +15, +30, +60, +90, with t = 0 representing the onset of an acute stressor. Female rats were only sampled 3 times at: -30, +30, +90. This was due to the smaller body weight of female rats and was in order to ensure that no more than 10% of total blood volume was taken. Samples were processed for plasma (see 2.3.2) and stored at -80°C until analysis. Blood glucose was measured directly from whole blood at each timepoint using an Aviva Accuchek glucose meter.

3.3.2.4 Diet Experiment

Male rats were housed in pairs with same sex litter mates at weaning and placed onto one of two diets: a diet consisting of ad lib access to standard rat chow (RM1, SDS) and water, hereafter referred to as the ‘bland’ diet; or an ad lib free choice of the standard rat chow, HFHS pellet, water and a 5% sucrose solution, hereafter referred to as the ‘choice’ diet. This resulted in a total of 4 experimental groups: Control rats on a bland diet; PNS rats on a bland diet; Control rats on a choice diet; PNS rats on a choice diet (N = 8 for all groups). Only one cage per litter was assigned to each group in order to prevent litter effects confounding the results. Body weight and daily food and water intake were measured once a week for 8 weeks.

As the results from the corticosterone sampling groups were inconclusive, at approximately 14 weeks of age, one rat from each cage was blood sampled before, during and after an acute 20-minute restraint stress, in order to obtain data on their corticosterone profiles as well as plasma leptin in order to examine whether differences in body weight could be attributed to increased fat mass. Rats were placed into a restraint tube and immediately a
blood sample was taken by tail nick. A sample was then taken at 20 minutes, immediately before the rat was removed from the tube, and a final sample was taken 60 minutes after the onset of the stressor. A 20 minute as opposed to 30-minute stressor was implemented in an attempt to reduce the severity of the stressor and prevent any stressor ceiling effects masking the effects of PNS on the stress response.

At the end of the experiment all animals were euthanized by an appropriate schedule 1 method.

3.3.2.5 Corticosterone and Leptin Assays

Plasma corticosterone was measured by radioimmunoassay (MP biomedical) in three separate assays carried out on the same day. All samples were assayed in duplicate. Intra-assay variation and inter-assay variation was under 10% for all assays.

Plasma leptin was analysed in duplicates from baseline samples obtained before the onset of the restraint stress using an ELISA (DRG diagnostics). Intra-assay variation was 4.4%.

3.3.2.6 Statistical Analysis

Data for the whole cage was divided by the number of rats per cage to obtain average data per rat. Normally distributed data were analysed using repeated measures, 2-way analysis of variance (ANOVA), with Sidak’s post hoc test; or unpaired student t test. For multiple t tests the Holm Sidak method was used to correct for multiple comparisons and determine statistical significance. Data not passing normality testing were analysed using non-parametric Mann Whitney U or Kruskal Wallis tests. In cases where data presented over time were not normally distributed, area under the curve (AUC) analysis was employed and then tested using parametric or non-parametric tests as appropriate.

For food choice analysis, daily Kcal data (averaged per rat) for each of the three foods available to rats on the choice diet was analysed using AUC analysis, and then divided to give an average daily value for each food.

Sucrose preference data was analysed by calculating daily sucrose preference (see section: 2.2.4.1) and then analysing area under the curve and dividing to give an average daily sucrose preference.
3.3.3 Results

3.3.3.1 Reproductive Parameters

Figure 3.8 shows gestational body weight and food intake data for both control and stressed dams. Two-way repeated measures ANOVA analysis illustrated no significant differences in absolute dam body weight between control and stressed dams from days 15-20 of gestation (Figure 3.8A, F(1, 24) = 0.28, P = 0.602). Similarly when percentage body weight increase from gestation day 15 was analysed, there were no significant differences between the two groups (Figure 3.8B; 2-way RM ANOVA; F(1, 24) = 4.2, p = 0.052).

There were no significant differences in total Kcal consumption (Figure 3.8C; 2-way RM ANOVA; F(1,21) = 0.015, p = 0.903) or water intake (Figure 3.8D; 2-way RM ANOVA; F(1,21) = 1.175, p = 0.291) between control or stressed groups from days 16 to 20 of gestation.

The mean attack score against the intruders over the 5 days of social stress was 53.6 ± 4.1.

Figure 3.8E illustrates the average daily stress scores for each of the intruder rats.

There were no significant differences in the total number of pups born to control and stressed dams (Figure 3.9A; unpaired t test; t(22) = 0.068, p = 0.946). There were also no significant differences in the average number of male and female pups born to stressed or control dams (Figure 3.9B; Males: t(22) = 0.739, p = 0.468; Females: U = 63, p = 0.615). For pup body weight shortly after birth (P3-4), there were no significant differences between PNS and control groups for male (Figure 3.9C; multiple t tests; t(22) = 0.693, p = 0.496) and female offspring (Figure 3.9C; multiple t tests; t(22) = 0.859, p = 0.399). Similarly, there were also no significant differences in body weight at weaning (P21) between control or PNS offspring for male (Figure 3.9D; multiple t tests; t(20) = 1.052, p = 0.305) or female pups (Figure 3.9D; multiple t tests; t(20) = 0.787, p = 0.441).

There were also no significant differences observed in body weight at P7 (data not shown; multiple t tests; males: t(22) = 1.259, p = 0.221; females: t(21) = 1.416, p = 0.171) or P14 (data not shown; multiple t tests; males: t(21) = 0.823, p = 0.419; females: t(20) = 1.159, p = 0.260) between control and PNS offspring.

3.3.3.2 Total Kcal Intake

There were no significant differences in total daily Kcal consumption between control and PNS rats on the bland diet for the 8 week testing period (Figure 3.10A; 2-way RM ANOVA;
F(1,14) = 0.806, p = 0.385). AUC analysis confirmed no significant differences between groups (Figure 3.10B; unpaired t test; t(14) = 0.925, p = 0.371). Similarly, for rats on the choice diet there were no significant differences between groups (Figure 3.10C; 2-way RM ANOVA; F(1,14) = 2.909, p = 0.110). Again, AUC analysis confirmed no significant differences between control or PNS offspring for rats on the choice diet (Figure 3.10D; unpaired t test; t(14) = 1.68, p = 0.115).

3.3.3.3 Food Choice

Figure 3.11 shows Kcal intake data for the control and PNS rats on the choice diet. Panel A shows the AUC data (averaged per day) for Kcal intake from the three calorie sources on the choice diet: the bland chow, the HFHS pellet and the sucrose solution. Multiple t tests found no significant differences in Kcal intake between control and PNS rats for the chow (t(14) = 0.291, p = 0.776), HFHS diet (t(14) = 1.204, p = 0.249) or sucrose solution (t(14) = 1.416, p = 0.179).

Figure 3.11B shows the daily Kcal consumption over time of the bland chow for control and PNS groups. Similarly to the AUC data, there were no significant differences observed between the control and PNS groups (2-way RM ANOVA; F(1, 14) = 0.0471, p = 0.831). There were also no significant differences between control and PNS groups for daily Kcal consumption of the HFHS diet over the 8 week testing period (Figure 3.11C; 2-way RM ANOVA; F(1,14) = 1.076, p = 0.317). Figure 3.11D illustrates the calories consumed from the solution sucrose over time. As data were not normally distributed an ANOVA could not be performed, however an increase in sucrose consumption over time was observed. This is likely due to an adaptation to neophobia, or simply due to the growth of the rats allowing for increased food consumption.

AUC analysis of average sucrose preference for control and PNS rats over the 8-week testing period illustrated no significant differences (Figure 3.11E; unpaired t test; t(14) = 1.068, p = 0.304).
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**FIGURE 3.8: DAM GESTATION PARAMETERS DURING SOCIAL STRESSOR**

All statistical tests were 2-way repeated measures ANOVA

A: Dam absolute body weight from days 15 – 20 of gestation. No significant differences were observed between stressed (n = 13) and control dams (n = 13) (p = 0.602).

B: Dam % body weight increase from gestation day 15 to day 20. No significant differences were observed between groups (p = 0.052, n: control = 13, PNS = 13).

C: Dams total Kcal intake from days 16-20 of gestation. No significant differences were observed between control (n = 11) and stressed (n=12) dams (p = 0.903).

D: Water consumption for stressed and control dams from days 16 – 20 of gestation. No significant differences were observed between the two groups (p = 0.291, n: control = 11, stressed = 12).

E: Average stress scores experienced by each intruder rat over the 5 days of social stress.
FIGURE 3.9: REPRODUCTIVE PARAMETERS FOR PUPS FROM STRESSED (PNS) AND CONTROL LITTERS

A: Total number of pups born to control (n = 12) and stressed (PNS, n = 12) litters. No significant differences were observed between the two groups (unpaired t test; p = 0.946).

B: Number of male and female pups per litter. No significant differences were observed between control and PNS males (unpaired t test; p = 0.468) or females (Mann Whitney U; p = 0.615).

C: Pup bodyweights shortly after birth. Multiple t test analysis showed no significant effect of stress for males (p = 0.496) or females (p = 0.399; n = 12 for all groups).

D: Pup body weight at weaning (P21). No significant differences were observed between control and PNS rats for males (multiple t tests; p = 0.305) or females (p = 0.441, males n: control = 10, PNS = 12; females n: control = 11, PNS = 12).
Figure 3.10: Total Kcal Intake for Control and PNS Rats on the Bland and Choice Diets

In all cases n = 8

A: Total daily Kcal intake per rat over the 8 weeks following weaning for rats on the bland diet. No significant differences were observed between control and PNS offspring over the 8-week period (2-way RM ANOVA; p = 0.385).

B: AUC analysis for the daily Kcal intake for the rats on the bland diet over the 8-week testing period. No significant differences were observed between control or PNS offspring (unpaired t test; p = 0.371).

C: Total Kcal intake for rats on the choice diet. No significant differences were observed between the two groups (2-way RM ANOVA; p = 0.110).

D: AUC analysis for daily Kcal for control and PNS rats on the choice diet. No significant differences were observed between groups (unpaired t test; p = 0.115).
FIGURE 3.11: TOTAL KCAL CONSUMPTION FROM THE DIFFERENT CALORIE SOURCES AND SUCROSE PREFERENCE FOR RATS ON THE CHOICE DIET

In all cases n = 8.

A: A comparison of AUC Kcal consumption for the three different Kcal sources on the choice diet for PNS and control offspring. No significant differences were observed between groups for consumption of the chow (p = 0.776), HFHS pellet (p = 0.249) or the sucrose solution (p = 0.179, multiple t tests). B: Kcal consumption per rat from RM1 over the 8-week measuring period, no significant differences were observed between the groups (2-way RM ANOVA; p = 0.831). C: Kcal consumption for the HFHS diet over 8 weeks. No significant differences were observed between control and PNS offspring (2-way RM ANOVA; p = 0.317). D: Data for sucrose consumption for control and PNS rats on the choice diet. As data was not normally distributed ANOVA analysis could not be performed. E: AUC analysis of average sucrose preference over the 8-week testing period. An unpaired t test illustrated no significant differences between control and PNS groups (p = 0.304).
3.3.3.4 Body Weight

Figure 3.12A shows body weight data for control and PNS rats on the bland diet. No significant differences were observed between groups (2-way RM ANOVA; F(1,14) = 0.157, p = 0.698). There were also no significant differences observed in body weight for the control and PNS rats on the choice diet (Figure 3.12B; 2-way RM ANOVA, F(1,14) = 0.464, p = 0.507).

Area under the curve analysis for all 4 groups illustrated a significant difference (Figure 3.12C; 1-way ANOVA; F = 43.18, p < 0.0001) with post hoc testing illustrating this difference lay between rats on the bland and choice diets.

3.3.3.5 Blood Sampling Results

3.3.3.5.1 Corticosterone Data

Figure 3.13 illustrates the plasma corticosterone data for male and female PNS and control rats. There were no significant differences observed between control and PNS male rats in plasma corticosterone concentration before, during and after an acute 30-minute restraint stress (Figure 3.13A; 2-way ANOVA; F(1, 10) = 1.911, p = 0.197). AUC analysis of plasma corticosterone from t(-1) to t(90) for control and PNS (both single and pair housed; Figure 3.13B) illustrated no significant differences between the three groups (Kruskal Wallis test; \( \chi^2 = 5.054, p = 0.076 \)).

There were also no significant differences observed between female control or PNS rats plasma corticosterone profiles when exposed to a restraint stressor (Figure 3.13C; 2-way RM ANOVA; F(1, 15) = 1.042, p = 0.324). This was confirmed by AUC analysis (Figure 3.13D; unpaired t test; t(15) = 1.4, p = 0.178).

3.3.3.5.2 Blood Glucose

No significant differences were observed in blood glucose between male control and PNS rats (Figure 3.14A, 2-way RM ANOVA; F(1,14) = 1.417, p > 0.999). There were also no significant differences in AUC data (from t(-1) to t(90) ) for male control and PNS (both group and single housed) rats (Figure 3.14B; 1-way ANOVA; F(2,20) = 0.339, p = 0.716).

For blood glucose during an acute stressor for female rats, there were no significant differences observed between control or PNS rats (Figure 3.14C; 2-way RM ANOVA; F(1,17)
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3.3.3.6 Diet Experiment Blood Analysis

3.3.3.6.1 Plasma Corticosterone

Figure 3.15A shows plasma corticosterone data for rats on the bland diet (after completion of the diet experiment) during an acute 20-minute restraint stressor. No significant differences were observed between control or PNS offspring (2-way RM ANOVA; F(1,12) = 0.035, p = 0.855).

3.3.3.6.2 Plasma Leptin

Figure 3.15B shows results for plasma leptin from control and PNS rats on the bland and choice diets. A one-way ANOVA showed a significant difference (F(3,23) = 3.901, p = 0.022) and post hoc testing showed this difference to lie between the PNS-bland and Control-choice groups.
Figure 3.12: Body weight data for control and PNS rats on the bland and choice diets

In all cases n = 8

A: Body weight over time for the 8-week testing period for rats on the bland diet. No significant differences were observed between control or PNS rats (2-way RM ANOVA; p = 0.698).

B: Body weight over time for control and PNS rats on the choice diet. No significant differences were observed between the two groups (2-way RM ANOVA; p = 0.507).

C: AUC data for average body weight for all four groups. A 1-way ANOVA showed a significant difference (p < 0.0001), and post hoc testing using Tukey’s multiple comparisons indicated a significant difference between the bland and choice diet groups.
FIGURE 3.13: PLASMA CORTICOSTERONE PROFILES DURING AN ACUTE 30 MINUTE RESTRAINT STRESS (SHADED AREA)

A: Corticosterone profile for male control and PNS rats. A 2-way, RM ANOVA showed no significant differences between groups (p = 0.197, n: control = 6, PNS = 6). B: AUC analysis for male control and PNS (group and single housed) for plasma corticosterone from t(-1) to t(90). No significant differences were observed between groups (Kruskal Wallis test; p = 0.076, n: control = 6, PNS = 8, PNS single = 7). C: Corticosterone profiles for female control (n = 9) and PNS (n = 8) rats. No significant differences were observed between groups (2-way RM ANOVA; p = 0.324). D: AUC data for female rats from t(-30) to t(90). No significant differences were observed between control (n = 9) or PNS (n = 8) groups (unpaired t test; p = 0.178).
Figure 3.14: Blood Glucose During an Acute Restraint Stressor (Shaded Area)

A: Glucose profile for male control (n = 8) and PNS (n = 8) rats. No significant differences were found between groups (2-way RM ANOVA; p > 0.999). B: AUC analysis for male control and PNS (group and single housed) for blood glucose from t(-1) to t(90). No significant differences were observed between groups (1-way ANOVA; p = 0.716; n: control = 8, PNS = 8, PNS single = 7). C: Glucose profiles for female control (n = 10) and PNS (n = 9) rats. No significant differences were observed between groups (2-way RM ANOVA; p = 0.771). D: AUC data for female rats from t(-30) to t(90). No significant differences were observed between control (n = 10) or PNS (n = 9) groups (unpaired t test; p = 0.758).
FIGURE 3.15: CORTICOSTERONE AND LEPTIN RESULTS FOR MALE RATS FROM THE DIET EXPERIMENT

A: Corticosterone profile of control and PNS male rats on the bland diet during an acute 20-minute restraint stressor (shaded area). No significant differences were observed between control and PNS groups (2-way RM ANOVA; p = 0.855, n: control = 6, PNS = 8)

B: Plasma leptin data for PNS and control rats on the bland and choice diets. A 1-way ANOVA showed a significant difference (p = 0.022, n: control-B = 8, PNS-B = 7, control-C = 7, PNS-C = 5), Post hoc testing using Tukey’s multiple comparisons indicated a significant difference between the PNS bland and control choice groups.
3.3.4 Discussion

3.3.4.1 Reproductive Parameters

Similarly to the study using prenatal restraint stress, there were no differences in dam body weight, or percentage body weight increase, as well as no differences in food and water consumption. As discussed previously, effects of prenatal stress on dam body weight are variable (see section: 3.2.4.1), however, these results are consistent with other studies using prenatal social stress, as there are generally no differences in dam body weight gain observed (Brunton et al., 2013, Brunton and Russell, 2010, Grundwald and Brunton, 2015).

Again, no differences were observed in total number of pups per litter. There were also no differences in the number of male or female pups per litter. Furthermore, no differences in body weight on postnatal day 3-4 or at weaning between control and PNS pups were observed. Other studies using prenatal social stress have found varying results in terms of pup birth weight, with Grundwald and Brunton (2015) finding no differences in birth weight, while Brunton et al. (2015) observed lower birth weights in both male and female pups. There is also evidence of sex specific effects of prenatal stress on birth weight, with some prenatal social stress studies illustrating lower birth weight only in female offspring (Brunton and Russell, 2010, Brunton et al., 2013). Overall birth weight in prenatally stressed offspring appears to be incredibly varied, even when using the same model, and is likely influenced by a variety of factors.

3.3.4.2 Total Kcal Intake and Food Choice

No significant differences were observed in total Kcal intake when rats were placed on either a bland or choice diet. Furthermore, no significant differences were observed between control and PNS rats in terms of food choice. Interestingly what can be seen from the figures showing food intake data over time (Figure 3.11B-D), is that while the consumption of the HFHS food remains stable, the increase in total calories consumed over time is a consequence of an increased intake of the bland chow and the sucrose solution.

The bland food, whilst containing essential nutrients, is likely not as palatable as the HFHS diet, as when placed on a ‘binge like’ feeding model where rats are given two hours of access to a HFHS diet, they will consume very little to no bland food during the two hour period (Bake et al., 2017). Furthermore, it has been illustrated that both adolescent and adult rats have a preference for high fat foods (Rockwood and Bhathena, 1990). However,
the increased consumption of bland food over time may be due to the nutritional requirements of the rats. For example, the bland diet may contain components essential to the rats’ diet which are lacking in the HFHS diet that drive the increase in consumption. Once again, no differences in sucrose preference were observed between control and PNS rats (see section: 3.2.4.3 for detailed discussion).

3.3.4.3 Body Weight

Similarly to the study using prenatal restraint stress, no differences were observed in body weight between PNS and control groups for rats on the bland or choice diets. There was, as expected, an increase in body weight for those rats on the choice diet due to the increased calorie consumption of rats on this diet. Few studies using prenatal social stress have reported body weight data, however Brunton et al. (2013) found an effect of PNS on body weight at 3 months of age, however this was only significant in females, and at 6 months body weight had normalised. As female rats were not tested in this experiment it cannot be concluded whether these findings are in line with those of Brunton et al. (2013).

3.3.4.4 Blood Sampling

3.3.4.4.1 Corticosterone

For this study a separate cohort of male and female rats were blood sampled during an acute restraint stressor in order to prevent the stressor impacting feeding behaviour for rats on the diet experiment. In these rats no differences in plasma corticosterone levels were observed. Although for this experiment the rats were habituated to being gently restrained in a towel once a day for a week prior to blood sampling, an increase in plasma corticosterone was observed between the two basal sampling points suggesting that the blood sampling procedure in itself was inherently stressful (although this was only measured in males). Therefore this could once again have led to maximal stress responses which would have masked the effects of prenatal stress (Weinstock, 2008).

Throughout the study rats were group housed in order to prevent any of the stress effects associated with individual housing having an impact on the rats’ corticosterone responses. As some PNS studies implement single housing (Lemaire et al., 2000, Lesage et al., 2004, Balasubramanian et al., 2015) a separate cohort of individually housed rats were also blood
sampled during an acute stressor to establish whether this could affect the rats’ corticosterone profiles. No differences in the corticosterone responses of group housed and singly housed prenatally stressed animals were observed, so it appears that the housing has no effect of corticosterone responses during an acute restraint stress.

After completion of the dietary experiment, blood samples were also obtained from these rats, however this time taking only one baseline sample (in order to attempt to reduce stress from the sampling procedure) and subjecting the rats to a 20-minute restraint stress, as opposed to a 30-minute restraint stress in order to reduce the peak stress response. Nevertheless, these results also illustrated no differences in plasma corticosterone between control and prenatally stressed rats. However, as peak corticosterone levels are generally observed around 30 minutes after the onset of a stressor (De Souza and Van Loon, 1982), by measuring corticosterone at 20 minutes following the onset of restraint, this may not provide an accurate value for peak corticosterone. Therefore, from this data it cannot be concluded whether these rats illustrated the HPA axis hyperactivity typical of PNS rats.

3.3.4.4.2 Glucose

Glucose is also a marker of stress, and many have observed elevated blood glucose after exposure to a restraint stress (Calvez et al., 2011). Brunton et al. (2013) found that PNS rats had a significantly higher blood glucose reading 15 minutes after the onset of a restraint stressor in comparison to control males, however by 30 minutes this difference was no longer significant. These differences in blood glucose were not observed in females. In this study no differences in blood glucose between control and PNS offspring were observed before, during or after an acute 30-minute stressor in both male and female rats.

Other studies have shown variable results on the effects of prenatal stress on blood glucose. Panetta et al. (2017) and Tamashiro et al. (2009) found no effects of PNS on a glucose tolerance test, while Lesage et al. (2004) found that glucose was higher in prenatally stressed rats during a glucose tolerance test at all timepoints examined. Although a glucose tolerance test was not performed in this study, no differences were observed in basal glucose levels, which is in line with findings from both Paternain et al. (2013) and Balasubramanian et al. (2015) who also observed no differences in basal blood glucose between control and PNS rats.
3.3.4.4.3 Leptin

For plasma leptin no significant differences were observed between control and PNS rats, however a significant effect of diet was observed, with control rats on the choice diet illustrating higher plasma leptin in comparison to PNS rats on the bland diet. Previous studies have also shown no effects of prenatal stress on plasma leptin (García-Cáceres et al., 2010), although rats on a high fat diet have been shown to demonstrate increased leptin (Panetta et al., 2017, Paternain et al., 2013). This usually associated with the increased body weight, and therefore fat mass, observed in rats consuming high fat diets (Handjieva-Darlenksa and Boyadjiowa, 2009). Lesage et al. (2004) found an increase in plasma leptin in 24 month old PNS rats, however as these rats were significantly older than those tested in this study, it may be that hyperleptinemia only develops in aged PNS rats.

3.3.4.5 Experimental Limitations

3.3.4.5.1 Prenatal Social Stress Procedure

One explanation to the lack of success in generating rats with an elevated corticosterone response may be that the dams did not receive a sufficient amount of stress in order to generate the PNS offspring. During the social stress sessions behaviour was video recorded and afterwards scored on a scale. The mean total attack score towards the intruder rats in this study was 53.6 ± 4.1. This score is lower than that observed by Brunton and Russel (2010) who found an average score of 80.0 ± 7.2. This suggests that perhaps the rats did not receive a sufficient level of stress to programme the offspring. However, as there are slight differences in these scoring systems, and scoring was performed by different experimenters, it is difficult to draw direct comparisons between these two results. This highlights the main problem with this model of prenatal stress in that is relies heavily on animal behaviour, and it is therefore difficult for each rat to receive consistent levels of stress over the 5-day stressor period.

Although this procedure has been very successful in generating PNS offspring in previous studies, the corticosterone responses during an acute stressor are not always reported. This could suggest that either this procedure is not always successful in generating animals expressing this enhanced stress response, or that an enhanced corticosterone response is not always exhibited in PNS animals.
3.4 Chapter Discussion

3.4.1 Summary of Findings and Hypotheses

The aims of these experiments were to establish whether prenatal stress would lead to an increase in food consumption when offered a HFHS diet, and whether prenatal stress could lead to alterations in food choice.

The hypotheses for these experiments were:

1 – Prenatal stress will lead to an increased calorie intake in comparison to control rats when offered a palatable diet

2 – Prenatal stress will lead to an increased preference for more energy dense food.

Body weight was also measured as a secondary outcome.

Overall no effects of prenatal stress were observed on Kcal consumption or on food choice, however in both of these studies, the HPA axis hyperactivity which is generally observed in PNS offspring was not replicated. This suggests that the stress paradigms used during pregnancy may have been insufficient to produce prenatally programmed offspring, or that the sampling procedures used during this experiment masked the expected changes in plasma corticosterone concentrations. Furthermore, it may be that previously reported protocols of prenatal stress are not as robust as often suggested in the literature.

3.4.2 Experimental Limitations

3.4.2.1 Blood Sampling Procedure

In both of these studies, the corticosterone results appear to suggest that the PNS rats were not programmed as they did not illustrate the expected stress hyperactivity. It may be that the stress paradigms used were not robust enough to result in programming or, it may be that the blood sampling procedure was masking the expected heightened stress effects.

Blood samples were obtained through a tail nick procedure. Although this technique is ideal for non-invasive, serial sample collections, it is apparent that the procedure itself acted as a stressor, as can be observed from the increase in plasma corticosterone from the first to second baseline measurement. This could be due to the gentle restraint of the rats in a towel during the tail nick procedure. Although the rats were habituated to this restraint for a week prior to sampling in the prenatal social stress experiment, it may be that this habituation time was insufficient, or that other factors of the blood sampling procedure
could also cause stress. This, along with the restraint stress, may have led to a ceiling effect in the plasma corticosterone levels, thereby masking the elevated corticosterone levels usually observed in PNS offspring (Weinstock, 2008). However, other studies have successfully used this blood sampling procedure to measure elevated plasma corticosterone in PNS rats (Viltart et al., 2006) which suggests that prenatal stress models may not be as robust for producing alterations to the HPA axis as often reported.

As an alternative to collecting samples via tail nick, cannulations could have been used to collect sequential samples. This may have reduced stress from the blood sampling procedure and also allowed for collection of larger volumes of blood which would have allowed examination of plasma ACTH as well as corticosterone, as some studies have indicated that the difference in ACTH levels between control and PNS offspring often show a clearer divergence than corticosterone (Brunton and Russell, 2010). However there are also problems associated with the use of cannulas as they can often become blocked (Parasuraman et al., 2010) and it has also been suggested that implantation of cannulas can result in chronic stress (Vahl et al., 2005) which would confound the measurement of stress hormones. Furthermore, as rats were group housed to reduce the stress effects of individual housing, this could have resulted in rats accidentally damaging cannulas during play interactions.

Furthermore, an alternative stressor could have been implemented to test the rats’ corticosterone responses. For example Grundwald and Brunton (2015) used systemic interleukin-1β as a physical stressor, and found a more pronounced effect of prenatal stress on both corticosterone and ACTH in females in comparison to when using a restraint stressor, although a restraint stressor appeared to give a clearer divergence in male offspring (however these experiments were carried out using F2 offspring).

3.4.2.2 Measuring Food Choice

One of the main limitations with this experiment is the use of the ‘choice’ diet model. One of the largest problems facing research into food choice is to develop an animal model that truly represents the scope and choice of different foods available to humans. The dietary choice model used in this study, whilst measuring preference for a bland food, HFHS sugar food or sucrose, is limited in that it does not provide a wide variety of foods. Although this model is good in that it offers a combination HFHS pellet, which is ecologically valid as many palatable foods consumed by humans are high in both sugar and fat (Drewnowski,
1998), a model such as that used by Schéle et al. (2016) using a choice of regular chow, lard and sucrose pellets, may be more efficient, as by separating the sucrose and fat component of the diet this allows for a closer examination of the exact nutritional preferences of the rat. (For further discussion on food choice models, see section: 6.2.2).

Another confound of this model is the presentation of sucrose in a solution. There is some evidence that carbohydrate consumption can vary when presented in different forms (Pan and Hu, 2011), with a trend towards higher consumption when in presented in a liquid (Apolzan and Harris, 2012). Although high sugar liquids are common in human diets, it could be argued that a sucrose solution cannot be classified as a ‘food’ and therefore this may confound this food choice model, as overconsumption of sucrose in solution may mask other food choice effects.

3.4.2.3 Age of Rats

Given that many of the effects of prenatal stress, such as anxiety behaviour, appear to be age dependent (see Weinstock, 2017), it is important to test across a variety of age ranges to ensure that age related effects of prenatal stress are not disregarded. Age dependent effects appear to be particularly relevant for those studies examining the effects of PNS on body weight and feeding (Schulz et al., 2011, Lesage et al., 2004), therefore it may be that the rats used in these studies were too young to fully illustrate these effects, and that the influence of prenatal stress on feeding and food choice would have become more pronounced with age.

3.4.2.4 Postnatal Handling

Regular postnatal handling during the pre-weaning period has been shown to illustrate some of the opposite behavioural and physiological effects to those observed in PNS offspring. While PNS rats were found to have higher anxiety levels, rats that had experienced postnatal handling during the first 3 weeks of life illustrated lower anxiety-like behaviours in comparison to controls (Vallee et al., 1997). Furthermore, it has been shown that whilst prenatal stress resulted in an enhanced corticosterone secretion in response to an acute stressor, postnatal handling exerted the opposite effects and resulted in a blunted corticosterone response that returned to baseline faster than control rats (Vallee et al., 1996, Vallée et al., 1999). The postnatal handling procedure in these studies involved daily handling during the pre-weaning period, and although in the studies performed in this
Chapter postnatal handling was only performed once weekly (in order to weigh pups and clean cages) it may be that this small amount of handling could have influenced the stress responses of our PNS offspring, perhaps resulting in a reduction in stress induced corticosterone secretion.

3.4.3 Prenatal Stress as a Model

3.4.3.1 Measuring PNS

3.4.3.1.1 Dam Parameters

As the mechanisms causing PNS are still unknown it is difficult to measure whether a stressor is effective in eliciting a sufficient stress response in the dam to produce programmed offspring. While both maternal glucocorticoids and maternal care have been implicated in the development of certain PNS characteristics (see section: 1.4.2.1.2), there is still no evidence for the precise mechanisms behind the development of the PNS phenotype. Therefore, measurement of parameters such as maternal corticosterone secretion during stressor application does not give a clear indication of the severity of the PNS offspring phenotype. Because of the likely complex interaction of factors contributing to the development of the prenatal stress phenotype, it is difficult to control and predict the outcomes of a maternal stressor on the offspring.

3.4.3.1.2 Corticosterone as a Measure of PNS

Whilst it is often suggested that HPA axis hyperactivity is the most significant characteristic of prenatally stressed rats, it may be that this effect is not as robustly observed as often suggested. Firstly, in studies of prenatal stress corticosterone measurements are not always reported, and therefore it cannot be guaranteed that offspring from all models illustrate this enhanced stress response. Furthermore, while there is a large amount of evidence showing this hyperactive corticosterone response (Maccari et al., 2003, Brunton and Russell, 2010, Viltart et al., 2006), there are also some studies that have illustrated no change in corticosterone levels (Brunton et al., 2013, McCormick et al., 1995), or even lower corticosterone levels after an acute stressor in PNS offspring (Van den Hove et al., 2005). These differences are likely dependent on the timing and duration of stressors applied during pregnancy and highlight the huge variation in prenatal stress models (further discussed below).
3.4.3.1.3 Anxiety Like Behaviours

Another particularly well-established effect of prenatal stress is increased anxiety like behaviours. These can be tested through a variety of behavioural tests such as: the open field test, elevated plus maze and light dark box.

Most studies have found an increase in anxiety like behaviour in both the elevated plus maze (Głombik et al., 2015, Palacios-García et al., 2015, Zohar et al., 2015) and the open field test (Said et al., 2015). The effects of prenatal stress on anxiety behaviour can be observed even without significant changes in HPA axis reactivity (Van den Hove et al., 2005, Richardson et al., 2006). Therefore, further behavioural testing could have been implemented in these studies to establish whether the PNS offspring presented the expected enhanced anxiety responses. This may then suggest that although the stress procedures may not have been sufficient in generating HPA changes, some programming effects were still in place.

3.4.3.2 Interpretation of Model

A reduction in birth weight, particularly in studies using prenatal restraint stress, can be associated with a form of intrauterine growth restriction (Lesage et al., 2004). Paternain et al. (2013) found a reduction in body weight in the stressed dams, which lead to PNS offspring of a lower birth weight offspring. Low birth weight has been shown to increase susceptibility to obesity (Simmons et al., 2001) and hyperphagia (Vickers et al., 2000). Therefore, it can be difficult to establish, particularly in studies measuring body weight, whether the effects observed are solely due to the stress during pregnancy, or whether they are a secondary outcome due to intrauterine growth restriction. However, as no birth weight differences were observed in this study it would appear that intrauterine growth restriction was not a factor in these experiments.

3.4.3.2.1 Model Reliability and Differences in Models

Given the difficulties in reproducing certain aspects of prenatal stress models, it may be that these models are not as robust as initially thought. One of the main flaws associated with prenatal stress as a model is the variability in offspring characteristics, ranging from reproductive parameters such as dam percentage weight gain and offspring birth weight, to
endocrine responses in the offspring such as differences in corticosterone responses, and even behavioural differences in PNS offspring such as anxiety like behaviour and learning and memory deficits (Weinstock, 2008, Weinstock, 2017).

One reason for a variety of these differences may be accounted for by the diverse types and application of stressors during pregnancy. Table 1.1 illustrates some of the different stress paradigms used in prenatal stress studies, and clearly demonstrates the range in severity, type and timing of stressors. Differences in prenatal stress models may also arise from strain differences (Stohr et al., 1998). Nevertheless, these model differences may also be considered an advantage in that they reflect the variation that is likely present in human cases of prenatal stress and may therefore illustrate the diverse effects and mechanisms of prenatal stress.

Richardson et al. (2006) found that repeated exposure to the same stressor (restraint) generated the most robust changes including increased anxiety related behaviours and a prolonged HPA axis response to stress in female offspring. Restraint males showed no differences in anxiety like behaviour but did show elevated basal ACTH and blunted HPA response to stress. On the other hand, prenatal exposure to varied, unpredictable stress did not produce as robust of an effect on HPA function highlighting the differences that can arise from different models of prenatal stress.

Whilst differences in the effects of prenatal stress can clearly arise between different PNS models, there is also evidence for differences in the effects of prenatal stress from the same model used across different laboratories. For example, Van den Hove et al. (2014) and Sun et al. (2013) both used the same model of restraint stress however whilst the former found that PNS offspring demonstrated lower birth weight than controls, Sun et al. (2013) found no differences. Furthermore, when testing anxiety using the elevated plus/zero maze, Sun et al. (2013) found a more pronounced anxiety phenotype in males, whilst Van den Hove et al. (2014) found increased anxiety in females. Although different tests of anxiety were used, the elevated plus maze and elevated zero maze have been found to be comparable measures of anxiety (Braun et al., 2011). Furthermore, Henry et al. (1994) found an elevated corticosterone response in male PNS offspring whereas Richardson et al. (2006) found lower corticosterone in PNS rats in comparison to controls, although both studies use the same prenatal restraint stress procedure. Similarly, when implementing the same model of prenatal stress Van den Hove et al. (2005) found no
difference in immobility in the forced swim test between PNS and control rats whilst Morley-Fletcher et al. (2003) illustrated increased immobility suggesting increased depressive like behaviour.

Overall, these papers and the results presented in this chapter suggest that models of prenatal stress may not be as robust and reproducible as often suggested in the literature, particularly in terms of generating offspring with robust alterations to the HPA axis, and highlights the importance of developing reliable and consistent experimental protocols.
4 The Effects of Neonatal Overnutrition on Food Choice

4.1 Chapter Background

Litter size manipulation is a well-established rodent model of early life nutrition. Rats from large litters are used as a model of under-nourishment, due to reduced access to milk in the pre-weaning period. Large litter rats often illustrate a reduced body weight gain (Bulfin et al., 2011) as well as reduced plasma leptin, and glucose stimulated insulin secretion (López-Soldado et al., 2006).

On the other hand, small litter rats are used as a model of over-nourishment due to their greater access to milk. Small litter rats show an increased body weight gain in comparison to control litter rats, which often becomes apparent in the pre-weaning period and persists throughout life (reviewed in Spencer, 2013). It appears that this increased body weight can, at least in part, be attributed to increased fat mass in small litter offspring (Stefanidis and Spencer, 2012, Mozes et al., 2014). Consistent with this increase in fat mass, small litter rats demonstrate increased plasma leptin (Stefanidis and Spencer, 2012) as well as hyperinsulinemia (Plagemann et al., 1999).

There is some evidence that the increased body weight observed in small litter offspring can be associated with an increase in food consumption (Spencer and Tilbrook, 2009, Rodrigues et al., 2009). Liu et al. (2013) found that when small litter rats were pair fed with control litter rats, their body weight was significantly reduced to levels similar to controls. However, once ad lib feeding was resumed on postnatal day 100, body weight returned to similar levels to ad lib fed small litter rats, suggesting a change in set-point. However, not all studies observe an increase in food intake (Noschang et al., 2014, Portella et al., 2015), particularly when food consumption is corrected for body weight (Mozes et al., 2014, Stefanidis and Spencer, 2012), which suggests there may be mechanisms other than increased food consumption leading to this increased adiposity, such as changes to metabolism and energy expenditure.

Although there is much evidence for alterations in food intake, evidence is still lacking to whether small litter rats have an altered food choice. Noschang et al. (2014) found that
when given a free choice between chow and sweet pellets over a 24 hour period, small litter rats consumed a similar amount of bland chow in comparison to control rats, but illustrated an increased intake of the sweet pellets. Conceição et al. (2016) gave rats access to both a high-fat and high-sugar diet for 12 hours and found that small litter rats showed an increased preference for the high-fat diet in comparison to controls. This suggests that small litter rats may have a preference for high energy foods, which could contribute to their increased body weight. Therefore, the aim for this chapter was to establish whether neonatally overfed rats, as modelled by litter size adjustment, demonstrated any alterations in food choice when on a long-term choice diet.
4.2 Experiment 1: The Effects of Small Litter Rearing on Food Choice

4.2.1 Background

Due to the evidence from Noschang et al. (2014) suggesting an increased preference for sweet foods in small litter rats, and Conceição et al. (2016) who found a preference for a high-fat diet in small litter rats, the hypothesis for the following experiment was:

- When given a food choice, small litter rats will show a preference for high energy foods such as lard and sucrose over bland chow.

A secondary outcome was to determine whether total Kcal consumption was increased in small litter rats, as current evidence for this is inconclusive.

As current data on food choice in small litter rats is limited to short-term tests under 24 hours (Noschang et al., 2014, Conceição et al., 2016), this study utilized a long-term food choice paradigm for a period of 10 weeks, in order to examine food preference over a longer period. The food choice model used by Schèle et al. (2016) was implemented, where rats are offered a free choice of chow, lard and sucrose pellets, as opposed to the previously implemented food choice model using a combined HFHS pellet, as it was hoped that this could provide a clearer indication of the rats exact macronutrient preferences.

Litter size manipulation was used as a well-established model of early life over-nutrition. In this model pups are cross-fostered after birth and litter sizes are adjusted to 4 pups for small litters, and between 10-12 pups for control litters. Cross-fostering was employed for control and test groups to ensure that the effects observed were purely due to the litter size manipulations and not differences in, for example, maternal care.

Shortly after weaning rats were placed onto either a bland diet or a choice diet and body weight, along with food and water intake, was measured for one day every week for 10 weeks.
4.2.2 Methods

4.2.2.1 Animals

28 nulliparous female Sprague-Dawley rats (bred in-house) were mated with a sexually experienced male. Rats were housed under standard conditions (as described in section 2.1.1). Pregnant rats were singly housed from approximately day 15 of pregnancy.

4.2.2.2 Litter Size Manipulation

The model of litter size manipulation was similar to that used by Sominsky et al. (2017b). On postnatal day 1 or 2, litters were weighed and sexed before being cross-fostered (see 2.1.3 for details). The dams received a mix of pups from different litters and none of her own pups during cross-fostering. In order to reduce the chance of rejection, pups were gently rolled in the bedding of their new dam before being placed in the new nest. A high level of rejection (lack of nest building, scattering of litter and infanticide) was observed during this study, with 9 dams (32%) rejecting their own litters prior to cross-fostering, and 1 dam rejecting her cross-fostered litter. This may be due to concurrent and unanticipated changes in animal unit staffing causing elevated stress which can contribute to litter rejection (Lane-Petter, 1968).

Small litters were composed of 4 pups and control litters contained between 10-12 pups. Due to the high level of pup rejection, litters were generally male biased to ensure a sufficient number of males were available for experimental testing. Pups and dams were weighed once weekly during the pre-weaning period but were otherwise undisturbed except for standard husbandry.

4.2.2.3 Experimental Groups

Pups were weaned on postnatal day 21 and housed in same-sex, litter-mate pairs to avoid stress effects of individual housing confounding food intake results. Only male offspring were tested. Two weeks following weaning, cages were randomly assigned to one of two diets: the ‘bland diet’ which consisted of *ad lib* access to a bland lab chow (RM1) and water, or the ‘choice diet’ which was based on a choice model used by Schéle et al. (2016) and consisted of *ad lib* access to bland chow, sucrose pellets, lard, and water.

Only one cage per litter was assigned to each group to prevent any trends due to intra-litter effects, and the whole cage was taken as the experimental unit. In total there were 8 cages
per group, although one cage was excluded from the experiment (Table 4.1; Control-choice group) due to an unexpected death shortly after weaning. Whole cage body weight, food and water intake was measured for each cage at the same time each day, for two consecutive days per week in order to provide a measurement for daily food and water intake, for a total of 10 weeks.

**Table 4.1: Abbreviations and N Numbers for Experimental Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Group Abbreviation</th>
<th>N number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control litter, bland diet</td>
<td>Control-B</td>
<td>8</td>
</tr>
<tr>
<td>Small litter, bland diet</td>
<td>Small-B</td>
<td>8</td>
</tr>
<tr>
<td>Control litter, choice diet</td>
<td>Control-C</td>
<td>7</td>
</tr>
<tr>
<td>Small litter, choice diet</td>
<td>Small-C</td>
<td>8</td>
</tr>
</tbody>
</table>

4.2.2.4 Data Analysis

All data from the whole cage measurements were averaged to give a value per rat prior to analysis. All data are plotted as mean ± SEM.

Data presented over time that passed normality testing were analysed using a 2-way repeated measures ANOVA, with Sidak’s multiple comparisons test used post hoc to establish the location of significant differences. If data were not normally distributed, AUC analysis was employed. AUC values were averaged to represent the average AUC for one day.

To test for differences between two groups that passed normality testing, an unpaired students t test was used. In the case where multiple t tests were performed, the Holm-Sidak method was used to correct for multiple comparisons and determine statistical significance. If data failed normality testing Mann Witney U testing was employed.

For normally distributed groups with two factors (e.g. litter size and diet) 2-way ANOVA analysis was performed, with Tukey’s multiple comparisons test to detect for significant differences.

In order to normalise food consumption to body weight, average Kcal consumption per rat was divided by the averaged body weight for the rat to give the total Kcal consumption per gram of body weight.
To obtain a one value index to represent food choice, the percentage of Kcal from the bland food of the total Kcal intake was calculated. This was then correlated with body weight to examine whether food preference was dependent on body weight.
4.2.3 Results

4.2.3.1 Pre-Weaning Body Weights

There were no significant differences observed in maternal body weight for dams of control and small litters from postnatal days 7 – 21 (Figure 4.1A; 2-way RM ANOVA; F (1, 16) = 0.262, p = 0.616). As expected, there was a significant difference in pup body weight between control and small litter rats during the pre-weaning period (Figure 4.1B; 2-way RM ANOVA; F (1, 16) = 16.20, p = 0.001). Sidak’s post hoc testing found this difference to lie at P14 and P21, with small litter rats weighing more in comparison to controls at these time points.

4.2.3.2 Body Weight

For rats on the bland diet, there was a significant difference in body weight between control and small litter rats over the 10-week testing period (Figure 4.2A; 2-way RM ANOVA; F (1, 14) = 5.989; -p = 0.028). Post hoc testing found this difference to be significant on weeks 9 and 10 of the experiment, with small litter rats illustrating an increased body weight in comparison to control rats. This was confirmed by area under the curve analysis, which illustrated a significant difference between the small and control litter rats on the bland diet (Figure 4.2B; unpaired t test; t(14) = 2.435, p = 0.029).

There was also a significant difference in body weight between control and small litter rats on the choice diet (Figure 4.2C; 2-way RM ANOVA; F (1, 13) = 6.226, p = 0.027) with small litter rats showing increased body weight for weeks 7-10 of the experiment (Sidak’s post hoc test). Again this was confirmed by AUC analysis, which illustrated a significant difference between small and control litter rats (Figure 4.2D; unpaired t test; t(13) = 2.487; p = 0.027).

When a 2-way ANOVA was performed on the body weight AUC data for all 4 groups (data not shown) a significant effect of litter size was observed as expected (F(1, 27) = 12.01, p = 0.002), however no effect of diet was found (F(1, 27) = 0.269, p = 0.608) illustrating that the different diets had no effect on body weight.

4.2.3.3 Total Kcal Consumption

Figure 4.3 illustrates data for total daily Kcal consumption over the 10-week testing period. Two-way RM ANOVA analysis showed a significant difference between control and small
litter groups for total daily calorie consumption for rats on the bland diet over the 10 weeks (Figure 4.3A; F (1, 14) = 5.108; p = 0.040). Post hoc testing showed this significance to lie during weeks 5 and 6. This difference was confirmed by AUC analysis (Figure 4.3B) which illustrated a significant difference (Mann Whitney U test; U = 11, p = 0.028), with small litter rats consuming more Kcal/day in comparison to control rats.

For rats on the choice diet, there were no significant differences in daily Kcal consumption between control and small litter rats (Figure 4.3C; 2-way RM ANOVA; F (1, 13) = 2.605, p = 0.131). This was confirmed by AUC analysis which showed no significant differences in Kcal consumption between control and small litter rats (Figure 4.3D; unpaired t test; t(13) = 1.612, p = 0.131).

When daily Kcal consumption was divided by body weight to provide a value for calorie consumption per gram body weight, there were no significant differences observed between control and small litter rats on either the bland (Figure 4.4A; 2-way RM ANOVA; F (1, 14) = 0.574, p = 0.461) or the choice diet (Figure 4.4B; 2-way RM ANOVA; F (1, 13) = 2.158, p = 0.166). Area under the curve analysis illustrated a significant difference between rats on the bland and choice diets, with rats on the choice diet consuming a significantly higher number of calories per gram body weight in comparison to rats on the bland diet (Figure 4.4C; 2-way ANOVA; F (1, 27) = 34.12, p < 0.0001, Tukey’s multiple comparisons).

No differences were observed in water intake between control and small litter offspring (Figure 4.4D; 2-way ANOVA; F (1, 27) = 0.529, p = 0.474) although a significant effect of diet was observed (F (1, 27) = 21.66, p < 0.0001). Tukey’s post hoc testing found this significance to lie between rats on the bland diet and the control choice group, with rats on the bland diet consuming more water than those on the choice diet.

### 4.2.3.4 Food Choice

Area under the curve analysis for Kcal intake for the three different foods available to rats on the choice diet (bland chow, sucrose pellets and lard, Figure 4.5A) illustrated a significant difference in calories consumed from the bland chow, with small litter rats consuming a larger number of calories from the chow in comparison to the control rats (Figure 4.5A; multiple t tests; t(13) = 3.072, p = 0.009). No significant differences were observed in calorie consumption from sucrose pellets (t(13) = 0.159, p = 0.876) or the lard (t(13) = 1.506, p = 0.156).
Data for Kcal consumption from the three diet sources over time confirms this, illustrating a significant difference in calorie consumption for the bland chow (Figure 4.5B; 2-way RM ANOVA; F (1, 13) = 9.137, p = 0.009). Sidak’s post hoc test found significance on weeks 3 and 8 of the experiment. No significant differences were observed in calorie intake over time from the sucrose (Figure 4.5C; 2-way RM ANOVA; F (1, 13) = 0.031, p = 0.862) or the lard (Figure 4.5D; 2-way RM ANOVA; F (1, 13) = 2.207, p = 0.161).

In order to confirm whether the preference for the bland chow was a product of body weight, the percentage of total calories consumed from the bland chow was calculated in order to give an index of food choice. No significant differences were observed between control and small litter rats in the percentage of Kcal consumed from the bland chow over the 10-week testing period (Figure 4.6A; 2-way RM ANOVA; F (1, 13) = 4.125, p = 0.063). AUC analysis also illustrated no significant differences between control and small litter rats in the percentage of calories consumed from the bland chow (Figure 4.6B; unpaired t test; t(13) = 2.058, p = 0.060). Linear regression analysis of the percentage of Kcal from the bland chow against body weight found no significant correlation for either control (Figure 4.6C; F(1,5) = 1.22, p = 0.319) or small litter rats (F(1, 6) = 0.049, p = 0.831).

When examining correlation between Kcal intake from bland chow and body weight, linear regression analysis illustrated a significant correlation between Kcal intake from the bland chow and body weight in control rats (Figure 4.6D; F(1,5) = 13.19, p = 0.015). A significant correlation was also observed in small litter rats (Figure 4.6D; F(1,6) = 6.974, p = 0.039).
Figure 4.1: Body weight for control and small litter rats from birth to P21

N: control litters: 8, small litters: 10. All tests are 2-way RM ANOVAs.
A: Body weight data for dams of small and control litters from postnatal days 7 – 21. No significant differences were observed between control and small litter dams (p = 0.616). B: Body weight data for pups from small and control litters from birth to weaning. A significant difference was observed between control and small litter pups (p = 0.001). Sidak’s post hoc testing found significance at P14 and P21.
**Figure 4.2: Body weight data for small and control litter rats on the bland and choice diets**

N: control-B = 8, small-B = 8, control-C = 7, small-C = 8.

A: Average body weight per rat for control and small litter rats on the bland diet. 2-way RM ANOVA analysis illustrated a significant difference between groups (p = 0.028) with post hoc testing showing these differences to lie in week 9 and 10. B: Area under the curve analysis for body weight data for rats on the bland diet. A significant difference was observed (unpaired t test; p = 0.029).

C: Body weight data for rats on the choice diet. A significant difference was observed between control and small litter rats (2-way RM ANOVA; p = 0.027), and post hoc testing illustrated this significance was in weeks 7-10. D: Body weight AUC analysis for rats on the choice diet. A significant difference was observed between small and control litter groups (unpaired t test; p = 0.027).
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Figure 4.3: Daily total Kcal consumption for rats on the bland and choice diets

N: control-B = 8, small-B = 8, control-C = 7, small-C = 8.

A: Daily Kcal consumption for small and control litters rats on the bland diet. A 2-way RM ANOVA found a significant difference between groups (p = 0.040) which post hoc testing found to be in weeks 5 and 6. B: AUC analysis for small and control litter rats on the bland diet. A significant difference was observed between groups (Mann Whitney U test; p = 0.028). C: Daily Kcal consumption for rats on the choice diet. No significant differences were observed between control and small litter rats (2-way RM ANOVA; p = 0.131). D: AUC analysis of daily Kcal for rats on the choice diet. There were no significant differences observed between control and small litter rats (unpaired t test; p = 0.131).
Figure 4.4: Daily Kcal consumption per gram body weight for rats on the bland and choice diets

A: Kcal consumption per g body weight for rats on the bland diet. There were no differences between control and small litter rats (2-way RM ANOVA; p = 0.461).

B: Kcal consumption per g body weight for rats on the choice diet. There were no differences between small and control litter rats (2-way RM ANOVA; p = 0.166).

C: AUC analysis for Kcal consumption per g body weight for rats on both the bland and choice diets. 2-way ANOVA analysis found a significant main effect of diet (p < 0.0001) but no effect of litter size (p = 0.098).

D: AUC analysis of water consumption over the 10-week testing period. 2-way ANOVA analysis illustrated no significant effect of litter size (p = 0.474) but did find a significant main effect of diet (p < 0.0001). Post hoc testing (Tukey’s) found the significance to lie between rats on the bland diet, and the control rats on the choice diet.
**Figure 4.5: Kcal Data from the Three Foods for Rats on the Choice Diet**

N: control-C = 7, small-C = 8.

A: AUC data for the three Kcal sources available to rats on the choice diet. Multiple t tests found a significant difference between control and small litter rats for the bland chow (p = 0.009) but not for the sucrose (p = 0.876) or lard (p = 0.156).

B: Kcal consumption from the bland chow over time for rats on the choice diet. A significant difference between groups was observed (2-way RM ANOVA; p = 0.009) which post hoc testing found to be significant for weeks 3 and 8.

C: Kcal consumption from sucrose for rats on the choice diet. No significant differences between small and control litter rats were observed (2-way RM ANOVA; p = 0.862).

D: Kcal consumption from lard for rats on the choice diet. No significant differences were observed between groups (2-way RM ANOVA; p = 0.161).
Figure 4.6: Percentage of Total Kcal from Bland Chow

N: control-C = 7, small-C = 8.

A: Percentage of total Kcal from the bland chow over the 10-week testing period. 2-way RM ANOVA analysis showed no significant differences between groups (p = 0.063). B: AUC analysis of % Kcal from the bland chow. No differences were observed between control and small litter rats (unpaired t test; p = 0.060). C: Linear regression analysis of % Kcal from bland chow (AUC) against body weight (AUC). No correlation was observed in control (p = 0.319, r² = 0.196, slope equation: Y = 0.084*X + 27.57) or small litter rats (p = 0.831, r² = 0.008, slope equation: Y = 0.016*X + 58.99). D: Linear regression analysis of Kcal from bland chow (AUC) against body weight (AUC). A significant correlation was observed between Kcal from bland chow and body weight in control rats (p = 0.015, r² = 0.725, slope equation Y = 0.1713*X - 3.945). A significant correlation was also observed in small litter rats (p = 0.039, r² = 0.538, slope equation: Y = 0.1623*X + 4.248).
4.2.4 Discussion

4.2.4.1 Reproductive Parameters

There were no differences observed in maternal body weight for dams of control and small litters from postnatal days 7 to 21. No attempt was made to assess maternal care, however previous studies have shown that small litter pups receive more care, with small litter dams illustrating higher levels of arched back nursing and licking in comparison to control dams (Carvalho et al., 2016). Differences in maternal care can influence emotionality (Dimitsantos et al., 2007) as well as the stress responsiveness of the offspring (Meaney, 2001).

As expected, a divergence in body weight during the pre-weaning period was observed between control and small litter rats, with small litter rats illustrating an increased body weight. This replicates previous studies (Sominsky et al., 2017b, Carvalho et al., 2016, Boullu-Ciocca et al., 2005) and is a well-established characteristic of small litter rats. This is likely due to the fact that not only do small litter rats have access to a larger quantity of milk, but also that the milk from small litter dams has a higher fat content in comparison to controls (Fiorotto et al., 1991).

4.2.4.2 Body Weight

An increased body weight was observed in small litter rats on both the bland and choice diets. This is as expected and has been reported several times in the literature (reviewed in Spencer, 2013). Although in this study body composition was not measured, previous data suggests that this increase in body weight is due to an increase in body fat (Mozes et al., 2014), which is further supported by the increased plasma leptin in small litter rats (Stefanianis and Spencer, 2012).

In previous experiments using models of food choice an increased body weight for rats on a choice diet has been observed (see 3.3.3.4). Interestingly, for this experiment no effect of diet on body weight was seen. Those studies using a food choice model and reporting an increase in body weight generally offer a sucrose solution as a component of the diet (Harris and Apolzan, 2012, Castonguay et al., 1981). Many have shown that rats will increase their calorie consumption when carbohydrate is offered as a liquid as opposed to a solid. Ramirez (1987) found that rats consumed more of a high carbohydrate diet when it was presented in a liquid form compared to when the same diet was offered as a solid. Furthermore, Sclafani (1987) showed that rats fed sucrose or glucose in addition to bland
chow consumed more calories when the carbohydrate was offered in solution in comparison to its powdered form. Ackroff et al. (2007) found that a sucrose solution was superior to solid fat to induce overeating, and also demonstrated that although nutritional intakes varied, total calorie consumption was very similar between rats offered chow with a sucrose solution and those given chow, a sucrose solution and fat. Therefore, for the development of obesity in rats on a choice diet, the presentation of sucrose in a liquid form rather than as a solid appears to be an important factor.

4.2.4.3 Total Kcal Intake

Although a difference in food consumption was observed on the bland diet, with small litter rats consuming significantly more Kcal in comparison to control litter rats, when this was normalised for body weight no significant differences were observed. No significant differences were observed in food intake for rats on the choice diet. Many studies using small litter rats as a model of neonatal overnutrition have reported hyperphagia (Spencer and Tilbrook, 2009, Rodrigues et al., 2009), however often these results are not corrected for body weight. Those which have normalised food intake to body weight have similarly found no differences in food consumption (Mozes et al., 2014, Stefanidis and Spencer, 2012).

These alterations in food intake between studies could be accounted for by minor differences in the models used. Whilst the majority use reduced litter size as a model of neonatal over-nutrition, not all papers employ the cross-fostering protocol used in this study, and the number of rats in control and small litters can differ, for example control litters can vary from 8 (Portella et al., 2015, Xiao et al., 2007) to 12 pups per litter (Liu et al., 2013, Velkoska et al., 2005). Furthermore, strain differences can have an impact on feeding behaviour (Walsh, 1980). These minor differences could perhaps account for the variation observed in terms of food intake in small litter rats.

Interestingly, whilst there is an increase in Kcal consumption per gram body weight for rats on the choice diet in comparison to those on the bland diet, no differences were observed in body weight of rats on the bland or choice diet. Larue-Achagiotis et al. (1992) found that when a choice diet was offered with three separate dietary components (protein, carbohydrate and fat) all in a solid form, rats consumed the same number of calories as chow controls, although they did illustrate a reduced body weight suggesting increased feed efficiency for rats on a chow diet (here defined as the efficiency with which calories
consumed are converted into body weight). This is in line with the experiment reported here as all the dietary components were offered in a solid form and may explain why although the rats on the choice diet in this study illustrated an increased calorie intake in comparison to those on the bland diet, no increases in body weight were observed. Furthermore, in this study energy expenditure and activity levels were not measured, so it may be that rats on the choice diet illustrated increased activity levels to compensate for the increased Kcal intake. Finally, as the rats on the bland diet are consuming more of the bland chow, they will likely have an increased protein intake in comparison to those on the choice diet. Tirapegui et al. (2012) found that rats on a high-protein diet illustrated an increased lean mass and decreased fat mass in comparison to a control group, while no differences in total body weight were observed. This may suggest that the rats on the bland diet have a higher lean mass and lower fat mass than those on the choice diet, which may explain why no differences in body weight between the two diets are seen. These data show that although small and control litter rats illustrate differences in body weight, rats on a choice diet are able to adjust their Kcal intake sufficiently to precisely maintain their body weight to similar levels to those on the bland diet.

A lower water consumption was observed between rats on the bland diet, and the control litter rats on the choice diet. This is likely due to the salt content of the two diets as both the sucrose and the lard contain minimal salt, and therefore the rats on the bland diet will have a higher overall salt consumption due to a relatively higher intake of the bland pellet, which would lead to an increased water intake (Stricker et al., 2003).

4.2.4.4 Food Choice

Interestingly in this study, an increased intake of bland chow was observed in small litter rats, while intake of sucrose and lard did not significantly differ from controls. This increased intake of chow did not result in an increased Kcal intake per g body weight. When the % of Kcal from the chow of the total Kcal was calculated, no significant differences were observed between groups although there was a trend for a larger % of Kcal from bland chow in the small litter group. When this value was correlated with body weight no correlations were found. However, when the Kcal from chow was correlated with body weight, a significant correlation was found for both control and small litter rats, suggesting that the increase in chow consumption may be due to the increased body weight of small litter rats. As no increases in lard or sucrose consumption are observed in small litter rats, it
is likely that the chow contains a component not present in the lard and sucrose that is required by small litter rats to maintain their increased body weight.

These results are similar to those observed by Schéle et al. (2016) who found an increased chow intake after ICV administration of ghrelin when using the same food choice model as implemented in this study. Furthermore, Schéle et al. (2016) showed that an increased chow intake was also observed after an overnight fast, which was almost completely reversed through the peripheral administration of a ghrelin antagonist. These results suggest that the effects of neonatal overfeeding on food choice may be driven by alterations in ghrelin signalling.

When measured at 80 days of age, lower serum ghrelin has been reported in neonatally over-nourished male rats (Fuente-Martín et al., 2012). Sominsky et al (2017a) also found lower serum ghrelin levels in small litter male rats, although this was only significant at P7. In both cases, this reduction in serum ghrelin was due to a decrease in des-acyl ghrelin, whilst acyl ghrelin levels remained similar to controls. Acyl ghrelin is the form of the peptide that acts at the GHSR1a receptor and has a role in the regulation of appetite and metabolism (Nakazato et al., 2001), as well as having a role in the development of hypothalamic feeding pathways (Steculorum et al., 2015). There is evidence to suggest an association between a higher acyl/des-acyl ghrelin ratio and obesity in both rodents (Delhanty et al., 2013) and humans (Barazzoni et al., 2007). Furthermore, Sominsky et al. (2017a) found that exogenous acyl ghrelin at P12 increased activation of the ARC and PVN in small litter rats but had no effect on control litter rats. This suggests that small litter rats, while demonstrating decreased serum ghrelin, may show enhanced sensitivity to the actions of ghrelin. This is further supported by Novelle et al. (2014) who demonstrated that small litter rats showed an enhanced orexigenic response to ICV ghrelin at P24 and P90.

The role of ghrelin in food choice in small litter rats could be tested by replicating this experiment in ghrelin receptor knockout rats and investigating whether the changes in body weight and food choice are replicated in this model (Zallar et al., 2018). Alternatively, administration of a ghrelin antagonist could also be implemented to investigate whether this is able to alter the food choices of small litter rats to similar levels of control litters rats (see section 6.3.2 for further discussion on future directions).

There is also some evidence for alterations of the reward system in small litter rats. In electrophysiological recordings from brain slices, Davidowa, Heidel and Plagemann (2002)
found increased inhibition of VMH neurons in response to dopamine in small litter rats. Portella et al. (2015) found that small litter rats less readily developed a conditioned place preference for sweet foods. Developing a place preference is dependent on the function of the mesolimbic dopamine system, and decreased levels of D2 receptors were reported in small litter rats (blockade of D2 receptors decreases the ability to form a place preference) (Portella et al., 2015). Furthermore, both leptin and insulin have been shown to act on the dopaminergic mesolimbic reward system, reducing sucrose self-administration and conditioned place preference, suggesting these peptides may act as inhibitors of the dopamine system resulting in reduced pleasure seeking (Khanh et al., 2014). Therefore, the hyperinsulinemia (Plagemann et al., 1999) and hyperleptinemia (Stefanidis and Spencer, 2012) often observed in small litter rats may alter the activity and output of VTA dopamine neurons to reduce motivation for palatable foods.

During early development, a surge in circulating leptin is involved in the development of hypothalamic feeding pathways (Bouret, 2010). Disruption to this surge in leptin can lead to disruptions in hypothalamic development and lead to permanent changes to feeding behaviour (Bouret and Simerly, 2004). As milk is one of the main sources of leptin for neonatal rodents (Oliver et al., 2002), those rats from smaller litters that have greater access to milk will be exposed to higher levels of leptin at an earlier timepoint in comparison to control neonates. It has been illustrated that small litter rats show increased leptin during the expected leptin surge at P14 (Stefanidis and Spencer, 2012, Sominsky et al., 2017b) which has been associated with increased AgRP and NPY density in the ARC (Sominsky et al., 2017b). Although these changes are not entirely leptin dependent (Sominsky et al., 2017b), it may be that increased leptin in neonatally overfed rats contributes to changes in the development of the hypothalamic feeding system that results in the development of obesity in small litter rats.
4.3 Experiment 2: The Effect of a 24 hour ‘bland chow fast’ on Food Choice in Small Litter Rats

4.3.1 Background
As an increased consumption of the bland chow was observed in small litter rats on the choice diet, it was investigated whether removing bland chow for a 24-hour period, and therefore presumably increasing motivation for this food, would lead to any changes in food choice upon refeeding. As well as investigating whether removing the bland chow would lead to changes in food choice following the fast, it was also investigated whether differences in sucrose or lard consumption would be observed between control and small litter rats when bland chow was not available.

As Conceição et al. (2016) found that small litter rats illustrated an increased preference for a high-fat diet over a high-sugar diet over a 12-hour period, it was hypothesised that when bland food was removed for 24 hours, the small litter rats would illustrate an increased preference for lard over sucrose.

4.3.2 Methods
Small and control litter rats on the choice diet from experiment 1 were used and housed under the same conditions (see section 4.2.2 for details on litter size adjustment, housing and diet). Bland chow was removed from the rats at 10am for a 24-hour period whilst maintaining *ad lib* access to the sucrose pellets and lard. After 24 hours the bland food was returned, and food and water consumption measured for the following 24 hours (food and water consumption were measured manually using a scale, see section 2.2 for further details). For this experiment the 24-hour period where bland chow was removed will be referred to as the ‘bland chow fast’ or ‘fast’.

After normality testing using the Shapiro-Wilk test, data were analysed using the appropriate parametric or nonparametric tests as described in section 2.8. Food consumption was normalised to body weight by calculating Kcal consumed per g of body weight as described in section 4.2.2.4.
4.3.3 Results

There were no significant differences observed in total Kcal consumption during the ‘bland chow fast’ period between control or small litter rats (Figure 4.7A; multiple t tests; t(13) = 0.253, p = 0.804). There were also no significant differences in the calorie consumption from sucrose (Figure 4.7A; t(13) = 1.896, p = 0.080) or lard (Figure 4.7A; t(13) = 1.417, p = 0.180) during this period.

For total Kcal consumption before and after the fast period, 2-way RM ANOVA analysis found a significant main effect of both time (F(1, 13) = 61.58, p < 0.0001) and litter size (F(1, 13) = 14.96, p = 0.002). Post hoc testing found that the difference between Kcal consumption between small and control litter rats was only significant before the fast, and that both small and control litter rats consumed fewer Kcal after the fast in comparison to before.

There was an increase in Kcal consumption from the bland chow in small litter rats for the 24 hour period before the fast (Figure 4.7C; multiple t tests; t(13) = 3.615, p = 0.003), however there were no significant differences in the consumption of sucrose (t(13) = 0.192, p = 0.850) or lard (t(13) = 0.373, p = 0.715). For the 24 hours following the chow fast there was also an increase in bland chow consumption in small litter rats in comparison to control rats (Figure 4.7D; multiple t tests; t(13) = 2.810, p = 0.015), but no significant differences in sucrose (t(13) = 1.497, p = 0.158) or lard consumption (t(13) = 1.438, p = 0.174).

When Kcal consumption per gram body weight was analysed for the 24 hours before and after the fast, there was no significant main effect of litter size (Figure 4.7E; 2-way RM ANOVA; F(1, 13) = 1.214, p = 0.291), however there was a significant effect of time (F(1,13) = 79.68, p <0.0001), with post hoc testing illustrating a lower Kcal consumption per gram body weight for both small and control litter rats for the 24 hours after the ‘bland chow fast’ compared to before the fast.

When food choice data was compared for control litter rats before and after the fast (data not shown), multiple t tests found no significant differences in the amount of bland chow (t(12) = 1.296, p = 0.219) or lard (t(12) = 0.631, p = 0.539) consumed before and after the fast, although there was a significant differences in sucrose consumption (t(12) = 4.121, p = 0.001) with control rats consuming less sucrose after the fast in comparison to before. This was also observed in small litter rats (data not shown) with no significant differences in
bland chow \( (t(14) = 0.981, p = 0.343) \) or lard \( (t(14) = 2.167, p = 0.047) \); Holm-Sidak method to determine statistical significance) consumption, although sucrose consumption was reduced following the fast \( (t(14) = 4.905, p = 0.0002) \).
Figure 4.7: Kcal intake before, during and after the 24 hours ‘bland chow fast’

N: control = 7, small = 8

A: Total Kcal consumption for lard and sucrose during the 24-hour ‘fasting’ period. Multiple t tests illustrated no significant differences in sucrose (p = 0.080), lard (p = 0.180) or total Kcal consumption (p = 0.804). B: Total Kcal consumption for the 24 hours before the ‘fast’ and after the ‘fast’. 2-way RM ANOVA analysis illustrated a significant effect of litter size (p = 0.002) and time (p < 0.001). C: Breakdown of the calorie consumption for the three foods before the ‘fast’. Small litter rats consumed a significantly larger amount of Kcal from the bland diet in comparison to control litter rats (multiple t tests; p = 0.003) but no significant differences were observed for either sucrose (p = 0.850) or lard (p = 0.715) consumption. D: Calorie consumption from the three foods for the 24 hours following the bland chow fast. Again, small litter rats consumed a significantly larger amount of the bland chow (multiple t tests).
tests; \( p = 0.015 \) but no significant differences were observed in calorie consumption from sucrose \( (p = 0.158) \) or lard \( (p = 0.174) \). E – When total Kcal consumption per g body weight was calculated, there was no significant main effect of litter size (2-way RM ANOVA; \( p = 0.291 \)), however there was an effect of time \( (p < 0.0001) \) with rats consuming fewer calories per g body weight after the 24-hour bland chow fast.
4.3.4 Discussion

4.3.4.1 Food Choice During the Fast

There were no significant differences in the total Kcal consumed during the ‘fast’ period between small and control litter rats. Furthermore, no differences were observed in the amount of sucrose of lard consumed during the chow fast. Previous studies have found a preference for sweet foods in small litter rats, showing increased consumption of sweet pellets in small litter rats when offered in addition to chow (Noschang et al., 2014).

Conceição et al. (2016) found that when offered 12 hours of access to both a high-fat and high-sugar diet, small litter rats consumed the same amount of the high-sugar diet as controls but consumed significantly more of the high-fat diet, suggesting that small litter rats may illustrate an increased preference for fat. However, these diets consisted of chow mixed with either sucrose or butter to alter the nutritional composition and were therefore still nutritionally complete unlike the pure sucrose and lard offered in this study which may have influenced the results.

4.3.4.2 Food Choice After the Fast

Small litter rats showed increased intake of the bland chow in comparison to control rats both before and after the bland chow fast. Interestingly, there was no compensation for the lack of chow consumption for 24 hours, with both small and control litter rats consuming a similar amount of chow after the ‘fast’ as before.

Compared to controls, small litter rats also showed an increased Kcal intake before the bland chow fast, however when normalised for body weight this was no longer significant. However, Kcal consumption per gram body weight was reduced in the 24-hour period after the fast compared to the 24 hours prior to the fast. This reduction in Kcal consumed after the fast is due to the reduced consumption of sucrose, as lard and chow consumption were not significantly different from consumption levels prior to the fast in both control and small litter rats. As rats slightly increased their sucrose consumption during the ‘fast’ period when no bland chow was available, this reduction in sucrose following the fast may be a compensatory mechanism for this increased consumption. Interestingly, although lard consumption was also increased during the fast period, lard consumption after the fast is similar to the standard consumption levels as seen before the fast. Previous studies have shown that rats are able to compensate for the increased calorie consumption when
offered limited access to a sugar solution or vegetable shortening by reducing their calorie intake from bland chow (Avena et al., 2009, Corwin et al., 1998). This study appears to illustrate that rats can compensate for an increased sucrose consumption by decreasing their consumption on the following day, however they do not compensate for an increased lard consumption.

Overall, the results from this study further support the data from experiment 1 suggesting that small litter rats show an increased preference for the bland chow in comparison to control rats.
4.4 Experiment 3: The Effect of Neonatal Overfeeding on Food Preference During ‘Binge’ Eating.

4.4.1 Background
Whilst the food choice model employed in experiment 1 does give a good indication of the exact nutrient preferences of the rat, it could be considered a less ethologically valid model. It is rare in real life that humans are exposed to foods composed exclusively of either fat or sugar – generally most palatable foods (and particularly ‘comfort foods’ which are associated with binge eating) contain a mixture of fat and sugar. Therefore, in order to examine the effects of offering a combined HFHS palatable pellet to small litter rats, rats were exposed to a daily 2-hour ‘binge eating’ style paradigm for 2 weeks. This is based on the model used by Bake, Hellgren and Dickson (2017) and involves providing access to a HFHS pellet for 2 hours a day, alongside regular chow, and examining food intake during this period. This model could be considered a non-homeostatic model of eating as providing a combined HFHS pellet during a short time frame encourages excessive food consumption (Berner et al., 2008).

Bake, Hellgren and Dickson (2017) found that ICV ghrelin injection altered rats preferences from a high-fat diet to chow during the 2 hour binge period. Therefore, as small litter rats illustrated similar food preferences on the chow/lard/sucrose diets as ghrelin injected rats, it was hypothesised that small litter rats would consume more chow and less of the HFHS diet in comparison to the control rats during the 2-hour binge period.

4.4.2 Methods
Rats originally on the ‘bland diet’ were exposed to a 2 hour ‘binge eating’ style paradigm, similar to that used by Bake, Hellgren and Dickson (2017). Rats were given 2 hours of access to a HFHS diet between the hours of 10:00 and 12:00 each day. This diet model generally leads to a 2-hour binge eating session where rats will consume a large amount of the HFHS diet, and usually very little or none of the bland food. Rats body weight and food consumption was measured every 24 hours (commencing at 10:00) and the total bland chow, HFHS pellet and water consumption was measured during the 2-hour ‘binge eating’ period.
Finally, following 2 weeks of exposure to the binge-eating paradigm, rats underwent a 24 hour fast in order to increase food motivation before receiving *ad lib* access to both the bland and HFHS pellets for 24 hours. Food intake was measured at 2 and 24 hours.
4.4.3 Results

4.4.3.1 Total Kcal Intake and Food Choice

During the 2-hour binge, there were no significant differences between groups in the consumption of bland chow (Figure 4.8A; multiple t tests; t(14) = 0.372, p = 0.715), although small litter rats did consume significantly more of the HFHS pellet (Fig. 9A; multiple t tests; t(14) = 2.589, p = 0.021), which led to a higher total Kcal consumption during the 2 hour period (Figure 4.8A; multiple t tests; t(14) = 2.489, p = 0.026). When Kcal consumption over 24 hours was examined, no significant differences were observed in chow consumption (Figure 4.8B; multiple t tests; t(14) = 1.304, p = 0.213), however the difference in total Kcal intake remained significant (Figure 4.8B; multiple t tests; t(14) = 2.524, p = 0.024).

When the total calorie intake per gram body weight was calculated, AUC analysis illustrated no significant differences (Figure 4.8C; unpaired t test; t(14) = 0.616, p = 0.548).

When the percentage of total calories from the HFHS diet was calculated as an index of food choice, there were no significant differences between control or small litter rats (Figure 4.8D; unpaired t test; t(14) = 1.501, p = 0.156).

When Kcal intake from the HFHS diet was correlated with body weight, a significant correlation was observed for control litter rats (Figure 4.8E; F(1,6) = 6.239, p = 0.047), however no significant correlation was observed for small litter rats (Figure 4.8E; F(1,6) = 1.672, p = 0.244).

4.4.3.2 Kcal Intake and Food Choice After Fasting

Figure 4.9 illustrates Kcal intake from the bland chow and HFHS pellet at 2 hours of refeeding following a 24 hour fast. Small litter rats consumed more of the bland chow in comparison to control rats during this period (Figure 4.9A; multiple t tests; t(14) = 3.629, p = 0.003). No significant differences were observed in Kcal consumption from the HFHS pellet (Figure 4.9A; multiple t tests; t(14) = 0.284, p = 0.220) or total Kcal consumption following 2 hours of refeeding (Figure 4.9A; multiple t tests; t(14) = 2.023, p = 0.063).

At 24 hours following refeeding, no significant differences were observed in Kcal consumption from the bland chow (Figure 4.9B; multiple t tests; t(14) = 2.078, p = 0.057), the HFHS pellet (t(14) = 0.301, p = 0.768) or in total Kcal consumption (t(14) = 1.960, p = 0.070).
FIGURE 4.8: FOOD CONSUMPTION DATA FOR RATS ON THE 2-HOUR BINGE FEEDING PARADIGM

N = 8 for all groups.

A: AUC analysis for Kcal consumption for the bland chow and the HFHS pellet during the 2-hour 'binge' period. There were no significant differences in Kcal consumption from the bland chow (p = 0.715), but there was a significant difference in consumption of the HFHS pellet (p = 0.021) and total Kcal consumption (p = 0.026, multiple t tests with Holm-Sidak correction for multiple comparisons). B: AUC analysis for total Kcal consumption per day. Once again there were no significant differences in bland chow consumption (p = 0.213), but a significant difference was observed for HFHS consumption (p = 0.021) and total daily Kcal consumption (p = 0.024, multiple t tests with Holm-Sidak correction for multiple comparisons). C: AUC analysis for Kcal per g body weight. No significant differences were observed between groups (unpaired t test; p = 0.548). D: Percentage of total daily Kcal from the HFHS diet (AUC analysis). No significant differences were observed between control and
small litter rats (unpaired t test; p = 0.156). D: Linear regression analysis for Kcal intake from the HFHS diet and body weight. In control litter rats there was a significant correlation between Kcal intake from the HFHS diet and body weight (p = 0.047; $R^2 = 0.510$; slope equation: $Y = 0.06860X - 0.6656$), however there was no significant correlation for small litter rats (p = 0.244, $R^2 = 0.218$; slope equation: $Y = 0.04773X + 14.22$)
N = 8 for all groups. All tests are multiple t tests with Holm-Sidak correction for multiple comparisons.

A: Kcal consumption 2 hours after food access following the 24-hour fast. There was a significant difference in bland chow consumption (p = 0.003), however no significant differences were observed in the consumption of HFHS diet (p = 0.220) or in total Kcal consumption during the 2-hour period (p = 0.063). B: Kcal consumption for the 24 hours period following the fast. No significant differences were observed in bland food consumption (p = 0.057) HFHS consumption (p = 0.768) or in total Kcal consumption (p = 0.070).
4.4.4 Discussion

4.4.4.1 Food Intake on the Binge Paradigm

In small litter rats, an increased Kcal intake from the HFHS diet was observed during the 2-hour binge, which led to a significantly increased total Kcal intake. This increase in total calorie consumption was significant at both the 2-hour binge and after 24 hours. No significant differences were seen in bland chow consumption between small litter and control rats. When the % of Kcal from the HFHS of the total Kcal was calculated no significant differences were observed between groups. This is likely due to that fact that there is also a marginal increased in bland food consumption (although this did not reach significance). When Kcal consumption from the HFHS pellet was correlated with body weight, a significant correlation between HFHS intake and body weight was observed in control litter rats, but no significant correlation was observed in small litter rats. However, the fact that there is no difference in Kcal intake per g body weight, or in the % of Kcal consumed from the HFHS diet suggests that the increase in Kcal intake observed in small litter rats is likely due to their increased body weight.

It may be that small litter rats illustrate a preference for the HFHS diet instead of the bland chow in this experiment because the HFHS pellet is more nutritionally complete in comparison to the separate lard and sucrose components used previously, and therefore there is no need to increase bland chow consumption for nutritional requirements (Table 2.1). It may also be that providing fat and sugar as a combined pellet is more palatable compared to the separate lard and sucrose components therefore promoting increased consumption.

Bake, Hellgren and Dickson (2017) used a similar ‘binge eating’ paradigm as the one implemented in this study. They found that acute ICV ghrelin directed food choice more towards chow during the 2-hour binge period. However, when rats received chronic ICV ghrelin treatment, they found no effect on chow intake, however did find an increase in the HFHS diet during the 2 hours scheduled feed. This is similar to what was observed in this study as small litter rats illustrated an increased consumption of the HFHS pellet during the 2-hour binge. This may again suggest that small litter rats illustrate an increased sensitivity to ghrelin.
4.4.4.2 Food Choice After Fasting

When rats were fasted for 24 hours, small litter rats demonstrated an increased consumption of the bland chow, although this was only apparent 2 hours after refeeding and was no longer significant by 24 hours. When *ad lib* access to the HFHS pellet and bland chow was given for 24 hours after the fast, no differences were observed in Kcal consumption from the HFHS pellet, or in total Kcal consumption over the 24-hour period. It has been shown that rats consume more calories when placed on a binge feeding model compared to *ad lib* access to a HFHS diet (Berner et al., 2008), and therefore it may be that the palatable pellet is more rewarding when only restricted access is offered. Therefore, it appears that a bland food preference in small litter rats is only apparent for a short period following a fast, however when food intake is measured over a full day following fasting this difference is no longer significant.

By fasting the rats in this experiment (and therefore presumably increasing ghrelin secretion, Bagnasco, Kalra and Kalra, 2002) a shift of food preferences towards bland chow 2 hours following refeeding was observed in the small litter rats, as was found by Bake, Hellgren and Dickson (2017) following acute ICV ghrelin injection. These results could again suggest that small litter rats illustrate an increased sensitivity to ghrelin that is able to influence food preferences.
4.5 Chapter Discussion

4.5.1 Experimental Findings and Hypotheses

It was hypothesised that small litter rats would show an increased preference for high energy foods such as sucrose and lard.

Unexpectedly, it was found that small litter rats increased their consumption of bland chow in comparison to control litter rats when offered an *ad lib* free choice of chow, sucrose and lard. This increase in chow intake correlates with body weight and therefore suggests that the increased chow consumption is due to a nutritional need to maintain the increased body weight of small litter rats. When offered a combined HFHS pellet on a daily 2-hour binge feeding schedule this preference for bland food was no longer apparent, and instead an increased consumption of the HFHS pellet during the 2-hour scheduled feed was observed in small litter rats. This increase is also likely due to body weight, and a preference of the HFHS pellet over chow may be observed as this pellet is more nutritionally complete in comparison to the separate sucrose and lard components. These alterations in food choice in small litter rats may be driven by an enhanced ghrelin sensitivity, changes to the reward system, or endocrine alterations affecting the development of the hypothalamic feeding pathways.

4.5.2 Experimental Limitations

4.5.2.1 Male Biased Litters

Due to the unexpectedly high number of pup rejections, the litters in this study (particularly the small litters) were male biased in order to ensure that there were enough male offspring for subsequent experimental testing. There is evidence that litter composition can have an impact on the offspring’s behavioural parameters. Michaels and Holtzman (2006) found that litters comprising of only male offspring had a reduced sucrose intake in comparison to mixed sex litters, although this effect was only observed following maternal separation stressor. This suggests the importance of ensuring mixed-sex litters to ensure that the effects observed are not influenced by litter composition.

4.5.2.2 Model Limitations

One of the problems associated with using litter size adjustment as a model of neonatal overnutrition is that changes in maternal behaviour can be observed dependent on litter
size, with dams raising small litters illustrating more maternal behaviours such as licking and arched back nursing in comparison to dams raising larger litters (Carvalho et al., 2016). As previously discussed, maternal behaviour can have an influence on the behaviour and physiology of the offspring, and therefore it is difficult to establish specifically which effects are due solely to the neonatal overnutrition. Furthermore, there may be problems associated with cross-fostering of litters and artificially changing litter sizes, as dams may cope better with the litter size that they produce rather than an artificially adjusted litter, as physiological and behavioural alterations that could affect maternal care and milk nutrition may have occurred during the prenatal period in accordance to litter size (Dimitsantos et al., 2007).

One way in which this could be prevented is through artificial hand rearing of pups to prevent the effects of maternal behaviour impacting on the offspring, such as the high carbohydrate milk model used by Patel and colleagues (reviewed in Patel and Srinivasan 2011). This method also allows precise control of milk and nutrient consumption during the pre-weaning period. However, artificial rearing can also impact on the offspring’s brain development (Auestad et al., 1990) and behaviour (Kaneko et al., 1996), which could be problematic when measuring certain outcomes.

### 4.5.2.3 Sex Differences

In this study only male offspring were tested. There is some evidence for sex differences in litter size manipulation models. Argente-Arizon et al. (2016) found that differences in body weight between control and small litters persisted for longer in male than female rats. Furthermore, it was also observed that increased food intake, whilst briefly observed in both sexes immediately after weaning, was only significant in male rats at around 19 weeks of age (Argente-Arizon et al., 2016). Sominsky et al. (2017a) found increased plasma ghrelin at P7 in small litter male rats, as well as increased c-Fos immunoreactivity in the ARC and PVN in response to exogenous acyl ghrelin. However when neonatally overfed female rats were tested, no differences in circulating ghrelin were observed, although alterations to the pituitary ghrelin system were found (Sominsky et al., 2017c). As there are also general sex differences in the feeding behaviour of rodents (Klump et al., 2013, Chen et al., 2015), it is important to further investigate whether the effects of litter size observed in this study are also present in female rats.
4.5.3 Overcoming the Effects of Small Litter Rearing

As well as examining the mechanisms behind the weight gain observed in small litter rats, it is also important to examine possible preventative measures that could prevent the effects associated with neonatal overnutrition.

There has been some evidence that calorie restriction from early post-weaning to adulthood is able to prevent some of the weight gain effects associated with small litters. Lui et al. (2013) illustrated that mild calorie restriction (by implementing pair feeding with control rats) was able to suppress some of the programming effects associated with small litter rats, however once ad lib feeding was resumed these rats experienced catch up growth to levels similar to ad lib fed small litter rats. However, moderate calorie restriction had a longer lasting effect on the suppression of the development of obesity once ad lib feeding resumed, although further evidence is required to examine how long term these suppression effects persist.

Sominsky et al. (2017b) found that early life administration of a leptin antagonist did not prevent increased body weight or leptin levels in small litter rats, although hypothalamic AgRP immunoreactivity was reduced. Therefore, although not all of the effects of small litter rearing are leptin dependent, treatment with a leptin antagonist is able to reverse some of the changes to hypothalamic circuitry observed in small litter rats. This suggests that the effects of small litter rearing are multifactorial and further investigations into factors that can mitigate these effects should be performed.
5 Positive Affect and Feeding

5.1 Chapter Background
There have been many links established between negative emotional states such as anxiety and stress and feeding behaviour, specifically the notion that certain negative states (such as anxiety and anger) can lead to an increased preference for high-fat and high-carbohydrate ‘comfort foods’ (Gibson, 2012). However, there is still very little evidence concerning the effect of a positive mood on food choice. While it is often asserted that a positive mood may lead to ‘healthier’ food choices, the experimental evidence is still conflicted. Gardner et al. (2014) found that participants in a positive mood consumed a larger proportion of healthier foods whereas those in a negative mood consumed a larger proportion of unhealthier foods. In contrast to this, Evers et al. (2013) found that those in a positive mood were more likely to consume more calories during a 10 minute taste test in comparison to control participants.

There are many problems associated with the study of positive mood in humans. Many studies rely on self-reporting or questionnaires to gain information on mood states which, due to the subjective nature of mood, may not lead to consistent results (Hammersley et al., 2014). Furthermore, induction of positive states is often achieved through presentation of pictures and films which, based on the participants previous experiences, can also lead to varying results (Burgdorf and Panksepp, 2006). Additionally, in humans food intake can be influenced by many other factors such as dietary restraint (Johnson et al., 2012) and social context (Patel and Schlundt, 2001). Therefore, the importance of developing a robust animal model of positive affect is crucial to allow for the in-depth study of neural pathways and behaviours related to positive affect.

Recently, ultrasonic vocalisations (USVs) have been used as a measure of affect in rats, with vocalisations of around 50 kHz associated with positive states such as sexual and play-like behaviours, whereas 22 kHz USVs have been associated with negative states such as aggression and drug withdrawal (Burgdorf et al., 2011). In 2000, Panksepp and Burgdorf found that by mimicking a rats rough and tumble play behaviour through a tickling interaction with the experimenter, an increase in 50 kHz USVs was observed, which is assumed to relate to an increased positive affect (Panksepp and Burgdorf, 2000). Evidence
suggests that this interaction is highly rewarding to rats as it has been well established that contact latency to the hand of the experimenter following tickling interaction is shorter in rats who have been tickled in contrast to light touch controls (Hori et al., 2009, Yamamuro et al., 2010). Additionally, tickled rats show a preference for an experimenters’ hand over an anaesthetised conspecific (Burgdorf and Panksepp, 2001) and show more 50 kHz USVs when exposed to an experimenter’s hand than rats receiving passive contact (Cloutier et al., 2012). Furthermore, tickling is able to induce stress resilience in rats (see section 1.5.3), further suggesting that it induces positive affect (Burgdorf et al., 2017).

Since the discovery of this model, it has been found that there are stable individual differences between rats and their levels of vocalisation (Mallo et al., 2007), as well as that heterospecific play leads to increased dopamine release in the nucleus accumbens (Hori et al., 2013a). Furthermore, Hori et al. (2009) found an increase in expression of genes regulating feeding behaviour in adolescent rats who had been exposed to repeated tickling stimulation, including genes encoding POMC, AgRP and NPY. However, although this model has been used in many studies of positive affect, the impact of positive affect on feeding behaviour and food choice has remained unstudied.

The aim of this chapter was to use a rat model of positive affect to investigate the effects of positive affect on food choice, as well as establish whether a food reward could induce positive affect.
5.2 Pilot Experiments

5.2.1 Background

Pilot studies were carried out prior to the feeding behaviour experiments to ensure replication of the rat model of positive affect. An increase in 50 kHz USVs was expected in rats exposed to daily heterospecific play (also referred to as ‘tickling’ or ‘social contact’). A social contact model based on that used by Burgdorf and Panksepp (2001) was implemented for these studies, and as a control a group of rats were exposed to passive contact which consisted of the experimenter’s hand resting passively in the cage.

5.2.2 Pilot Experiment General Methods

All rats were housed under standard conditions as described in section 2.1.1.

In order to group rats according to USV characteristics, a 5-minute baseline recording was made for all rats prior to the start of the experiment (see 2.6.2 for details).

The recording arena consisted of an empty cage with a microphone positioned approximately 30 cm above the floor of the cage (see section 2.6.1). Recording was carried out using an Avisoft-UltraSoundGate 116H recorder with microphone using Avisoft RECORDER USGH.

Rats in the ‘contact’ group were placed in the recording arena and received 30 seconds of passive contact. This was then followed by 30 seconds of contact from the experimenter which mimicked the natural rough and tumble play behaviour of the rat (this involved vigorous, whole body stimulation of the rat, including repeated pinning, with care taken not to harm the rat). This cycle was repeated 5 times, leading to a total recording time of 5 minutes. Control rats received an equal amount of time of ‘passive contact’ whereby they were recorded for a period of 5 minutes with the experimenter’s hand resting passively in the cage.

As a measure of motivation to receive social contact, contact latency was also recorded at the beginning and end of the experiments. Contact latency was measured by placing the rat in one corner of the recording arena and placing the experimenters hand in the opposite corner of the arena, total time taken for contact (front paws or nose) was recorded in seconds. If there was no contact at 180 seconds the recording was stopped.
5.2.2.1 Data Analysis

USVs were scored manually from recordings following the social contact sessions from spectrograms generated using Avisoft SASLab Lite (see section 2.6.4 for further details). Number of USVs was divided by total recording time to provide a value for number of 50 kHz USVs/min.

USV data were tested for normality and normally distributed data were analysed using 2-way RM ANOVA or unpaired t testing. For data not fitting a normal distribution, AUC analysis was implemented, and parametric or non-parametric statistical testing performed as appropriate.

Contact latency data were analysed using 2-way RM ANOVAs, with Sidak’s post hoc testing to determine the location of any significant differences.
5.2.3 Pilot Experiment 1 – Sprague Dawley
The first pilot experiment consisted of 10 male Sprague Dawley rats of approximately 7 weeks of age (bred in-house). Rats were group housed in a group of 4 (control) and two groups of 3 (contact). Rats received daily 5-minute social contact sessions starting at 10am for a period of 8 days.

5.2.3.1 Results

Figure 5.1A shows the number of 50 kHz USVs per minute over the 8 days of simulated social contact/passive contact. Two-way repeated measures ANOVA analysis illustrated no significant differences between control and contact groups (F (1, 8) = 0.474, p = 0.511). This was confirmed by AUC analysis which also illustrated no significant differences between groups (Figure 5.1B; Mann Whitney U test; U = 10, p = 0.724).

There was an increase in 22 kHz USVs observed in the contact group, particularly during the first 4 days of social contact (Figure 5.1C), however AUC analysis found no statistically significant differences between groups (Figure 5.1D; t(8) = 1.322, p = 0.223, unpaired t test).

Contact latency for control and contact groups on day 0 (before social contact) and day 8 (after social contact) is displayed in Figure 5.1E. No significant differences were observed between groups (2-way RM ANOVA; F(1,8) = 0.419, p = 0.536) or before and after the social stimulation (F(1,8) = 3.814, p = 0.087), although a trend for a reduced contact latency after the experiment, particularly for those in the contact group, was observed.
**Figure 5.1: Ultrasonics Data for Pilot Experiment 1 (Sprague Dawley Rats, 7 Weeks of Age)**

- **A:** Total number of 50 kHz USVs per minute over the 8 days of simulated social contact. Two-way RM ANOVA analysis illustrated no significant differences between control or contact rats (p = 0.511).

- **B:** AUC analysis for the number of 50 kHz USVs per day. No significant differences were observed between groups (p = 0.724, Mann Whitney U).

- **C:** Number of 22 kHz USVs per minute over the 8 days of testing (no statistical testing performed as data were not normally distributed.)

- **D:** AUC data for the number of 22 kHz per day. No significant differences were observed between groups (p = 0.223, unpaired t test).

- **E:** Average contact latency for both groups before and after receiving 8 days of simulated social contact. Two-way RM ANOVA analysis found no significant differences between groups (p = 0.536) or before and after the contact (p = 0.087).

n: control = 4, contact = 6
5.2.3.2 Discussion

This first pilot experiment was unable to replicate the increase in 50 kHz USVs generally exhibited when rats are subjected to contact simulating social play. Previous experiments have shown a significant increase in vocalisations after only a few sessions of simulated social contact (Panksepp and Burgdorf, 2000, Burgdorf and Panksepp, 2001, Mallo et al., 2007), however this experiment found no significant differences even after 8 days of the social contact paradigm. Other researchers have had success in recording USVs from Sprague Dawley rats (Cloutier et al., 2012, Cloutier et al., 2013), however some have reported that Sprague Dawley vocalisation levels are lower than observed in other literature, and can take up to 14 days to stabilise (Popik et al., 2012).

There was a trend for an increased number of 22 kHz USVs emitted by the rats in this experiment. As these are often expressed in negative situations (Brudzynski, 2013) this may suggest that the rats were averse to the tickling procedure. However, there was a trend for a decrease in contact latency in both groups (particularly the contact group) which suggests that the rats were motivated to receive the social contact.
5.2.4  Pilot Experiment 2: Long Evans

Following on from the first pilot study, Long Evans rats were tested using the same social contact paradigm. Long Evans rats are regularly used in USV research by Burgdorf, Panksepp and colleagues and show a robust increase in 50 kHz USVs when receiving simulated social contact (Burgdorf and Panksepp, 2001, Burgdorf et al., 2008, Panksepp and Burgdorf, 2000). Furthermore, as there is evidence that rats in individual housing emit larger numbers of 50 kHz USVs when ‘tickled’ in comparison to rats in group housing (Panksepp and Burgdorf, 2000), for future experiments all rats were individually housed.

5.2.4.1  Methods

6 male Long Evans rats (bred in-house) at around 9 weeks of age, were individually housed (control n = 2; contact n = 4). These rats received 5 minutes of daily simulated social contact (or passive contact) at 10am for 7 days.

5.2.4.2  Results

Figure 5.2A and Figure 5.2B illustrate the number of 50 kHz USVs per minute between control and contact groups over the 7 days of the experiment. Due to small n numbers no statistical analyses were performed, however upon observation there appears to be no differences between the two groups. Figure 5.2C & D show the number of 22 kHz USVs per minute for control and contact rats over the course of the experiment. Statistical analyses were not performed due to low n numbers, however there appears to be a slight increase in 22kHz USVs in the contact group, particularly during the first 3 days of social contact, although the variability between rats was very large.

Contact latency was measured on day 0 (before social contact) and after 7 days of social contact (Figure 5.2E). No statistical analyses were performed due to low n numbers, although there does appear to be an increase in contact latency at the end of the experiment for both control and contact rats.
Figure 5.2: Ultrasonics Data for Pilot Experiment 2 (Long Evans Rats, 9 Weeks of Age)

N: control = 2, contact = 4
No statistical analyses performed due to low n numbers
A: 50 kHz USVs per minute over the 7-day testing period. B: AUC data for 50 kHz USVs over the week of testing. C: 22 kHz per minute over the 7 days of testing. D: AUC data for 22 kHz USVs. E: Contact latency for control and contact groups on day 0 (before social contact) and day 7 (after social contact).
5.2.4.3 Discussion

This pilot study using Long Evans rats at 9 weeks of age was also unable to replicate the expected increase in 50 kHz USVs during simulated social contact. Similarly to the first pilot experiment, an increase in 22 kHz USVs was observed suggesting an aversion to the tickling, although by day 4 of social contact there were no more 22 kHz USVs present. Furthermore, there was an increase in contact latency from the end of the experiment compared to the beginning. This also suggests that the contact was not rewarding as the rats were less motivated to receive contact from the experimenter’s hand.

Many studies have successfully used Long Evans rats with a similar tickling protocol (Burgdorf and Panksepp, 2001, Burgdorf et al., 2008, Panksepp and Burgdorf, 2000), however in these cases the rats were younger than those used in this pilot experiment (between 30-50 days of age). There is evidence that adolescent rats vocalise more than older rats during simulated social contact (Panksepp and Burgdorf, 1999), likely due to the fact that rat conspecific play peaks at 30-40 days of age and then declines with sexual maturity (Salchner et al., 2004, Pellis and Pellis, 1990). Therefore, to ensure that it was not age influencing the number of USVs observed, the next pilot experiment used rats of a younger age.
5.2.5 Pilot Experiment 3: Wistar

Due to difficulties in sourcing young wild-type Long Evans rats, Wistar rats were used for the third pilot experiment. Wistars have been successfully used in previous USV research (Wohr and Schwarting, 2007, Wöhr et al., 2009). Furthermore, the length of this pilot study was increased to ensure that there was a sufficient amount of time for the rats to habituate to the social contact.

5.2.5.1 Methods

For the final pilot study, 12 male Wistar rats (Charles River UK) around 4 weeks of age were individually housed (control = 6, contact = 6). Rats were tickled (or received passive contact) for 5 minutes a day for 15 days.

5.2.5.2 Results

Although there were no differences in the number of 50 kHz USVs for over a week of contact, at approximately 12 days of contact an increase in 50 kHz USVs was observed in the contact group (Figure 5.3A) and when AUC analysis was performed a significant difference was observed between control and contact groups (Figure 5.3B; Mann Whitney U test; U = 0, p = 0.002). An increase was also observed in the number of 22 kHz USVs per minute in the contact group (Figure 5.3C) although AUC analysis showed that this difference was not significant between control and contact groups (Figure 5.3D; Mann Whitney U test; U = 9, p = 0.182).

Both groups illustrated a reduced contact latency after the experiment compared to before the experiment (Figure 5.3E). Statistical analysis found no significant effect of group (2-way RM ANOVA, F(1,10) = 0.909, p = 0.363) although a significant main effect of time was observed (F(1,10) = 32.16, p = 0.0002). Sidak’s post hoc testing determined this significance for rats in the contact group before and after the 15 days of tickling stimulation.
**Figure 5.3: Ultrasonics Data for Pilot Experiment 3 (Wistar Rats, 4 Weeks of Age)**

N: control = 6, contact = 6  
A: 50 kHz USVs per minute over the 15-day testing period. (Not normally distributed, no statistical testing).  
B: AUC data for 50 kHz USVs. A significant difference was observed between control and contact groups (p = 0.002, Mann Whitney U test).  
C: 22 kHz per minute over the 15 days of testing. (Not normally distributed, no statistical testing).  
D: AUC data for 22 kHz USVs. No significant differences were observed between groups (p = 0.182, Mann Whitney U test).  
E: Contact latency for control and contact groups on day 0 (before contact) and day 15 (after contact). Two-way RM ANOVA analysis found no significant differences between groups (p = 0.363) but did find a significant effect of time (p = 0.0002). Sidak’s post hoc testing confirmed this significance for the contact group before and after the social stimulation.
5.2.5.3 Discussion

This experiment found an increase in the number of 50 kHz USVs exhibited by the contact group during the stimulated social contact sessions, although this was not expressed until 12 days of social contact. However, there was considerable variability in this data, with not all rats illustrating this behaviour. Furthermore, the number of 50 kHz USVs observed per minute, whilst increased did not reach levels previously described, where rats have been shown to emit as many as 50 USVs during 15 seconds of social contact (Burgdorf et al., 2001, Panksepp and Burgdorf, 2000, Burgdorf and Panksepp, 2001).

Similarly to the previous pilot experiments, some of the rats in the contact group exhibited an increase in 22 kHz USVs, although this was extremely varied between individual rats. Upon closer inspection of the raw data, only one rat in the contact group consistently emitted 22 kHz USVs, although these had reduced by day 10 of the tickling procedure. This may suggest an aversion to the tickling procedure. It has been previously reported that USVs call rates in response to simulated social contact can vary between rats (Mallo et al., 2007). Low-call rats have been shown to be less motivated to receive social contact from an experimenter, and illustrate increased 22 kHz call rates, whereas high-call rats exhibit very few 22 kHz USVs (Panksepp and Burgdorf, 2000). Therefore, the differences observed in this study may simply be due to inherent individual differences of the rats.

In this study, both groups showed a trend for a reduced contact latency at the end of the experiment compared to the start, with the contact group showing a significant reduction in contact latency in comparison to the start of the experiment. This is expected as the rats in the contact group are receiving the rewarding stimulation from the experimenter and should therefore have a higher motivation to approach the experimenters hand in comparison to the control group.

Although the results in this pilot study were varied amongst rats, it is likely that had the tickling procedure been continued for a further few weeks, more of the rats would have illustrated increased levels of 50 kHz USVs, and numbers of 50 kHz USVs would likely have also increased. Furthermore, as contact latency was reduced in the contact group this suggests that these rats found the simulated social contact rewarding. Therefore, due to the results from this pilot experiment, Wistar rats of around 4 weeks of age were used for all other experiments measuring ultrasonic vocalisations.
5.2.6 Pilot Studies Summary

Overall the evidence from these pilot studies suggests that there may be an effect of strain and age on the likelihood of exhibiting an increased number of 50 kHz USVs during simulated social contact. Furthermore, it appears that it can take longer than described in previous literature to reliably induce an increase in vocalisations.
5.3 Experiment 1: The Effect of a Palatable Food Reward on USVs.

5.3.1 Background

The rewarding properties of certain food types has been well established (Berthoud et al., 2011). This is linked to the phenomenon of comfort eating whereby foods, often high in fat and sugar, are consumed due to particular emotional states (generally negative states) as opposed to eating due to hunger (Spence, 2017). Consumption of palatable foods following a period of stress has been shown to be able to blunt the stress response (Dallman et al., 2005), and therefore it is often assumed that consumption of certain rewarding foods are able to elicit a positive mood, however there is still very little evidence for this.

Therefore, the aim of this first experiment was to establish whether consumption of a food reward in rats can induce a positive affect (as measured by 50 kHz USVs). Previous evidence has illustrated that rats with access to sweetened condensed milk (SCM) show an increase in 50 kHz USVs with presentation of a tone indicating the start of milk access (Brenes and Schwarting, 2014). This suggests an induction of positive affect in anticipation of a food reward. There is also evidence of increased 50 kHz USVs with food presentation after a period of fasting (Burgdorf et al., 2000). However, there is still a lack of evidence to show whether consumption of a palatable food reward in a satiated state can induce a positive affect. Therefore, this experiment involved schedule feeding rats sweetened condensed milk for 10 days and measuring USVs before, during and after consumption to investigate whether an increase in USVs would be observed after consumption of SCM. Furthermore, recordings were continued for one day following the scheduled feeding of SCM to observe whether not receiving an expected food reward would have an impact on USVs.

It was hypothesised that 50 kHz USVs would be increased before and after SCM consumption in comparison to controls, as both the anticipation and consumption of SCM would induce a positive affect.
5.3.2 Methods

5.3.2.1 Animals

16 male Wistar rats obtained from Charles River at approximately 4 weeks of age were singly housed and maintained on an *ad lib* diet of standard lab chow (RM1) and water. Before the experiment a 5-minute baseline recording was made from each rat, and rats were grouped into two groups of 8 (control and SCM) to ensure an equal spread of USV characteristics across groups (see 2.6.2).

5.3.2.2 SCM Access

Rats were habituated to individual housing and handling for 5 days prior to the experiment. For 48 hours prior to SCM access, the SCM group were habituated to having a small glass bowl (for SCM presentation) in their cage. Following this, the SCM group received 10 minutes of access to 5ml of sweetened condensed milk (diluted 50% with tap water) for 10 days at the same time each day. Food and water was removed 15 minutes before SCM access to act as a cue and USV recording from the rat’s home cages began 5 minutes before milk access, continued throughout milk access and for 5 minutes following (20 minutes of recording in total). Control rats had their food and water removed and USVs were then recorded 10 minutes following food removal, for a total of 10 minutes. USVs were recorded on days 1, 3, 7 and 9 of milk access.

On day 11 following the milk access, SCM was withheld from rats in the SCM group to determine the effects on food anticipation. USVs were recorded 10 minutes following food removal for a total of 20 minutes. Control rats were recorded as usual.

5.3.2.3 Data Analysis

Body weight and water data were analysed using 2-way RM ANOVA. Kcal intake from bland chow during SCM access was analysed by comparing average values for AUC.

USVs were manually counted from recordings using Avisoft SASLab Lite. The total number of 50 kHz USVs was divided by the time to give counts for the total number of USVs per minute before, during and after milk consumption. USV data was analysed using Kruskal Wallis tests.

Two rats in the SCM group did not consistently consume all of the SCM and were therefore excluded from all analysis.
5.3.3 Results

5.3.3.1 Body Weight and Food Intake

There were no significant differences observed in body weight between control and SCM groups (Figure 5.4A; 2-way RM ANOVA; F (1, 12) = 0.021, p = 0.887). There were also no significant differences in water consumption between control and SCM groups over the experiment (Figure 5.4B; 2-way RM ANOVA; F (1, 12) = 0.031, p = 0.863). Finally, there were no differences observed in food consumption (Figure 5.4C) which was confirmed by AUC analysis (Figure 5.4D; unpaired t test; t(12) = 1.392, p = 0.189).

5.3.3.2 SCM Access and USVs

When the number of USVs was examined on Day 9 of milk access, no differences were observed in the number of 50 kHz USVs between controls and the SCM group either before, during or after milk access (Figure 5.5A; Kruskal Wallis test; F = 4.552, p = 0.208).

5.3.3.3 USVs With Expected SCM Access

There were no differences in the number of USVs between the control group or the SCM groups during anticipation of SCM access, or when access was not given at the anticipated time (Figure 5.5B; Kruskal Wallis test; F = 0.086, p = 0.963).
FIGURE 5.4: BODY WEIGHT, FOOD AND WATER DATA

N: control = 8, SCM = 6. Shaded area indicates the 10 days of SCM access.
A: Body weight data over the 24 days of measurement. No significant differences were observed between groups (p = 0.887, 2-way RM ANOVA). B: Water consumption over the course of the experiment. No significant differences were observed between groups (p = 0.863, 2-way RM ANOVA). C: Daily Kcal consumption from bland chow. (Not normally distributed, no statistical testing). D: AUC analysis for daily Kcal consumption during 10 days of SCM access (shaded area). No statistically significant differences were observed between groups (p = 0.189, unpaired t test).
Figure 5.5: Ultrasonics Data for Experiment 1

N: control = 8, SCM = 6. Statistics are Kruskal Wallis tests.
A: Number of 50 kHz USVs per minute observed in the control group and the SCM group before, during and after SCM access on Day 9 of milk access. No significant differences were observed between groups (p = 0.208).
B: Number of 50 kHz USVs in control rats and SCM group before expected SCM access and when no SCM was provided at the anticipated time (no SCM). No significant differences were observed between groups (p = 0.963).
5.3.4 Discussion

5.3.4.1 Kcal Consumption

No differences were observed in body weight or water consumption between control and SCM groups. A decrease in Kcal intake from bland food in the SCM groups may be expected as it has been previously shown that rats are able to compensate for the calories provided by a small food reward by reducing their bland food consumption (Hume et al., 2016), however in this experiment no differences in food consumption were observed.

5.3.4.2 USVs in Response to Food Reward

No differences were observed in the number of 50 kHz USVs in SCM rats either before, during or after consumption of a palatable food reward. This is not in line with previous evidence which has found that 50 kHz USVs increased during the presentation of a tone which indicated the start of 30 minutes of access to SCM (Brenes and Schwarting, 2014). As USVs were not recorded immediately after the removal of bland food, 15 minutes prior to milk consumption, it may be that any increase in USVs in response to this cue were missed. However, prior to recording, the cage was moved to the recording area and the cage lid removed which should have acted as a further cue for receiving SCM, however there was still no effect observed. It may be that the cue used in this study (removing bland food) was not strong enough to induce this increase in 50 kHz USVs and perhaps a more robust cue immediately before food presentation (such as a buzzer) may have been more successful.

50 kHz USVs were also not increased after the consumption of palatable food, which suggests that the presentation of a food reward (in the form of sweetened condensed milk) is not sufficient to induce positive affect in the rat. This may be due to the fact that the rats were in a satiated state, which may reduce the reward value of the SCM (Cameron et al., 2014) as previous evidence has shown that access to food after a period of fasting leads to increased levels of 50kHz USVs (Burgdorf et al., 2000).

5.3.4.3 Baseline 50 kHz USVs

In this study, an increased number of 50kHz USVs was observed in the rats at baseline compared to the pilot studies. For example, the control rats in this study illustrated an average of around 7 USVs per minute which is much larger than observed in the pilot studies. This may be because in this study, all rats were recorded in their home cages,
rather than in the recording arena previously used. This increase in USVs is likely due to the presence of bedding in their home cages as Natusch and Schwarting (2010) illustrated that rats vocalised more in cages containing bedding in comparison to empty cages. It may also be that the novelty of the recording arena was suppressing 50 kHz USVs due to increased stress (Schwarting and Wöhr, 2012). This could also explain why it took longer to induce an increase in the number of 50 kHz USVs during simulated social contact in the pilot studies. Therefore, for all further USVs experiments, ultrasonics recording was carried out in the rat’s home cages where possible.
5.4 Experiment 2: Positive Affect and Food Choice

5.4.1 Background

It is often asserted that a positive mood is associated with ‘healthier’ food choices, although evidence is conflicting. Some have found reduced consumption of ‘unhealthy’ foods in subjects in a positive mood (Gardner et al., 2014, Turner et al., 2010) whilst others have found increased food intake (Evers et al., 2013, Macht et al., 2002). As these experiments have all been carried out on human subjects and can therefore be influenced by variety of external factors, the aim of this experiment was to use a rat model of heterospecific play to examine the short-term effects of positive affect on food choice.

Following 10 days of simulated social contact, rats were given access to a sucrose solution for 1 hour and sucrose and water consumption was measured in order to examine any differences in sucrose intake or sucrose preference. To ensure concurrence with previous USV studies, the tickling procedure as detailed by Burgdorf and Panksepp (2001) was implemented in this study. This consists of a 2-minute protocol alternating between tickling and passive contact every 15 seconds as opposed to the 5-minute protocol used previously in the pilot studies.

There is evidence to suggest a role for oxytocin in the regulation of mood states. Both systemic and central administration of carbetocin, an oxytocin analogue, has antidepressant actions when measured in the forced swim test, an effect which is prevented by administration of an oxytocin receptor antagonist (Chaviaras et al., 2010). Oxytocin receptor activation has also been suggested to play a role in the affective state produced by the recreational drug MDMA (Broadbear et al., 2011). In rats, stroking like stimulation has been shown to increase emission of 50 kHz USVs, as well as inducing expression of c-Fos in oxytocin neurons of the PVN and SON (Okabe et al., 2015). Central oxytocin administration has been shown to act as an anorexigen, an effect which is particularly potent for sweet tasting carbohydrates (Olszewski et al., 2016). Therefore, the hypothesis for this experiment was that a positive affect, as induced by heterospecific play, would lead to a decreased consumption of sucrose, and therefore a reduced sucrose preference, in comparison to passive contact controls.
5.4.2 Methods

5.4.2.1 Power Analysis

Post hoc power analysis was performed on data from pilot experiment 3 to determine the sample size required for greater than 80% probability of detecting a significant difference in the number of USVs between control and contact groups. It was estimated that a sample size of 6 was required, and therefore a sample size of 8 rats per group was chosen to ensure significant power in the event that any rats were excluded from analysis.

5.4.2.2 Animals

16 male Wistar rats from Charles River at approximately 4 weeks of age were singly housed (in large cages to ensure adequate space for social contact) and maintained on an ad lib diet of standard lab chow (RM1) and water. Before the experiment a 5-minute baseline recording was made from each rat (in their home cages), and rats were grouped into two groups of 8 (control and contact) to ensure an equal spread of USV characteristics across groups (see section 2.6.2).

5.4.2.3 Experimental Procedure

For 3 days following habituation to the individual housing, all rats were given 1 hour of access to a 5% sucrose solution along with their normal food and water in order to overcome neophobia (Modlinska et al., 2015). For 10 days following this, the contact group were subjected to a 2-minute tickling procedure. The experimenters hand was placed in the cage for 15 seconds before they began ‘tickling’ the rats. In short this involved vigorous finger movements across the whole body of the rat and included pinning down and tickling of the stomach. Whilst the stimulation was firm, care was taken not to harm the rat. This procedure was repeated 4 times leading to a 2-minute recording time in total. For control rats the experimenter rested their hand passively in the cage for 2 minutes. Tickling took place in the rat’s home cages and was completed at the same time every day.

On days 11-13 of social contact, rats were given 1-hour access to a sucrose solution directly after the tickling stimulation/passive contact. Consumption of sucrose solution and water was measured in this time.
5.4.2.4  Data Analysis

Body weight, Kcal intake and water intake over time was analysed using 2-way repeated measures ANOVA. Percentage change for Kcal intake from baseline (day 4) was calculated for daily Kcal intake during the contact period. This was calculated for each rat for each day and then averaged to obtain an average value per rat.

USVs were manually analysed from recordings using Avisoft SASLab Lite to count the total number of 50 kHz USVs emitted in the 2-minute recording period. Counts were averaged to give the number of USVs per minute. USVs over time were analysed using AUC analysis.

Contact latency data was analysed using 2-way repeated measures ANOVA.

Delta values were calculated for sucrose consumption data. In order to achieve this, values for sucrose consumption and sucrose preference at baseline were subtracted from values obtained on day 13 (final day of social contact) for each rat. The differences between groups were then analysed using Mann Whitney U and t-tests (as appropriate after testing for normality).
5.4.3 Results

5.4.3.1 Body Weight, Food and Water Data

Over the 18-day testing period, there were no significant differences observed in body weight between control and contact groups (Figure 5.6A; 2-way RM ANOVA; F (1, 14) = 0.006, p = 0.941). There were also no significant differences observed between control and contact rats for daily Kcal intake (Figure 5.6B; 2-way RM ANOVA; F (1, 14) = 1.552, p = 0.233), however when percentage change was calculated for each day during the contact period compared to baseline (day 4) and averaged for each rat (Figure 5.6C), a significant difference was observed (unpaired t test, t(14) = 2.171, p = 0.048) with contact rats consuming a higher percentage of Kcal during the contact period in comparison to controls. No significant differences were observed for water consumption between control and contact rats (Figure 5.6D; 2-way RM ANOVA; F (1, 14) = 0.015, p = 0.906).

5.4.3.2 Ultrasonics Data

There was an increase in the number of 50 kHz USVs per minute in the contact group over the 13-day contact period (Figure 5.7A) which was significantly different when compared to control rats with AUC analysis (Figure 5.7B, Mann Whitney U test; U = 0, p = 0.0002).

Contact latency measures before and after the experiment are displayed in Figure 5.7C. Two-way ANOVA analysis found no significant differences between control and contact groups (F(1, 14) = 0.181, p = 0.677) although a significant main effect of time was observed (F(1,14) = 9.449, p = 0.008). Sidak’s post hoc testing was unable to determine the location of this significance although it appears that both control and contact groups have a lower contact latency after the tickling stimulation in comparison to before.

5.4.3.3 Sucrose Consumption

When delta was calculated for sucrose consumption, no differences were observed in sucrose consumption for control or contact rats (Figure 5.8A; Mann Whitney U test; U = 27, p = 0.934). When comparing delta values for sucrose preference (Figure 5.8B), contact rats showed a significantly lower sucrose preference in comparison to controls (unpaired t test, t(13) = 2.259, p = 0.042). No significant differences were observed between groups for water intake during the sucrose preference test on day 3 (unpaired t test; t(13) = 0.004, p = 0.996) or day 13 (Mann Whitney U; U = 16, p = 0.179, data not shown).
Figure 5.6: Body weight, food and water consumption for rats during Experiment 2

Shaded area represents period of daily social contact. Blue lines represent days where sucrose solution was given.

N: control = 8, contact = 8. All tests used are 2-way RM ANOVAs unless otherwise stated.

A: Body weight data over the testing period. No significant differences were observed between control or contact groups (p = 0.941).

B: Daily Kcal intake over the experiment. No significant differences were observed between control and contact groups (p = 0.233).

C: Average percentage change in Kcal consumption from baseline (day 4) in control and contact rats during the contact period (shaded). Contact rats consumed a significantly higher percentage of Kcal in comparison to baseline during the contact period than control rats (unpaired t test, p = 0.048).

D: Daily water consumption during the experiment. No significant differences were observed between control and contact rats (p = 0.906).
Figure 5.7: Ultrasonics Data for Experiment 2

N: control = 8, contact = 8

A: Number of 50 kHz USVs over the 13 days of contact. The number of 50 kHz USVs increased in the contact group over time (not normally distributed, no statistical testing). B: AUC analysis for 50 kHz USVs over the 13 days of the experiment. A significant difference was observed between groups (p = 0.0002, Mann Whitney U test). C: Contact latency for control and contact groups before and after the experiment. 2-way ANOVA analysis found no significant differences between groups (p = 0.677) although a significant effect of time was observed (p = 0.008), however Sidak’s post hoc testing was unable to establish the location of this difference.
**Figure 5.8: Sucrose Data for Experiment 2**

N: control = 8, contact = 7 (1 rat excluded from contact group due to bottle leaking).

A: Difference in sucrose consumption (delta) from baseline to day 13. No statistically significant differences were observed between groups (Mann Whitney U, p = 0.934). B: Difference in sucrose preference (delta) from baseline to day 13. A significant difference was observed between control and contact groups (unpaired t test, p = 0.042).
5.4.4 Discussion

5.4.4.1 Body Weight, Food and Water Intake

No significant differences were observed between control and contact rats for body weight or water consumption over the course of the experiment. While no differences were observed for Kcal consumption during the experiment between control or contact groups, when percentage change in Kcal intake from baseline was calculated for each day during the contact period and then averaged for each rat, a significantly larger percentage increase was observed in contact rats in comparison to controls, suggesting that during the contact period contact rats increased their intake of bland food. This may be due to an increased energy expenditure, as during the social contact sessions there was increased activity (i.e. increased running and jumping) in contact rats when compared to the passive contact controls. This would also explain how no differences in body weight were observed even though Kcal intake increased.

5.4.4.2 Positive Affect

This study was able to robustly replicate a model of positive affect (as measured by an increase in 50 kHz USVs) in response to simulated social contact by the experimenter. After around 10 days of a daily 2-minute social contact paradigm, these USVs reached a plateau and on following sessions remained at similar levels.

The fact that this study was able to replicate this model in a much shorter timeframe, and with a larger increase in USVs compared to the pilot study is likely due to the fact that the rats received social contact in their home cages, and therefore there was bedding present which has been shown to increase USVs (Natusch and Schwarting, 2010). This highlights how important environmental factors are in order to be able to fully replicate this model of positive affect.

5.4.4.3 Contact Latency

Contact latency in both control and contact groups decreased from the start to the end of the experiment, and although a robust increase in 50 kHz USVs was observed in the contact group, no differences in contact latency were observed between contact and control rats. This is unexpected, as it would be assumed that rats in the contact group would show a larger reduction in contact latency, as they have been receiving the rewarding stimulation,
and therefore would have increased motivation to interact with the experimenter’s hand. Previous studies using this measure have tested contact latency in rats directly after they have received social contact (Burgdorf and Panksepp, 2001), whereas in this experiment, contact latency was measured at least 24 hours after rats had previously received social contact stimulation. This could explain the differences observed in this study and previous literature as another study testing contact latency after a delay from tickling also found no effects of the social contact (Cloutier and Newberry, 2008).

### 5.4.4.4 Sucrose Preference

In this study, when sucrose consumption for each rat was compared at baseline (before receiving any social contact) and after social contact (day 13 of contact) no differences were observed in sucrose consumption between control or contact rats. There was an increase in total sucrose consumption for both groups at the end of the experiment when compared to baseline, however this is likely due to the increased body weight of rats at the end of the experiment, meaning they are able to consume an increased amount of food.

Interestingly, although there were no significant differences in sucrose or water consumption between control and contact groups, when comparing the difference in sucrose preference at baseline and after social contact, contact rats illustrated a lower difference in sucrose preference in comparison to control rats. This may be due to a marginal decrease in sucrose consumption and slightly increased water consumption (which did not reach significance) in contact rats during the sucrose preference test resulting in an overall reduced sucrose preference when compared to control rats. The data suggests that whilst sucrose preference increased over the course of the experiment for the control rats, sucrose preference remained at similar levels to baseline in the contact group.

As stroking-like stimulation in rats has been shown to increase 50 kHz USVs and hypothalamic oxytocin neuron activation (Okabe et al., 2015), it is likely that heterospecific social play also leads to increased activation of oxytocin neurons. VTA administration of oxytocin has been shown to suppress intake of a sucrose solution, and VTA administration of oxytocin receptor antagonists increased sucrose intake (Mullis et al., 2013). These data may implicate a role for oxytocin signalling in the reduced sucrose preference observed rats in the contact group, as it may be that oxytocin release induced by the social interaction reduces preference for the sucrose.
5.4.4.5 Limitations

One of the problems in this study is the use of a variation on a sucrose preference test as a test of food choice. The sucrose preference test is generally used as a measure of anhedonia and often used as a test of depressive like behaviour in rodents (Mateus-Pinheiro et al., 2014), and therefore may not be a suitable model for this study where positive affect is being tested. Furthermore, as rats can behave differently when consuming sucrose in a solution compared to its solid form (Pan and Hu, 2011, Apolzan and Harris, 2012), it may be that by offering a solution, this could mask any effects that may be seen when offered a solid food.

There is little evidence for how long an increase in USVs lasts following exposure to simulated social contact. Therefore, it may be that by providing the sucrose solution for an hour any differences in consumption at the beginning of the hour period where positive affect is likely still high could be masked by the extended time frame of the testing period. Therefore, a study similar to this should be repeated using a similar contact paradigm and examining how long following the contact session an increase in 50 kHz USVs is observed. Furthermore, during the food choice testing period, food should be weighed regularly over the hour period to ensure that any effects directly following the contact session are not overlooked.
5.5 Experiment 3: The Effect of Fasting and Food Reward on Motivation to Receive Social Contact.

5.5.1 Background

It has been established that heterospecific social interaction is able to induce a conditioned place preference (Burgdorf and Panksepp, 2001). However, there is little evidence as to whether this place preference can be altered by food-related stimuli. Fasting, for example, is a negative stimulus whereas a food reward is a positive stimulus.

There is evidence that negative states can lead to a reduction in 50 kHz USVs, as when rats are tickled one hour after a restraint stress, this leads to a reduction in 50 kHz USVs (Popik et al., 2012). Furthermore, Schwarting, Jegan and Wohr (2007) found that food deprived rats emitted fewer 50 kHz USVs compared to ad libitum fed subjects, which suggests that fasting may lead to a suppressive effect on 50 kHz USVs.

Therefore, the aim of this experiment was to establish whether either a negative or positive stimuli was able to influence the rewarding nature of tickling, and alter a rats motivation to receive social contact.

Fasted rats are highly motivated to consume food, and it was therefore hypothesised that fasted rats would show reduced motivation to receive social contact as they would be more motivated to consume food.

By providing access to sweetened condensed milk rats would be receiving a rewarding stimulus which may lessen the rewarding nature of the social contact. Therefore, it was hypothesised that presentation of a food reward would also reduce motivation to receive simulated social contact.

Furthermore, it was hypothesised that when rats received social contact after a period of fasting, they would emit fewer 50 kHz USVs in comparison to control rats.
5.5.2 Methods

5.5.2.1 Animals

30 Male Wistar Rats (approx. 3-4 weeks of age) were singly housed (in large cages to ensure adequate space for social contact) under standard housing conditions (see 2.1.1).

5.5.2.2 Protocol

Prior to commencing the experiment baseline USVs were recorded for 5 minutes in order to sort rats into groups (see 2.6.2). Rats were also subjected to a baseline CPP test where they were placed in the CPP apparatus for 15 minutes and video recorded. This was then analysed to ascertain the amount of time spent in each chamber (see section 2.7 for further information on CPP testing).

Rats were allocated into 1 of three groups: control (n = 8), fasted (n = 8) and SCM (sweetened condensed milk, n= 14).

All rats had their bodyweight, as well as food and water consumption measured daily. From days 3-14 of the experiment rats were tickled for 2 minutes in their home cage (using the same 2-minute paradigm as described by Burgdorf and Panksepp, 2001). This was done to ensure that the rats found the interaction rewarding, so that they would not entirely avoid the experimenters hand when placed in the CPP apparatus. From days 15 – 31 rats received social contact in the CPP apparatus. Rats were placed in the compartment for which they had originally show the least preference and immediately tickled for 15 seconds before receiving 15 seconds of passive contact (repeated for 2 minutes). If a rat at any point left the ‘contact chamber’ and then re-entered they were immediately tickled and a 15 second on/off cycle would resume until they had been the CPP apparatus for a total of 2 minutes.

On day 31 rats in the fasted group had their food removed and were fasted for 24 hours. As a further measure of motivation to receive social contact, contact latency was also measured in all rats on day 31. This was measured 3 times consecutively and then averaged.

On day 32, rats were tested for conditioned place preference. First, contact latency was again measured 3 times before rats were placed in the CPP apparatus for 15 minutes and video recorded. Immediately following the CPP test, rats were then tickled in the CPP apparatus following the same 2-minute pattern as before and USVs were recorded. Rats in
the SCM group had approx. 15ml of 50% SCM placed in their cage an hour before the CPP test. Fasted rats were re-fed immediately after completion of the CPP test and tickling procedures (see Figure 5.9 for summary of experimental procedures).

5.5.2.3 Data Analysis

2 rats in the SCM group did not consume any SCM on day 32 of the experiment and were therefore excluded from analysis.

For body weight, food and water consumption, AUC was calculated from days 1-31 and averaged to provide an average value per day. Differences between groups were analysed using 1-way ANOVA analysis.

USVs were counted manually using Avisoft SASLab Lite. USVs resembling 22 kHz USVs were not included in the analysis (see Chapter Discussion 5.6.3 for more details). Total number of USVs per contact session were averaged to provide the number of 50 kHz USVs per minute. AUC was then analysed to enable statistical testing.

To measure place preference, CPP tests were video recorded and manually scored by an experimenter. This involved using a stopwatch to count the total amount of time spent in one of the chambers by the rat (a rat was counted as having entered a chamber when both their head and front paws crossed the central threshold). This value was then compared to the total time of the test to evaluate the percentage of time spent in a certain chamber.

In order to provide one value for contact latency, the average contact latency on day 32 was subtracted from the average value obtained on day 31 for all groups to provide a value denoting the difference in contact latency over the two days.
Day 1
- CPP Box habituation

Day 2
- CPP Baseline measurement

Days 3-14
- 2 mins daily social contact in home cage

Days 15-30
- 2 mins daily social contact in CPP box

Day 31
- Social contact in CPP box
- Contact latency measurement
- Food removed from fasted group

Day 32
- CPP box test (after fasting or SCM access)
- Contact latency measurement
- Social contact in CPP box

**Figure 5.9: Timeline of Experimental Procedures**
5.5.3 Results

5.5.3.1 Body Weight, Food and Water Intake

Over the course of the experiment no significant differences were observed in body weight between control, fasted and SCM groups (Figure 5.10A, 1-way ANOVA, $F (2, 25) = 2.097, p = 0.144$). There were also no significant differences observed in daily Kcal intake (Figure 5.10B; 1-way ANOVA; $F (2, 25) = 2.133, p = 0.140$) or water consumption (Figure 5.10C; Kruskal-Wallis test; $U = 3.323, p = 0.190$).

5.5.3.2 Ultrasonics Data

When the rats experienced simulated social contact in their home cages at the start of the experiment, this led to an increase in the number of 50 kHz USVs per minute for all three groups. No significant differences were observed between groups (Figure 5.11A; 2-way RM ANOVA, $F (2, 25) = 0.380, p = 0.688$) which was confirmed with AUC analysis (Figure 5.11B, 1-way ANOVA, $F (2, 25) = 0.394, p = 0.678$).

When transferred to the CPP apparatus for social contact, there was a continued increase in 50 kHz USVs which stabilised by day 7 (Figure 5.11C). AUC analysis found no significant differences between control, fasted and SCM groups (Figure 5.11D; 1-way ANOVA; $F (2, 25) = 0.692, p = 0.510$).

5.5.3.3 CPP Data

When place preference was tested at baseline in the CPP apparatus, all rats (apart from 1) showed a preference for the checked chamber. However, the percentage of time spent in the least preferred chamber did not differ between the three groups (Figure 5.12A; 1-way ANOVA; $F (2, 25) = 0.510, p = 0.606$). When tested after fasting/SCM access, there was also no significant difference between the three groups in the percentage of time spent in the contact chamber (Figure 5.12B; 1-way ANOVA, $F (2, 25) = 0.675, p = 0.519$).

When experiencing social contact in the CPP apparatus after fasting/SCM access, there were no significant differences in the number of 50 kHz USVs exhibited by the three groups during the social contact (Figure 5.12C; 1-way ANOVA; $F (2, 25) = 0.053, p = 0.949$).

There were also no significant differences in contact latency difference between groups when tested before and after fasting/SCM access (Figure 5.12D; 1-way ANOVA; $F (2, 25) = 1.506, p = 0.241$).
Figure 5.10: Body weight, Food and Water Consumption

N: control = 8, fasted = 8, SCM = 12
A: AUC body weight from experimental days 1-31. No significant differences were observed in body weight between the three groups (1-way ANOVA, p = 0.144). B: AUC analysis of daily Kcal intake from experimental days 1 – 31. No significant differences were observed between control, fasted or SCM rats (1-way ANOVA, p = 0.140). C: AUC analysis of water intake from days 1-31 of the experiment. No significant differences were observed between groups (Kruskal-Wallis Test, p = 0.190).
Figure 5.11: Ultrasonics Data for Home Cage and CPP Box

N: control = 8, fasted = 8, SCM = 12
A: Number of 50 kHz USVs per minute during daily social contact in the rat’s home cage. There were no significant differences between control, fasted and SCM groups (2-way RM ANOVA, p = 0.688). B: AUC analysis of the number of 50 kHz USVs per minute for social contact in the rat’s home cage. No significant differences were observed between groups (1-way ANOVA, p = 0.678). C: Number of 50 kHz USVs per minute for social contact in the CPP apparatus (not normally distributed, no statistical testing). D: AUC analysis of number of 50 kHz USVs per minute during social contact in the CPP apparatus. No significant differences were observed between groups (1-way ANOVA, p = 0.510).
Figure 5.12: CPP Apparatus Data

N: control = 8, fasted = 8, SCM = 12

A: Baseline CPP apparatus data showing the % of time spent in least preferred chamber. No significant differences were observed between the three groups (1-way ANOVA, p = 0.606).

B: % of time spent in contact chamber during the CPP test on day 32 after fasting/SCM access. No significant differences were observed between groups (1-way ANOVA, p = 0.519).

C: Number of 50 kHz USVs during contact session in CPP box on day 32 following fasting/SCM access. No significant differences were observed between groups (1-way ANOVA, p = 0.949).

D: Difference in contact latency when comparing the day before fasting/SCM access (day 31) and day 32. No significant differences were observed between groups (1-way ANOVA, p = 0.241).
5.5.4 Discussion

5.5.4.1 Ultrasonic Vocalisations

When experiencing social contact in their home cages at the beginning of the experiment, all three groups illustrated an increase in 50 kHz USVs over time, with no differences observed between groups. When transferred into the CPP apparatus for contact sessions on day 15 of the experiment there was a slight reduction in the number of USVs, likely due to the lack of bedding (Natusch and Schwarting, 2010), however after a week of daily contact sessions in the CPP apparatus, the number of 50 kHz USVs had stabilised. Again, as expected, there were no differences between groups in the number of USVs per minute during the contact session in the CPP apparatus.

5.5.4.2 Conditioned Place Preference Test

There were no differences in the percentage of time spent in the least preferred chamber (designated the contact chamber) between the three groups at the start of the experiment. However, all three groups did show an overall preference for the checked side of the CPP chamber. This is likely due to the fact that this side of the CPP apparatus was slightly darker due to the increased amount of dark tape on the walls (see section 2.7) as rats prefer darker environments (Garcia et al., 2008). However, this preference was overcome by using a bias experimental design, whereby rats received simulated social contact in their least preferred chamber (in all but one case this was the striped chamber).

On day 32 (following fasting/SCM access for the appropriate groups) there were also no differences between the three groups in conditioned place preference. This suggests that both fasting, and access to a food reward have no effect on rat’s motivation to receive simulated social contact. On day 32 the percentage of time spent in the contact chamber is only around 50%, which suggests no preference, however as this is an increase in the percentage of time spent in this chamber from the beginning of the experiment, it can be assumed that the social contact received in the CPP box was rewarding and did produce a place preference.

Contact latency was used as a secondary measure of motivation to receive social contact and was performed on day 31 and day 32. When the differences in contact latency were examined, there were no significant differences between control, fasted or SCM groups. This further suggests that both fasting and a food reward have no effect on a rat’s
motivation for social contact. However, a slight increase in contact latency was observed in all three groups from day 31 to 32 which suggests a decrease in motivation to obtain contact from the experimenter. As this was observed in all three groups, including the control group, it is likely that these changes are due to an environmental difference, and may be due to the different timings of contact latency measurements over the 2 days.

After the conditioned place preference test on day 32, rats received social contact in the CPP apparatus and USVs were recorded as on previous days. No differences were observed in the total number of 50 kHz USVs between the three groups during these sessions, which suggests that fasting and SCM access have no effect on the generation of positive affect from simulated social contact. However, the SCM group, having already experienced the CPP test, wouldn’t have consumed any SCM for at least 15 minutes (likely longer) and therefore this may impact the results. It would have been expected that rats may produce fewer 50 kHz USVs following a period of fasting, as other negative stimuli, such as restraint stress, lead to a reduction in 50 kHz USVs when tickled (Popik et al., 2012) and Schwarting, Jegan and Wohr (2007) found a reduction in the number of 50 kHz USVs in food deprived rats, however this was not observed in this experiment.

By providing contact sessions in the CPP box, it was difficult to standardise the amount of time spent ‘tickling’ each rat. When receiving social contact in their home cage, a 2 minute 15 second on/15 seconds off paradigm is used, however when in the CPP box rats were able to avoid the simulated social contact by moving to the other chamber. This will have resulted in each rat receiving a slightly different amount of social contact each day, depending on their movement from different chambers. This could have perhaps been prevented by changing the paradigm to ensure that all rats received an equal amount of contact in the chamber.

During the contact sessions throughout the study, rats received contact at a roughly similar time each day. However, on the final test day (day 32), due to also testing the conditioned place preference, many of the rats received social contact at a different time. It may be that this could have affected the rat’s behaviour, as they would have been anticipating social contact at a different time to which it was received.
5.6 Chapter Discussion

5.6.1 Experiment Summary and Hypotheses

This chapter has shown that in order to accurately reproduce a model of increased 50 kHz USVs from simulated social contact, it is important to take note of strain, age and housing conditions, as these could all have an impact on the reproducibility of this model.

Furthermore, it has been illustrated that daily access to a food reward does not lead to an increase in 50 kHz USVs either in anticipation of the food reward, or after consumption. It was also demonstrated that inducing a positive affect through simulated social contact does not lead to an alteration in sucrose solution consumption, although a decreased sucrose preference after social contact was observed in comparison to controls. Finally, both a food reward (sweetened condensed milk) or an aversive stimulus (fasting) had no impact on conditioned place preference for receiving simulated social contact or the number of 50kHz USVs emitted during tickling.

The four hypotheses for this chapter were:

1. 50 kHz USVs will be increased before and after SCM consumption in comparison to controls, as both the anticipation and consumption of SCM will induce a positive affect (experiment 1).
2. Positive affect, as induced by heterospecific play, will lead to a decreased consumption of sucrose, and therefore a reduced preference for sucrose (experiment 2).
3. Both fasting and access to a food reward will lead to a reduced motivation to receive social contact, as measured by conditioned place preference (experiment 3).
4. When rats received social contact after a period of fasting, they will emit fewer 50 kHz USVs in comparison to control rats (experiment 3).

Although a robust and reliable model of positive affect in rats was generated, the majority of hypotheses in this chapter were rejected. However, there was evidence for differences in sucrose preference between control and contact rats, with rats receiving social contact illustrating lower sucrose preference in comparison to control rats receiving passive contact. This may suggest that social contact, and therefore positive affect, is able to influence food choice, however further experiments need to be carried out to fully establish these effects.
5.6.2 Experimental Limitations

5.6.2.1 Experimental Testing

For experiments in which simulated social contact was taking place, these procedures occurred in the same room as which the rats were housed. As it has been suggested that USVs may have a communicative function (Brudzynski, 2009) and the fact that playback of vocalisations is able to influence behaviour (Sadananda et al., 2008, Wohr and Schwarting, 2007) it may be that vocalisations of rats undergoing the social contact could influence the behaviour of other rats in the same housing room. Ideally for USV studies recordings should be carried out in a separate room to ensure that USVs don’t influence the behaviour of other rat’s present. Unfortunately, due to space constraints this was not possible in these studies, however on observation USVs emitted by rats undergoing stimulated social contact did not appear to influence the behaviour of rats not being tested.

5.6.2.2 Model Limitations

As the exact function and meaning of USVs are still unknown, this model of positive affect works under the assumption that 50 kHz USVs denote positive affect. Whilst the behaviour of the rats observed during simulated social contact does suggest that the interaction is rewarding, it is still debatable whether the number of 50 kHz USVs can be correlated with the level of positive affect experienced. Recently, there has been some debate over whether 50 kHz USVs are a predictor of affective state, with the alternative hypothesis suggesting that these vocalisations are produced by the biomechanical forces on the thoracic area generated during movement (Burke et al., 2017). Others have also observed 50 kHz USVs in situations that may not necessarily be considered rewarding such as separation from cage mates (Wöhr et al., 2008) and during aggressive encounters (Sales, 1972). This may suggest that 50 kHz USVs are not exclusively an indicator of positive affect and could lead to problems when interpreting the model of heterospecific social play.

Furthermore, as found when carrying out pilot experiments in this thesis, there can be many difficulties in establishing a model of positive affect, and factors such as housing conditions, age and strain can lead to differences in the number of 50 kHz USVs produced. These factors could lead to questions over the reproducibility and accuracy of the model. Table 5.1 illustrates shows the strain and ages of rats used in tickling studies, as well as the average number of 50 kHz USVs per minute emitted during the tickling interaction, and
highlights the differences observed across studies. However recently Schwarting (2018) performed a comparative analysis between Wistar, Sprague Dawley and Long Evans rats, and found no differences between strains for total call time, call number, amplitude or peak frequency. There were some differences in types of call, with Long Evans and Sprague Dawley rats illustrating significantly higher numbers of step calls in comparison to Wistar rats. These data suggest that although there may be minor differences in USV characteristics between these strains of rats they are all suitable for use in studies measuring ultrasonic vocalisations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Laboratory</th>
<th>Strain</th>
<th>Age</th>
<th>Avg. peak number of 50 kHz USVs/min</th>
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<td>Long Evans</td>
<td>7 weeks</td>
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<td>Long Evans</td>
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<td>(Popik et al., 2014)</td>
<td>Popik</td>
<td>Sprague Dawley</td>
<td>7-8 weeks</td>
<td>~220</td>
</tr>
<tr>
<td>(Rygula et al., 2012)</td>
<td>Popik</td>
<td>Sprague Dawley</td>
<td>6-7 weeks</td>
<td>~120</td>
</tr>
<tr>
<td>(Cloutier et al., 2013)</td>
<td>Newberry</td>
<td>Sprague Dawley</td>
<td>4-6 weeks</td>
<td>~100</td>
</tr>
<tr>
<td>(Cloutier et al., 2014)</td>
<td>Newberry</td>
<td>Sprague Dawley</td>
<td>12 weeks</td>
<td>~100</td>
</tr>
<tr>
<td>(Hori et al., 2013b)</td>
<td>Ichitani</td>
<td>Fischer</td>
<td>4 weeks</td>
<td>~210</td>
</tr>
<tr>
<td>(Hori et al., 2014)</td>
<td>Ichitani</td>
<td>Fischer</td>
<td>4 weeks</td>
<td>~270</td>
</tr>
</tbody>
</table>
5.6.2.3 USV Analysis

In order to analyse USVs for these experiments, USVs were counted manually and scored for frequency, amplitude, length and shape. This could be potentially problematic concerning differences in how USVs are analysed by different experimenters as this could result in slightly different counts from the same experiments. Although some laboratories have analysed USVs digitally (Reno et al., 2013) this can also create problems, particularly when it comes to scoring the shape of the vocalisations. Automated analysis of USVs is desirable not only due to the labour-intensive nature of manual analysis, but also because it can overcome observer bias.

For the purpose of these experiments, USV analysis was carried out based on the methods described by Brudzynski (2013) counting either flat or frequency modulated (FM) 50 kHz USVs and then further categorising the FM vocalisations as trill, step, step trill or other. However, Wright, Gourdon and Clarke (2010) have further evaluated these categories to generate 14 distinct call categories. As there is still very little evidence on the function of specific call types aside from the distinction between flat and frequency modulated calls, the rationale behind such in depth analysis is unclear, however for continuity across experiments there should be some effort to standardise the analysis of USVs.

Few have analysed inter and intra experimenter reliability with repeated counting of USVs. Wright et al. (2010) found an inter experimenter reliability of 96% and an intra experimenter reliability of 97%. It would be of interest to examine variability in USV analysis between different labs as because of the lack of standardised counting methods there is likely much variation between research groups.

5.6.3 30-40 kHz USVs

Throughout these studies, particularly experiment 2 and experiment 3 where large numbers of USVs were observed in response to social contact, a class of USVs was observed that have previously not been described in detail. These are at around 30-40 kHz, are generally flat and relatively long, similar to 22 kHz USVs but slightly shorter and at slightly higher frequency (see Figure 5.13 for examples). These were excluded from analysis. It may be that these are a form of lower frequency 50 kHz USVs as these do tend to vary widely across different frequency ranges (Brudzynski, 2013). Wright et al. (2010) states in her categorisation that any flat USVs with frequency greater than 30 kHz would be categorised as flat 50 kHz USVs, whereas 22kHz USVs are defined as vocalisations between 20-25 kHz.
Given this definition these calls could be categorised as 50 kHz USVs, however this would likely vary across different experimenters.

There is some evidence for emission of 22 kHz during social contact sessions (Burgdorf et al., 2008, Mallo et al., 2007) as well as following ejaculation (Hull and Dominguez, 2007). This may suggest that 22 kHz USVs do not always indicate a negative state and therefore the presence of low frequency calls observed during the tickling stimulation in this experiment may not indicate aversion to the procedure.
Figure 5.13: Examples of 30-40 kHz USVs

A: Examples of the 30-40 kHz USVs. These are generally flat, although may occasionally include a step or some frequency modulation. B: Examples of 22 kHz USVs for comparison. These are much longer and flatter, as well as appearing at a lower frequency.
6 General Discussion

6.1 Overview of Findings

Much research has been carried out into the understanding of energy-balance related signals and circuits and how these can influence behaviour. Many of these circuits are now relatively well understood, particularly in rodents (for review see: Timper and Brüning, 2017). However, less has been done to understand how more subtle behaviours, such as food choice, are controlled, and how other systems under hypothalamic control such as stress, as well as early life interventions can interact with the homeostatic and hedonic appetite systems. This project set out to explore two understudied aspects of appetite control. Firstly, how food choice may be influenced by early life experience and secondly, how positive affect can impact on food preference.

The effects of prenatal stress on feeding were investigated using two well established models: prenatal restraint stress and prenatal social stress. In both cases, no effects on total calorie intake or food choice were observed, however the expected alterations in corticosterone response were not replicated in the PNS offspring from either model. This suggests that the dysregulation of the HPA axis often observed in PNS offspring may not be as robust as often cited in the literature. However, it may also be that the blood sampling procedure used in these experiments induced a high level of stress which masked the real impact of prenatal stress on food choice.

Secondly, using small litter rats as a model of neonatal overnutrition, the effects of early life overfeeding on food choice were investigated. When offered an *ad lib* free choice of chow, sucrose pellets and lard over a period of 10 weeks, small litter rats illustrated an increased consumption of bland chow in comparison to control litter rats, whilst maintaining similar consumption of sucrose and lard. This is likely due to the increased body weight of the small litter rats and suggests that the chow diet contains nutritional components not available in the lard and sucrose necessary for small litter rats to maintain their increased body weight. When offered a high-fat high-sugar (HFHS) pellet on a two-hour daily binge feeding schedule alongside *ad lib* chow, small litter rats illustrated an increased consumption of the HFHS pellet during the scheduled feed whilst maintaining similar chow consumption to controls. This increased consumption is again likely a product of body
weight, and the changes in preference from chow to HFHS diet in this feeding paradigm may be due to the fact that the HFHS diet is more nutritionally complete and palatable in comparison to the separate lard and sucrose components.

One major outcome of this project was to highlight limitations in animal models in current use. For some models, such as using litter size manipulations as a model of neonatal overnutrition, similar effects to those reported in the literature were observed. However, when using models of PNS the expected outcomes were not seen. This raises important questions about the reproducibility in this field and highlights the importance of describing and sharing precise experimental protocols across laboratories.

Another area in food research that has been neglected is the impact of positive mood on feeding behaviour. In this project a novel approach was implemented, using a rodent model of heterospecific social play to investigate the effects of positive affect on food choice. Using 50 kHz ultrasonic vocalisations as a measure of positive affect in rats, it was found that daily scheduled access to a food reward (sweetened condensed milk) led to no alterations in the number of 50 kHz USVs emitted either in anticipation of the food, during consumption, or after consumption. It was also investigated whether induction of positive affect using heterospecific social play could lead to alterations in food choice. While no differences in total sucrose consumption were observed between control and contact groups, a difference in sucrose preference was found, with those rats receiving social contact illustrating a lower sucrose preference following interaction with the experimenter in comparison to passive contact controls. Finally, it was investigated whether fasting or access to a food reward would lead to any changes in motivation to receive social contact, however no differences in conditioned place preference for social contact were observed. These data suggest that positive affect may have a role in determining food choice, although further studies are necessary to fully establish these effects. This is the first time a model of heterospecific social play has been implemented to study the impact of positive affect on feeding behaviour, and while many of the hypotheses were not supported by experimental outcomes, these data are still of use to the field and provide a basis for the further study of positive affect on food choice.
6.2 Experimental Limitations

6.2.1 Animal Models

Animal models are widely used in research, particularly in the study of early life programming. However, a problem with the use of rodents in research highlighted in this thesis is the reproducibility of certain animal models. Reproducibility of research and experimental outcomes can be influenced by a variety of factors such as experimenter bias (Holman et al., 2015), laboratory environments and different testing apparatus (Crabbe et al., 1999). Reproducibility seems to be a particularly large problem in the field of prenatal stress, with many laboratories highlighting different findings even when implementing the same model of PNS (see section: 3.4.3.2.1). Boersma and Tamashiro (2015) have suggested that various factors such as genetic background and differences in stress vulnerability can vary the outcomes of prenatal stress, even determining whether PNS exposure can lead to adverse or protective outcomes. The varying outcomes of prenatal stress, as well as problems in accurately reproducing many aspects of the model, raises important questions around the degree of physiological relevance and generalisability of animal models of early life influences. It may be that factors such as stress-coping style are more relevant to the development of adverse outcomes than the prenatal stress procedure and could therefore provide a useful avenue for further research into early life programming.

There are also more general problems with the use of animal models in research. Often, animal strains used in behavioural studies are inbred and therefore do not represent a heterogenous population (Jensen et al., 2016). This can be particularly problematic in studies examining food choice as taste preferences can alter between strains (Smith et al., 2000, Lewis et al., 2005). Furthermore, individual strains can also have different propensities for developing obesity (Bray et al., 1987) and show different metabolic effects when fed a high fat diet (Marques et al., 2016). Food choice in humans can be influenced by many factors such as social context (Herman, 2015), culture (De Castro, 1997) and personal attitudes towards health concern (Sun, 2008). As many of these factors cannot be effectively modelled in rodents, it can be difficult to fully model all the determinants of food choice. However, this can be advantageous as it allows for the study of more precise influences on feeding without unwanted extraneous variables, such as dietary restraint, affecting the outcomes. Furthermore, the ability to carry out precise experimental manipulations, as well as test therapeutic interventions, in rodents is particularly important.
in the field of early life programming and allows for the detailed study of mechanisms otherwise impossible in the human population

6.2.2 Food Choice Models in Rodents
Many studies investigating the effects of obesity in rodents often implement a high-fat diet feeding paradigm (Hariri and Thibault, 2010). While this can be effective in inducing obesity in rodents, this model is not particularly ecologically valid. Humans have access to a very food rich environment and therefore it is important to develop rodent models of food choice which accurately reflect this environment in order to be able to more effectively model diet-induced obesity. Some have observed that rodents with access to a selection of foods consume more calories and illustrate increased body weight gain in comparison to those on a high-fat diet alone, particularly if a sucrose solution is offered as part of the diet (Rolls et al., 1983, Harris and Apolzan, 2012), illustrating the importance of variety in the increased consumption of food.

In this thesis, two models of long term food choice were implemented. Firstly, access to a HFHS pellet and a sucrose solution alongside chow and water was implemented for the studies on prenatal stress (Chapter 3). Although this model is effective at inducing obesity, it can be difficult to establish exact nutrient preferences due to the combined nature of the HFHS pellet, as well as the fact that excessive consumption of calories from the sucrose solution could mask subtle food preferences. For studies on neonatal overnutrition (Chapter 4), a second food choice model was implemented, consisting of bland chow, sucrose pellets, lard and water. While this diet does not induce increases in body weight in comparison to control rats, it is more effective for examining exact nutrient preferences, as it discriminates between fat and sugar preference. However, this model is not as ecologically valid as it is rare that humans will consume foods high in purely fat or sugar. While it can be argued that neither of these models is a truly valid representation of the human food environment, both provide useful insight into differing food preferences in the rat and provide a basis for the further study of food choice.

Alternative models of food choice in the literature include the cafeteria diet. Cafeteria diets, which consist of feeding rats a variety of commercially available foods, leads to a large increase in caloric consumption, up to 50-100% which is much larger than the 15-30% increase for those placed on high-fat or high-carbohydrate diets (Sclafani, 1989). It has also been suggested that cafeteria fed rats are a better model for human metabolic syndrome
compared to high-fat diet fed rats as they illustrate more robust symptoms such as glucose intolerance and inflammation of white and brown adipose tissue (Sampey et al., 2011). But while providing an excellent model of diet induced obesity, the cafeteria diet may contribute less insight into food preferences due to the mixed nature of the foods often provided in these diets. Therefore, an important avenue for future research is to develop a selection of food choice models which can be selectively implemented dependent on the interests of the researchers.

When designing models of food choice it is important to ensure that the foods provided meet the animals minimal nutritional requirements in order to prevent over consumption of certain foods to maintain those requirements (Hariri and Thibault, 2010). Furthermore, the composition of foods can also have an impact on consumption and therefore must be considered. There is evidence that rats will consume more sucrose in a liquid form in comparison to its powdered form (Sclafani, 1989). Furthermore, rats consuming a mixed high-fat high-sugar emulsion consumed more calories than those offered only a high-fat or high-sugar emulsion (Lucas and Sclafani, 1990). Additionally, when evaluating food choice models in rodents it is important to consider whether they are modelling taste or nutrient preferences. It has been suggested that rats alter their food choice to maintain a particular dietary composition regardless of food palatability. Castonguay, Hirsch and Collier (1981) offered rats carbohydrate solutions of varying type and caloric density (therefore presumably differing in relative palatability) and found that all groups adjusted their chow and carbohydrate solution intake to maintain a nutrient composition of around 10-15% protein and 55-65% carbohydrate. However, in this study all groups offered a carbohydrate solution consumed more calories than chow controls, suggesting that the palatability of a sugar solution can influence food consumption. Therefore, while discriminating taste and nutrient preferences in rodent studies can prove difficult, it is likely that both factors play a key part in food choice.

6.2.3 Sex Differences

Although in the prenatal restraint stress experiment in Chapter 3 both males and females were tested, the remainder of the experiments carried out in this thesis were performed only on male rats in order to increase experimental power and reduce the incidence of false positives from multiple comparisons. Sex differences have been reported in prenatal stress models (Brunton and Russell, 2010, Zuena et al., 2008, Sickmann et al., 2015) and sex specific effects of prenatal stress have been similarly suggested in human studies (Van den
Bergh et al., 2008, Li et al., 2010a). However, while female rats sometimes illustrate a more pronounced effect of PNS on HPA activity (Weinstock et al., 1992) the behavioural and physiological alterations associated with prenatal stress exposure are generally more pronounced in males (Brunton, 2013). Neonatal overnutrition studies have also suggested sex differences in litter size manipulation models (Argente-Arizon et al., 2016) highlighting the importance of investigating the effects of sex on early life manipulations. For investigations into positive affect it is also important to consider differences between males and females as emotional eating is generally found to be more prevalent in females (Gibson, 2012). Male rats were used in studies measuring USVs as during adolescence male rats emit more 50 kHz USVs during social contact in comparison to female rats (Panksepp and Burgdorf, 2003). Overall there is still little evidence for robust sex differences in USVs, and many studies have used both male and female rats in the same analysis (Burgdorf and Panksepp, 2001, Mällo et al., 2009).

More broadly speaking, it is essential to carry out experiments in both male and female models when researching feeding behaviour as there are sex differences in obesity development in the human population. For example females are more likely to develop obesity, and there is also evidence to show that sex steroids such as oestrogen can impact on feeding behaviour (Lovejoy et al., 2009). Furthermore, fat distribution in men and women is different, with men carrying more visceral fat whereas females carry more subcutaneous fat. Additionally, males and females show differences in their responses to certain hormones, with females being more sensitive to leptin and males showing higher sensitivity to insulin (Vickers et al., 2011).

### 6.2.4 Experimental Testing

For the experiments in this thesis, all experimental procedures were carried out during the light period. Rats are nocturnal animals and consequently the majority of their food consumption and activity takes place in the dark phase (ter Haar, 1972, Borbély and Neuhaus, 1978). Therefore, the presentation of certain food choice paradigms during the light period, such as the sucrose preference test and the 2-hour binge feeding paradigm, could influence the amount of food consumed. Nevertheless, some laboratories have found few effects when comparing testing during the light and dark phase (Yang et al., 2008) which may suggest that these factors have relatively little influence on behaviour. Furthermore the HPA axis is influenced by a circadian rhythm (Spiga et al., 2014), and therefore when measuring corticosterone levels during an acute restraint stress in the
prenatal stress studies, the timing of blood sampling may have impacted on the results.
During testing samples were taken throughout the full day, however care was taken to
ensure a mix of rats from each group were tested at each time point to ensure that timing
of the stressor did not bias the results.

6.3 Future Directions

6.3.1 Prenatal Stress

Although the experiments undertaken in this thesis were unable to replicate the HPA axis
dysregulation often observed in PNS offspring, overall the results indicate that prenatal
stress has no effect on feeding behaviour and food choice. As basal corticosterone levels of
PNS rats are generally unchanged (Richardson et al., 2006, Brunton et al., 2013), this may
explain why no changes in feeding behaviour are observed under a basal condition.
Therefore, future studies should examine the feeding behaviour of PNS rats when placed
under stress, as it is under these conditions that differences in corticosterone responses are
generally observed. This could be achieved through subjecting control and PNS rats to a
chronic stress paradigm for 2-3 weeks and examining their food intake and food
preferences when exposed to a choice diet.

Furthermore, future work should focus on the robustness and generalisability of animal
models of PNS. Efforts should be made to record similar parameters across different lab
groups and to standardise these models in order to generate more robust paradigms for
use in future studies.

6.3.2 Neonatal Overnutrition

The experiments in this thesis found that small litter rats consumed more bland chow than
controls, but no differences were seen in lard and sucrose consumption. As these effects
are similar to those observed with ICV injection of ghrelin (Schéle et al., 2016), future
studies should aim to further examine alterations in ghrelin signalling in small litter rats. As
current evidence is still lacking, further efforts should aim to characterise serum ghrelin
levels in small litter rats under differing conditions (such as hunger/satiety). The effects of
fasting on food preference is similar to those observed with ICV ghrelin administration, and
peripheral administration of a ghrelin antagonist is able prevent these effects (Schéle et al.,
2016). Therefore, experiments should be carried out whereby small litter rats are
administered with a ghrelin antagonist in order to observe whether this can prevent the
increased chow consumption seen in small litter rats, as this may indicate whether ghrelin signalling is implicated in these changes in food preference.

As there is evidence to suggest that moderate calorie restriction can ameliorate the effects of small litter rearing on increased body weight (Liu et al., 2013), it would also be interesting to observe whether a moderate calorie restriction can alter food choice in small litter rats when ad lib feeding is resumed. This would indicate whether the changes in food preferences of small litter rats are permanently programmed, or whether they can be altered by appropriate early interventions. Moreover, further research into reversing the effects of neonatal overnutrition in rats may provide data for the potential development of treatments or preventative measures that can be used to minimise the effects of early life overfeeding.

Furthermore, there is evidence for an altered stress response in small litter rats (Spencer and Tilbrook, 2009). Portella et al. (2015) found an increased consumption of sweet food in small litter rats in response to an acute tail pinch stressor, but the effects of chronic stress on feeding in small litter rats have yet to be elucidated. Therefore, small litter rats should be subjected to a chronic stress paradigm, and their feeding behaviour and food intake examined, as it may be that stress could enhance the obesogenic effects of small litter rearing.

In contrast to small litter rats, rats reared in large litters have shown resistance to obesity development (Patterson et al., 2010). Therefore, future research should also investigate the impact of large litter rearing on food choice.

6.3.3 Ultrasonic Vocalisations
As was observed by the USV pilot studies carried out in this thesis, age, strain and environmental differences can have a large impact on the number of USVs emitted by rats during heterospecific play. More in-depth research should be carried out into the possible strain and age differences in vocalisations and response to heterospecific play as this is still lacking in the literature and would enable researchers to make appropriate decisions for future studies.

While there is evidence to show that a stroking interaction is able to activate hypothalamic oxytocin neurons (Okabe et al., 2015), there is still no evidence as to whether heterospecific social play with the experimenter is able to induce oxytocin release.
Therefore, a simple experiment could be set up using immunohistochemistry to measure c-Fos expression in oxytocin neurons following social contact.

As the results obtained in this thesis suggest that positive affect may influence food choice, further studies should be carried out to fully establish these effects. Firstly, it is important to establish how long lasting the increase in 50 kHz USVs is after the tickling stimulation in order to allow food choice measures to be designed appropriately. It may be that the hour-long food choice test implemented in these studies was too long, and that by reducing this time-frame more significant effects may be observed. Additionally, further measures could be made during the food choice period, such as latency to eat following social contact.

Furthermore, as the use of a sucrose preference test may have been problematic as a measure of food choice (see 5.4.4.5) the impact of positive affect on food choice should be further studied using alternative models of food choice, for example, providing a short-term choice of sucrose pellets, chow and lard. Providing an increased selection of foods would be particularly interesting when examining the hypothesis that oxytocin release during social interaction influences food choice. As it has been suggested that the anorexigenic effects of oxytocin may be specific to sweet carbohydrates (Olszewski et al., 2016), it may be that the effects of tickling-induced positive affect on food choice are only specific to sweet foods.

6.4 Wider Perspectives

The importance of early life programming in determining feeding behaviour and obesity development is becoming increasingly well studied. Case studies have highlighted some of the effects of prenatal stress on humans which include alterations to the HPA axis (Davis et al., 2011) as well as increased incidence of some psychiatric disorders such as depression (Watson et al., 1999), attention deficit hyperactivity disorder (Li et al., 2010a) and schizophrenia (Fineberg et al., 2016). There is also evidence from humans that experiencing stress during pregnancy can lead to higher BMI values in the offspring (Li et al., 2010b).

Whilst these case studies provide a basis for the study of prenatal stress, it is impossible to manipulate specific conditions in these studies which is why the use of animal models is essential to enable researchers to elucidate the mechanisms by which prenatal stress exerts its effects. This may then lead to the development of interventions to reduce the impacts of prenatal stress. One such avenue may be increasing intake of antioxidants during pregnancy as there is evidence to suggest that treatment with antioxidants may lead to reductions in some of the effects of prenatal stress (Lipton et al., 2017).
In humans, rapid weight gain in early life is associated with an increased risk of overweight and obesity later in life (Stettler et al., 2002, Biro and Wien, 2010) and therefore by establishing the mechanisms behind how neonatal overnutrition can lead to an increase in weight gain, it could help in designing early life interventions to overcome these effects and assist in lowering the childhood obesity epidemic. An alternative model of neonatal overnutrition, the high carbohydrate model, highlights the importance of preventing excessive carbohydrate intake during infancy as this may predispose to obesity development (Patel and Srinivasan, 2011). Studies illustrating the impacts of early life overnutrition could pave the way for early life interventions to minimise the effects of neonatal overfeeding, such as altering the nutritional content of infant foods.

From a therapeutic point of view the study of how early life events, such as prenatal stress and neonatal overfeeding, influence obesity susceptibility is particularly relevant as unlike other factors such as genetic influences, they can be mitigated against with appropriate early intervention. Using animal models to elucidate the mechanisms by which prenatal stress and neonatal overnutrition impact on the offspring is key to defining the timing and nature of therapeutic and policy interventions.

### 6.5 Concluding Remarks

The ever-expanding global problem of obesity has made it more important than ever to fully elucidate the mechanisms which can contribute to energy imbalance. As well as over consumption of foods, alteration of food choice, specifically a preference for foods high in fat and sugar, could be a contributing factor to weight gain. The importance of prenatal and early postnatal environmental changes in influencing feeding behaviour is becoming increasingly well examined and merits further investigation as it could lead to the development of potential interventions aimed at minimising the negative impact of early life programming effects. Furthermore, there are many potential influences on feeding that have been relatively understudied such as the impact of positive affect. It is important to fully investigate these factors as it could lead to further understanding of how feeding behaviour and food choice are controlled. By fully understanding the mechanisms leading to alterations in food consumption and choice, this may enable development of preventative measures that can help slow or even reverse the global obesity epidemic.
7 References


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