Impacts of perinatal high fat diet and prenatal stress exposure on the programming of the Hypothalamic-Pituitary-Adrenal axis and anxiety-like behaviour in rats

by

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Abstract

The maternal environment has a profound effect on the development of offspring, including responses to stress mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Maternal diet and stress are two environmental factors that program the HPA axis in a manner that persists throughout adulthood, however there are no reports of their joint effect on the development of the HPA axis and anxiety-like behaviour. The objective of this thesis was to examine the effects of maternal obesity in the form of high fat diet (HFD) consumption and maternal stress in the form of exposure to chronic variable stress (CVS) on stress-related behavioural, endocrine, and molecular measures in neonatal and adult offspring in rats. I hypothesized that combined effects perinatal HFD and prenatal CVS would have synergistic effects on programming of stress-related phenotypes. I observed strong main effects of perinatal HFD on offspring anxiety-like behaviours and HPA axis physiological responses. Interestingly, CVS normalized the effects of perinatal HFD on anxiety-like behaviour in adult female offspring and physiological stress response in neonate offspring. I also characterized maternal physiological and behavioural responses to HFD consumption and CVS exposure and found increased sensitivity to CVS in HFD dams. Further, examining the interaction between the dam and neonatal offspring using maternal behaviour and pup ultrasonic vocalization (USVs) characteristics, I found impaired pup retrieval in HFD dams and altered USV characteristics in HFD neonates. Finally, due to the main effects of perinatal HFD on behavioural and physiological phenotype, I examined differential effects of maternal HFD consumption on offspring gene expression and the epigenome in brain

regions that regulate the HPA axis. I observed increased Crh expression in the PVN and decreased Nr3c1 expression in the ventral hippocampus in neonates along with differential methylation of genes involved in neuronal development in the amygdala. In adulthood, HFD induced differential DNA methylation in genes involved in protein phosphorylation, specifically GTPase related genes. Findings in this thesis provides several avenues for future studies in both mother and offspring, including possible intervention studies targeting dam-pup interaction and molecular mechanism underlying the programming effects of perinatal HFD in offspring.

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instilled in me the love for science and knowledge and always encouraged me to pursue my dreams and whose prayers are always with me, I dedicate this work.

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List of Abbreviations

11b-HSD	11beta- hydroxysteroid dehydrogenase
5-hmC	5-hydroxymethylcytosine
a-MSH	a-melanocyte-stimulating hormone
AB	Arched-back nursing
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AG	Anogenital licking
ANOVA	Analysis of variance
Anxa6	Annexin A6
AVP	Arginin vasopressin
BAT	Brown fat tissue
BDNF	Brain derived neurotrophic factor
CaMKII	Ca2+/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CB	Clean bedding
CBG	Corticosteroid-binding globulin
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cGMP-PKG	cGMP-dependent protein kinase G
CHD	Chow diet
Cort	Coticosterone
CpG	Cytosine and guanine
CRH	Corticotropin-releasing hormone
CVS	Chronic variable stress
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase
DOHaD	Developmental Origins of Health and Disease
E	Embryonic day
ECM	Extra cellular matrix
EPM	Elevated plus maze
FACS	Fluorescence-Activated Cell Sorting
FDR	False discovery rate
Fkbp5	FK506 binding protein 5
GABA	Gamma-Aminobutyric Acid
GAD1	Glutamic acid decarboxylase 1
GADD45b	Growth arrest and DNA damage inducible beta
GC	Glucocorticoid

GD	Gestation day
GLM	General linear models
GnRH	Gonadotropin-releasing hormone
GO	Gene Ontology
GR	Glucocorticoid receptor
HAB	High anxiety-like behaviour
HFD	High fat diet
HPA	Hypothalamic pituitary adrenal
IL-1B	Interleukin 1 beta
LAB	Low anxiety-like behaviour
LMM	Linear mixed models
LPS	Lipopolysaccharide
LSD	Least significant difference
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MB	Male bedding
MBT	Marble burying test
mCG	Methylated CpG
mCH	non-CpG methylated DNA
MeCP2	Methyl CpG binding protein 2
MeDIP	Methylated DNA immunoprecipitation
MR	Mineralocorticoid receptor
NFκB	Nuclear factor κB
NGFI-A	Nerve-growth factor-inducible factor A
NIH	Novelty induced hypophagia
OF	Open field
PFC	Prefrontal cortex
PND	Postnatal day
POMC	Proopiomelanocortin
PVN	Paraventicular nucleus
qRT-PCR	Quantitative real-time reverse transcriptase-polymerase chain reaction
Rab5a	Ras-related protein Rab-5A
RNA	Ribonucleic acid
RRBS	Reduced representation bisulfate sequencing
SNP	Single nucleotide polymorphism
TBC1D10	TBC1 domain family member 10b
USV	Ultrasonic vocalizations
WGBS	Whole genome bisulfate sequencing
Zfp423	Zinc finger protein 423

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Chapter 1
General Introduction

This chapter was adapted from:

Abuaish, S., McGowan, P.O., 2017. Impacts of Maternal High-Fat Diet on Stress-Related Behaviour and the Endocrine Response to Stress in Offspring, in: Rajendram, R., Preedy, V.R., Patel, V.B. (Eds.), Diet, Nutrition, and Fetal Programming. Springer International Publishing, Cham, pp. 213–225. doi:10.1007/978-3-319-60289-9_17

1 General Introduction

1.1 The hypothalamic pituitary adrenal axis: a mediator of early life environments

Animals are exposed to windows of critical developmental periods where they are most vulnerable to environmental impacts priming them for subsequent health consequences. The organism adapts to early life events through generating stable alterations in their phenotype, which is explained by the term early life programming (Xiong and Zhang, 2013). The brain starts developing during gestation and continues postnatally throughout the early period of life to adolescence, where it goes through rapid development and is especially influenced by the environment (Lupien et al., 2009; Marco et al., 2011; Matthews, 2002; Weinstock, 2008). The hypothalamic pituitary adrenal axis (HPA) (Figure 1.1), a circuitry that orchestrates a hormonal cascade to regulate the stress response and maintain homeostatic equilibrium, seems to be highly impacted by and act as a sensor of early life environment that later shapes the organism's response to stress and consequently modulates its mental health (Matthews, 2002; Xiong and Zhang, 2013).

Upon stress, the paraventricular nucleus (PVN) of the hypothalamus receives several neuronal inputs from different stress monitoring areas in the brain leading to the release of corticotropin releasing hormone (CRH) that reaches the anterior pituitary gland inducing the release of adrenocorticotropic hormone (ACTH). Through the systemic circulation, ACTH reaches the adrenal cortex stimulating the release of the downstream effecter of the HPA axis, glucocorticoid (GC) (Smith and Vale, 2006; Trollope et al., 2012). GC, cortisol in humans and corticosterone (Cort) in rodents, has a wide range of effects on the body in response to stress and leads to a negative feedback to different components of HPA axis through its interaction with its receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (McEwen, 2017). Chronic stress and high levels of GC leads to several health-debilitating effects on the immune system, the cardiovascular system, and the nervous system (Kadmiel and Cidlowski, 2013).

Several brain regions are involved in the regulation of the HPA axis and the stress response including the hippocampus, amygdala, and the prefrontal cortex, which are also implicated in

several psychological disorders (Smith and Vale, 2006). The hippocampus is essential in terminating the HPA axis response to stress through GR and Cort interaction. It sends signals to GABAergic interneurons that in turn inhibit the hypothalamic PVN (Cullinan et al., 1993). Similarly, the prefrontal cortex has an inhibitory effect on the HPA axis and is rich in GR, which indicates its role in mediating Cort negative feedback signal (Herman et al., 2016). The amygdala is another important limbic region that is known as a major regulator of emotions and a main target of Cort, where it has opposite effects there relative to the hippocampus. It contains a number of nuclei two of which are implicated in the activation of the HPA axis; the central nucleus and the medial nucleus, each activated by either physiological (i.e. hemorrhage) or psychological (i.e. predator exposure) stressors, respectively (Herman et al., 2016). Cort signaling in the amygdala causes an increase in CRH expression potentiating the effect of a stressor on the HPA axis through direct projections into the hypothalamus (Canteras et al., 1995; Makino et al., 1994).

There are several evidences demonstrating that epigenetic modification could explain these long lasting changes in the HPA axis (Burdge and Lillycrop, 2010; McGowan et al., 2009; Monk et al., 2012; Oberlander et al., 2008; Paternain et al., 2012; Sosnowski et al., 2018). Epigenetic modifications cause an alteration in the expression patterns of genes without changing the underlying sequences (McGowan and Roth, 2015). These epigenetic regulations include DNA methylation, histone modifications, and non-coding RNA (Figure 1.2) (McGowan and Roth, 2015). DNA methylation has been extensively studied and has been considered a relatively stable epigenetic modification. Recent research has indicated that, in certain conditions, DNA methylation may remain dynamic throughout the lifespan (McGowan and Roth, 2015). DNA methylation of cytosine and guanine (CpG) rich sites at gene promoters, where a methyl group is added to the 5' cytosines, leads, in many cases, to repression of gene expression (Bird, 2002). Recently, 5-hydroxymethylcytosine (5-hmC), thought to be an intermediate DNA modification between fully methylated and unmethylated DNA, may contribute to the dynamic nature of DNA modifications (McGowan and Roth, 2015). As discussed above, the HPA axis appears to be highly impacted by and act as a sensor of early life environment that later shapes the organism's response to stress and consequently modulating its mental health (Xiong and Zhang, 2013).

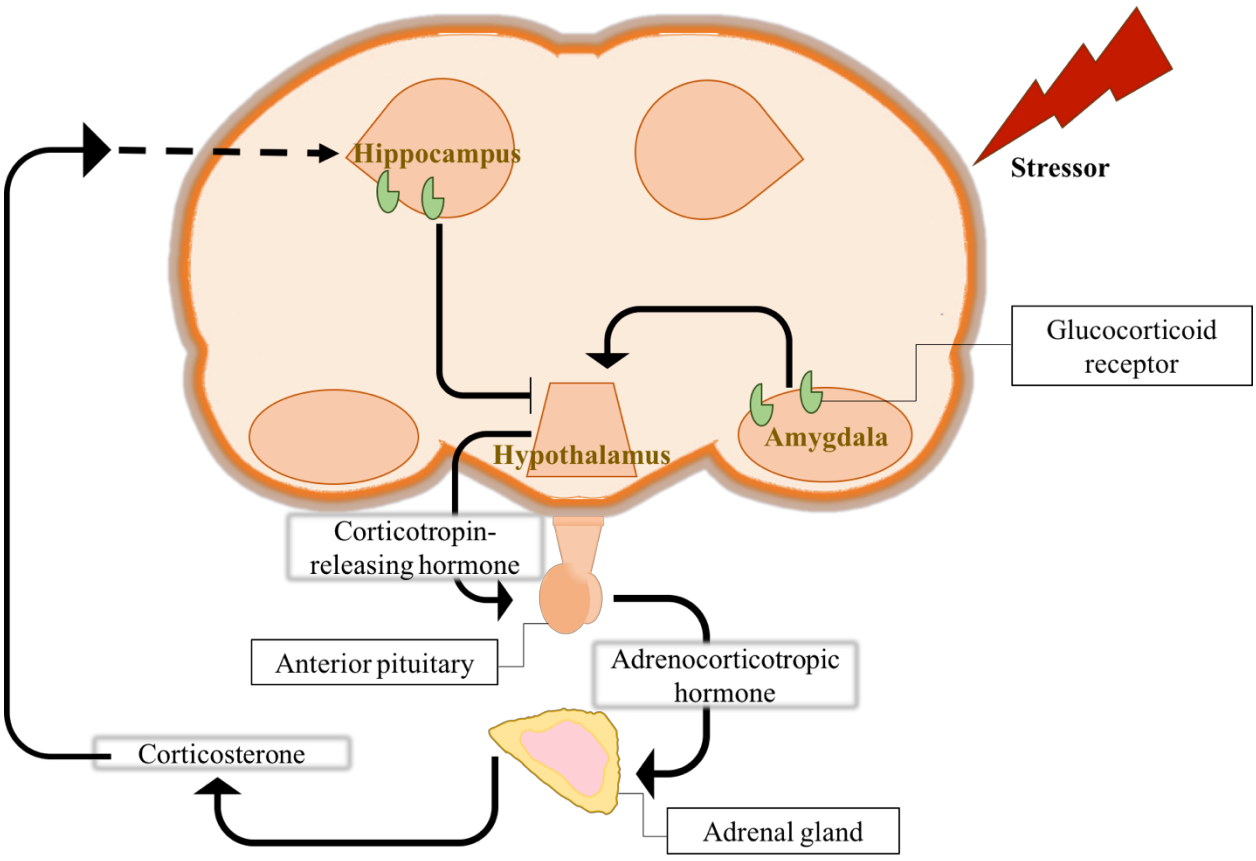

Figure 1.1 The hypothalamic-pituitary-adrenal axis. The hypothalamic pituitary adrenal axis (HPA) orchestrates a hormonal cascade to regulate the endocrine stress response and maintain homeostatic equilibrium. Upon exposure to a stressor, the paraventricular nucleus (PVN) of the hypothalamus receives coordinated neuronal inputs from different stress monitoring brain regions, leading to the release of corticotropin-releasing hormone (CRH) that reaches the anterior pituitary gland and induces the release of adrenocorticotropic hormone (ACTH). Through systemic circulation, ACTH reaches the adrenal cortex, stimulating the release of the downstream effecter of the HPA axis, corticoserone. Corticosterone has a wide range of effects on the body in response to stress and leads to feedback through its interaction with corticosteroid receptors, including the glucocorticoid receptor (GR). (Modified from Robert Sanders. *New neurons help us remember fear.* Available from http://news.berkeley.edu/2011/06/14/new-neurons-help-to-remember-fear/)

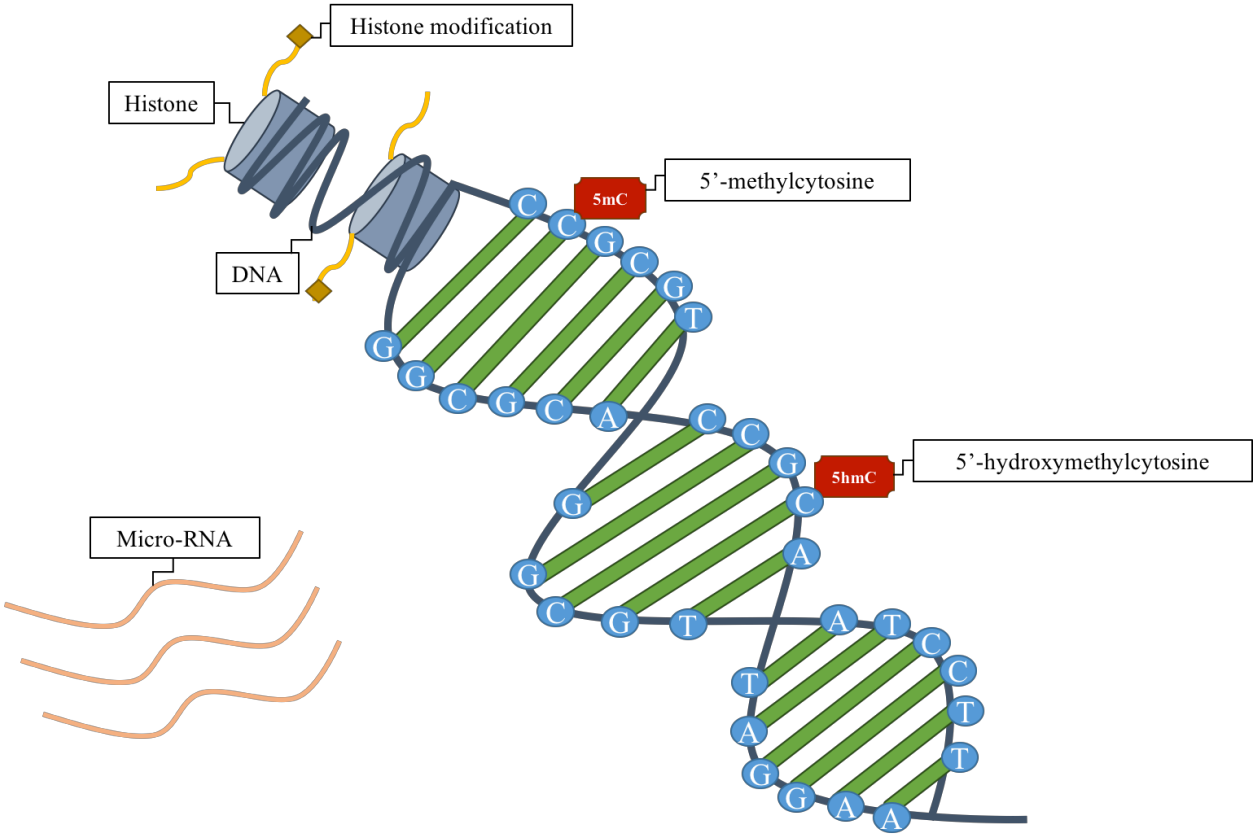

Figure 1.2 Epigenetic regulation of transcription. There are a number of epigenetic mechanisms the cell uses to regulate gene expression. These include the modification of histone tails, DNA methylation modifications at the 5' end of cytosines in cytosine and guanine rich regions, and the expression of non-coding RNAs.

Studies have shown that this sensitivity to early life is mediated by the methylation of GR gene (Nr3c1) in the brain's limbic regions in many models of early life stress (McGowan and Roth, 2015).

1.2 Early life factors impacting HPA axis programming

Stress and obesity are risk factors to several health issues, including metabolic, vascular and mental. Studies have reported association between chronic stress, such as job demand and socioeconomic stress, and obesity (Rosmond and Björntorp, 1999). Stress and food intake regulation have reciprocal effects on one another and on the HPA axis that could be explained in part by shared anatomical brain structures and circuitry (Spencer, 2013; Warne, 2009). Obesity and chronic stress are associated with a hyperactivated HPA axis and elevated GC levels (Dallman et al., 2004; Rosmond et al., 2000). Diet induced obesity in rodents is a model used in an effort to understand the effect obesity has on the different systems in these animals. Different brain systems including the HPA axis in these animals were reported to show dysregulation accompanied with behavioural abnormalities (Abildgaard et al., 2011; Evans et al., 2013; Heyward et al., 2012; Lavin et al., 2011; Sharma et al., 2013; Sharma and Fulton, 2013; Sivanathan et al., 2015). Prenatal and early postnatal psychological or nutritional environmental factors can cause alterations in the HPA axis leading to the development of cognitive, emotional, and social adaptations in human and animal models (Painter et al., 2005; Raygada et al., 1998; Richardson et al., 2006; Sasaki et al., 2014; Sasaki et al., 2013; Sullivan et al., 2010; Watson et al., 1999; Wright et al., 2011). This is also accompanied by alteration in the brain structure and physiology (Martínez-Téllez et al., 2009; Murmu et al., 2006).

1.3 Maternal obesity impacts on HPA axis phenotype

Obesity is recognized as a major threat to public health and a risk factor for many chronic diseases. The prevalence of obesity has risen dramatically worldwide in the last 30 years, including in women of reproductive age (Fisher et al., 2013; Ng et al., 2014). A number of studies in humans have indicated that maternal obesity is a major factor in predisposing the offspring to develop metabolic disorders (Dearden and Ozanne, 2015). There is also evidence

linking maternal obesity to the development of a number of neuropsychological disorders in the offspring including anxiety and depression, schizophrenia, ADHD, and cognitive impairments (Rivera et al., 2015).

1.3.1 Rodent models of maternal obesity

A number of rodent models have been used to study the Developmental Origins of Health and Disease (DOHaD), including the impact of the early life nutritional environment on behaviour, physiology, and gene regulatory mechanisms. Generally, increasing the fat content of a diet to over 30% has been demonstrated to induce obesity in rodents, which is also associated with a dysfunctional HPA axis and related behavioural dysfunction (Hariri and Thibault, 2010; Sharma and Fulton, 2013; Sivanathan et al., 2015). However, several variations of the diet exist under the broad umbrella of high fat diet (HFD), which likely contribute to the variability in results found in the field of HFD-induced obesity. For example, commercially available diets offer a number of purified HFDs with a range of between 20% to 60% fat content. In addition, there is a substantial variation in the type of fatty acid in the diet, ranging from primarily animal sources (ie. Lard, beef tallow, fish oil) to plant sources (ie. Corn, soybean, olive) (Buettner et al., 2007; Hariri and Thibault, 2010; Lai et al., 2014). For example, a recent report (Hryhorczuk et al., 2015) has shown that saturated and monounsaturated fat have different effects on mesolimbic function in rats. Another paradigm that has been used to induce obesity in rodents is “Cafeteria diet” or “Junk food diet”, where a combination of highly palatable human food (ie. Potato chips, cookies, chocolate, cheese, etc) is given to the animal with *ad libitum* access to the regular chow diet. This type of diet has received some criticism due to the difficulty of accurately measuring nutrient intake and the variation in caloric content and type (Buettner et al., 2007). While acknowledging the different types of HFD regimen used to study diet-induced obesity in the literature and their possible contribution to variation in the results across studies, here we will explore the effects of maternal consumption of HFD on the development of HPA axis and emotionality in the offspring. However, we will begin by discussing the effects of the HFD on the mothers, who are the first level of interface between the offspring and their environment.

1.3.2 HFD effects on the mothers

In many species, offspring are exposed to their early environment primarily through the mother. Therefore, in order to understand how early environments affect the offspring, the effects of

environmental factors that influence offspring development should be examined in the mother. There are a number of paradigms of HFD exposure that have been developed to study the effect of the time of exposure of this diet during offspring development and to distinguish between effects of maternal obesity and the diet itself.

One way in which maternal obesity has been modelled in rodents is by feeding females HFD for at least 4 weeks prior to gestation and continued throughout gestation and lactation. The pregestational exposure to the HFD leads to an increase in body weight of the females and an increase in their caloric intake (Bellisario et al., 2015; Bilbo and Tsang, 2010; Rolls and Rowe, 1982; Sasaki et al., 2014; Sasaki et al., 2013; Vogt et al., 2014; White et al., 2009). Also, there is evidence of an increase in glucose levels in the blood and development of insulin resistance (Vogt et al., 2014). In our laboratory, we have shown (Sivanathan et al., 2015) that adult females exposed to HFD for 8 weeks develop anxiety-like behaviour and a decrease in glucocorticoid receptors in the brain, while others have shown (Ressler et al., 2015) an increase in basal levels of Cort in plasma. These findings indicate that these animals already have an altered HPA axis prior to gestation.

Once pregnant and still maintained on the HFD, the dams continue to gain more weight and consume more calories (Rolls et al., 1984; Sasaki et al., 2013). In addition, obese dams show a decrease in basal locomotor activity which could contribute to their body weight gain (Bayol et al., 2007; Bellisario et al., 2015). HFD consumption lead to an elevated basal Cort levels during gestation (Bellisario et al., 2015; Rodriguez et al., 2012), which was accompanied by a decrease in placental 11beta- hydroxysteroid dehydrogenase (11β-HSD) type 2, an enzyme that rapidly metabolises maternal Cort to deactivate it before entering the fetal circulation (Bellisario et al., 2015). These effects are similar to effects of prenatal stress (Jensen Peña et al., 2012), which could suggest that HFD is acting as a stressor during this sensitive period of development.

Obese dams have been reported in some studies (Buonfiglio et al., 2016; Rolls et al., 1984; Rolls and Rowe, 1982; Wright et al., 2011) to lose weight during lactation – a finding that is not fully understood. However, one study (Wright et al., 2011) suggested an increased investment in energy in milk production in these dams compared to control dams could explain the higher weight loss. Indeed, in studies of milk composition (Purcell et al., 2011; Rolls et al., 1986; Sun et al., 2012), it was found that maternal milk is higher in energy provided by higher fat and protein

content in obese dams compared to those on a control diet. One study reported (Bertino, 1982) that HFD dams are more active during the dark phase of the circadian cycle during lactation. Thus, the increase in locomotor activity during the dark phase might account, in part, for the weight loss observed in obese dams. Also, dams during late gestation and lactation become hyperphagic and accumulate visceral fat in order to meet the demands of lactation, and this is regulated in part by an increase in prolactin, a key hormone for milk production, during this period (Woodside, 2007). A recent study (Buonfiglio et al., 2016) indicated that obese HFD mice are unresponsive to prolactin signaling in both mammary glands and the hypothalamus during lactation and that this unresponsiveness is mediated by the high levels of leptin in obese animals, a common feature seen in obese dams (Bilbo and Tsang, 2010; Buonfiglio et al., 2016). Interestingly, one study (Marco et al., 2014) reported that HFD dams' weight loss during lactation was correlated with demethylation of the proopiomelanocortin (POMC) gene in the arcuate nucleus where it functions to inhibit food intake. In addition, the weight loss was also correlated with the increased expression of the growth arrest and DNA damage-inducible beta (GADD45b) gene, which has been associated with the active demethylation of DNA (Marco et al., 2014).

A few studies that use mice to study maternal HFD have reported increased pup cannibalism, reduced lactation, and reduced maternal retrieval behaviour, all of which contribute to a lower survival rate of litters. These effects may also be explained in part by the insensitivity to prolactin, which plays a role in maternal behaviour and pup retrieval (Bellisario et al., 2015, 2014; Buonfiglio et al., 2016; Terkel et al., 1979). HFD mouse dams also show lower c-fos expression in the olfactory bulb during gestation, which could explain the increased cannibalism and reduced retrieval of their pups, since olfaction plays an important role in identifying the pup and initiating maternal behaviour (Bellisario et al., 2015; Fleming and Rosenblatt, 1974). On the other hand, at least two studies in rats (Bertino, 1982; Purcell et al., 2011) have reported increased maternal behaviour in HFD dams, seen by an increase in arched back nursing and a decrease in time away from the pups. HFD rat dams have shown delayed lactation during a weigh-suckle-weigh test, where the pups were separated from their dams for four hours and weighed then returned to their mothers for 30 min and weighed again to measure milk yield. The HFD dams had lower milk yield compared to controls on postnatal day (PND) 1, however by PND 2 the milk yield was comparable to between HFD and control dams (Hernandez et al.,

2012). This was accompanied by an increase in inflammatory cytokine production in mammary glands. An earlier study (Bilbo and Tsang, 2010) showed that HFD dams have higher pro-inflammatory cytokine in their plasma during lactation, indicating a systemic inflammation. Inflammation is highly influenced by altered HPA axis as we will discuss below. Alterations in the HPA axis in lactating HFD dams have recently been reported, where the dams showed a low basal Cort level, an increase in their reactivity to ACTH injection and increased anxiety-like behaviour compared to control dams (Perani et al., 2015). This was associated with an alteration adrenal lipid supply and steroidogenesis. As evident by the results discussed above, HFD and maternal obesity alter several aspects of maternal physiology and behaviour, which in turn appear to contribute to the programming of offspring phenotype.

1.3.3 Maternal obesity effects on offspring HPA axis and stress-related behaviours

Work in our laboratory and others (Bilbo and Tsang, 2010; Kang et al., 2014; Peleg-Raibstein et al., 2012; Rodriguez et al., 2012; Sasaki et al., 2013) has demonstrated that maternal obesity leads to an increase in anxiety-like behaviour in adult offspring in novelty-induced approach/avoidance tasks. We found (Sasaki et al., 2013) that the increase in anxiety-like behaviour was accompanied by an increase in Nr3c1 gene expression in the amygdala, a brain region where GR activation potentiates the HPA response to stress (Joels et al., 2008). In tandem, we found that adult female offspring exhibited a more reactive HPA axis, as they released higher levels of Cort in response to restraint stress. A recent study (Lin et al., 2015) measuring HPA axis reactivity to a repeated restraint stress reported a higher Cort response in HFD adult offspring of HFD dams and showed an impaired habituation of the HPA response to repeated restraint after 4 restraint trials compared to the controls. The same study also measured depressive-like behaviour in the adult offspring after 14 days of chronic unpredictable mild stress, where the animals were randomly exposed to nine different mild stressors, and found an increase in anhedonia and learned helplessness in offspring, which was also present prior to the chronic unpredictable mild stress in young adult HFD offspring. CRH, a key activator of the HPA axis, was highly expressed in the PVN of the hypothalamus of adult offspring exposed to HFD during development (Chen et al., 2009).

Perinatal HFD effects may lead to a differential anxiety-like behaviour profiles between adolescents and adults, where a decrease in anxiety-like behaviour and an accompanying

increase in Nr3c1 mRNA levels in the hippocampus were observed in adolescent offspring (Sasaki et al., 2014). The hippocampus is a primary region through which GR inhibits HPA axis activity, which could explain the decrease in anxiety-like behaviour in adolescent animals (Sasaki et al., 2014). The decrease in anxiety-like behaviour in adolescents has been reported previously in several studies investigating other forms of early life stress, and could potentially be an indication of an increased impulsivity in these animals (Sasaki et al., 2014). Risk-taking behaviour is considered one of the hallmarks of adolescent behaviour and it has been suggested that early life environment could augment the display of this behaviour (Jacobson-Pick and Richter-Levin, 2010; Sasaki et al., 2014). A recent study (Grissom et al., 2014a) showed that adult offspring of obese dams had an increased impulsivity when tested on a 5-choice serial reaction time test.

An examination of Cort levels in offspring of obese dams at different ages revealed low basal Cort level at birth compared to control animals that later changed to higher basal Cort levels at 3 weeks of age and in adulthood in HFD offspring compared to control offspring. However, these animals also had higher weights in adulthood compared to controls, a finding that is not always observed (Desai et al., 2014; Sasaki et al., 2013; Tamashiro et al., 2009) and could potentially explain the high basal Cort levels. In contrast, we have previously found evidence of (Sasaki et al., 2013) low basal Cort levels in the HFD offspring and an increase in MR gene (Nr3c2) expression in the amygdala. At basal levels, MR binds Cort with high affinity, which might explain the decreased basal Cort levels seen in the HFD offspring (Joels et al., 2008). Anxious behaviour was also seen in HFD juvenile female offspring of non-human primates (Sullivan et al., 2010).

Cort and Nr3c1 expression are believed to modulate inflammatory responses in the body and the brain, which are upregulated as mentioned above in response to developmental HFD exposure (Bilbo and Tsang, 2010; Sasaki et al., 2013; Sorrells et al., 2014; White et al., 2009). Consequently, some work has revealed (Bilbo and Tsang, 2010; Kang et al., 2014; Sasaki et al., 2013; White et al., 2009) an increase in inflammation in the brains of HFD offspring, revealed by an activation in microglia and increased pro-inflammatory cytokines. In addition, increased inflammation in the limbic regions of the brain has been linked to anxiety and depression (Maes et al., 2002). Increased oxidative damage has been observed in HFD offspring (Tozuka et al., 2010; White et al., 2009). Nuclear factor κB (NFκB), a pro-inflammatory transcription factor,

which was upregulated in the amygdala of HFD offspring, is known to induce oxidative stress in neurons (Hovatta et al., 2010; Sasaki et al., 2013).

Oxidative stress has also been reported in a number of anxiety disorders in humans (Hovatta et al., 2010). An oxidative stress mediated reduction in brain-derived neurotrophic factor (BDNF) in the hippocampus of HFD offspring has been reported, which was associated with decreased neurogenesis and impaired arborization of hippocampal neurons (Tozuka et al., 2010). Hippocampal plasticity that is governed by BDNF levels is highly sensitive to the HPA axis and is altered in anxiety disorders and depression (Lupien et al., 2009; Roth et al., 2011).

Overall, this evidence suggests that developmental exposure to maternal obesity has an impact on the HPA axis of the offspring, reflected at least in part in an alteration in their behaviour, HPA axis physiology, and stress-related gene expression, though additional mechanisms are also involved. Procedural variations in the timing of HFD exposure during development can lead to differential effects on the offspring HPA axis and behaviour, as we will discuss below.

1.3.4 Timing of HFD exposure during development and its effects on the offspring

Other dietary paradigms have been developed to try to understand the effects of maternal HFD consumption in the presence or absence of maternal obesity at different developmental stages, during gestation and/or lactation. For instance, in one study (Grissom et al., 2014b), the authors investigated whether obesity prior to conception and not during gestation and lactation could program the offspring brain. Using embryo transfer from obese or lean donor mice into obese or lean pseudopregnant dams, pregestational obesity alone was found to alter gene expression of the μ-opioid receptor, which plays a role in reward signalling in the nucleus accumbens. However, obesity during gestation and lactation had a more pronounced impact on increasing gene expression in other brain regions in the same study. Others also showed (Wright et al., 2011) that pregestational obesity alone could impact behaviour in the offspring seen by reduced activity in the elevated plus maze and open field tests.

Two procedures that have been used to examine the effect of gestational and/or lactational HFD effects on offspring are diet intervention during gestation or lactation and cross-fostering. In the diet intervention procedure, the dams are switched from HFD in gestation to control diet in lactation or from control diet in gestation to HFD in lactation. In the cross-fostering procedure,

offspring from HFD dams are cross-fostered to control diet dams and vice versa. While these study designs have been used in a number of studies investigating metabolic programming in the offspring (see (Dearden and Ozanne, 2015) for review), there are limited studies looking at the effect of these manipulations on behavioural outcomes in offspring. One study (Desai et al., 2014) investigating the effects of maternal HFD on offspring HPA axis has shown that gestational HFD causes an elevation of basal Cort levels in the offspring that was not rescued by control diet during lactation. Another study (Kang et al., 2014), found that gestational HFD leads to increased anxiety-like behaviour in female offspring that was not observed in offspring exposed to the HFD during lactation alone. In addition, while increased microglial activation and pro-inflammatory cytokine levels were observed in female offspring of dam exposed to gestational HFD, offspring switched onto control diet during the dams' lactational period did not show this inflammatory response (Kang et al., 2014). There is evidence suggesting that adult male offspring exposed to maternal HFD during the dams' lactational period alone exhibit reduced anxiety-like behaviour (Wright et al., 2011). Another report (Balsevich et al., 2015) found reduced anxiety-like behaviour in adult male offspring when exposed to HFD during the dams' gestational period alone. However, at 12-months of age, offspring had an increase in anxiety-like behaviour due to the gestational HFD exposure (Balsevich et al., 2015). Lactational exposure to HFD alone leads to a blunted HPA axis response to stress in neonates from HFD litters, which was suggested to be mediated via the high levels of leptin in the offspring, as earlier reports indicated that leptin inhibits the ACTH response to stress in neonatal rats (Oates et al., 2000). However, in adolescence, the offspring had a heightened HPA axis response to stress compared to control rats, and their leptin levels were normalized at that age (Trottier et al., 1998). Leptin, in addition to its role in food intake and energy homeostasis, plays a role in regulating the HPA axis, where it downregulates CRH mRNA levels in the PNV and increases Nr3c1 levels in the hippocampus and hypothalamus (Oates et al., 2000; Spencer, 2013; Trottier et al., 1998). At the same time, at high levels, Cort is believed to stimulate the secretion of leptin, indicating a reciprocal relationship between the two hormones (Slieker et al., 1996). It is clear that HFD has differential effects depending on the developmental timing of exposure. More research is necessary to disentangle the mechanisms by which these effects occur, especially given the complex relationship between the HPA axis and hormones that regulate diet and energy homeostasis. In the next section, we discuss evidence suggesting that the long-term effects of maternal HFD on offspring behavioural phenotype may, at least in part, involve

epigenetic modifications.

1.3.5 Epigenetic mechanisms of maternal programming in offspring

There is evidence that the maternal diet is associated with alterations in the epigenome of the offspring and offspring phenotype. For instance, supplementing obese Agouti dams, an animal model of obesity, with methyl donors in their diet led to the methylation and silencing of the Agouti gene in offspring, sparing them the obese phenotype (Waterland et al., 2008). Maternal HFD has also been reported to impact the offspring epigenome. Adult offspring of HFD dams exhibited global DNA hypomethylation in the hypothalamus and the prefrontal cortex (PFC) compared to control offspring, which was reversed when dams were supplemented with methyl donors in their diets (Carlin et al., 2013; Zivjena Vucetic et al., 2010). This was associated with an overexpression in the DNA methyltransferase DNMT1 in the PFC, which was positively correlated with impulsive behaviour in adult offspring (Grissom et al., 2014a). Increased methylation was observed in the POMC gene promoter, a precursor to ACTH, and α-melanocyte-stimulating hormone (α-MSH), an anorexigenic neuropeptide, in the arcuate nucleus of the hypothalamus of HFD offspring (Marco et al., 2014). This reduction could indirectly affect the HPA axis. For example, POMC is also a precursor to β-endorphin which has an inhibitory effect on CRH release (Weinstock, 1997). Global hypomethylation was observed in HFD female placentas, associated with a downregulation of DNMT3l, a de novo DNA methyltransferase (Gabory et al., 2012; Gallou-Kabani et al., 2010). This exciting field is still in its infancy and future work will help elucidate the epigenetic alterations associated with maternal HFD in brain regions and genes regulating HPA axis function.

1.3.6 Consideration of relevance of rodent models as translational models: strengths and limitations

Animal models have been successful in recapitulating some aspects of maternal obesity effects in human offspring, such as the alteration in metabolic outcomes (Dearden and Ozanne, 2015). More work is needed to characterize the emotional and behavioural phenotypes in both human and animal models, however, important parallels have emerged between the human and animal literature. Maternal obesity in humans is associated emotional dysregulation, as children from obese mothers were reported to have increased fear and sadness (Rivera et al., 2015). In animals, as discussed above, offspring from HFD dams exhibit increased anxiety and depression-

like behaviours in a number of studies (Chen et al., 2009; Peleg-Raibstein et al., 2012; Sasaki et al., 2013). However, a few considerations need to be taken into account when comparing studies of animal models of maternal HFD consumption. First, the timing of exposure varies across studies. For example, many of the studies start HFD feeding in the dam prior to gestation. However, the duration of the pregestational exposure varies among studies, ranging from HFD exposure from weaning age to mating to a few weeks prior to mating. This difference in the duration of maternal exposure might yield different outcomes. For instance, starting the HFD regimen from the weaning period in the prospective dams, was reported to result in higher body weights in the offspring that were maintained until adulthood along with elevated Cort levels (Desai et al., 2014), a result not found by a number of studies using more restricted maternal exposures to HFD (Sasaki et al., 2013; Tamashiro et al., 2009; Vucetic et al., 2012). Second, as discussed earlier, lactational compared to gestational exposure to HFD is associated with differential behavioural outcomes. Maternal metabolic hormones such as insulin and leptin, which depend on the duration of HFD feeding in the dams, are either unreported or variable across studies. Insulin and leptin act as growth factors in the brain, and could influence the development and maturation of neurocircuitry in the brain of the offspring, which in turn could influence the behaviour and physiology of the offspring (Dearden and Ozanne, 2015; Vogt et al., 2014). In order to better characterize the phenotype of the offspring, we suggest measuring and reporting maternal leptin and insulin levels as a way to validate the HFD exposure protocol used in the study and to help compare the results across models of maternal HFD. Consequently, this will help improve our understanding of how these maternal hormones could play a role in mediating the effects of the HFD on the offspring. Figure 1.3 provides a summary of the effects reported in dams and offspring in response to maternal HFD consumption.

It is essential to acknowledge the difference in brain development when translating rodent finding to humans. For example, the prenatal period is a time of heightened neurogenesis, while important milestones in neurocircuit development and changes in connectivity occur during the early postnatal period of development in rodents, and during the third trimester in humans (Dearden and Ozanne, 2015; Vogt et al., 2014). Thus, the early postnatal period in rodents diverges from conditions during human development, where these milestones occur in utero.

The timing of the developmental expression of Nr3c1 in the brain between the rodent and humans is also different. In the fetal rat brain, Nr3c1 is first detected at embryonic day (E) 13 at

low levels that increase rapidly after birth (Xiong and Zhang, 2013), however, Nr3c1 expression in humans is detected in the hippocampus between 23-34 weeks of gestation and its level stays stable in the early postpartum period (Xiong and Zhang, 2013). It is important to be cognizant of limitations in directly translating the results from rodent studies to humans, despite the obvious advantages of rodent studies vis-à-vis a causal and mechanistic understanding of dietary effects.

1.4 Prenatal stress impacts on the HPA axis phenotype

Rodents have been a useful model to study the impact of early life stress on their behaviour, physiology and genetics. Multiple paradigms have been developed to study prenatal stress effects in rodents including exposure to restraint, saline injection, predator exposure, shock, or a combination of multiple stressors several times during gestation (St-Cyr and McGowan, 2018; Weinstock, 2008). The timing of exposure to early stress has a differential effect on mental health outcomes in adulthood, which could be governed by the periods of plasticity during brain development (Davis and Sandman, 2010; Huizink et al., 2003; Lupien et al., 2009; Watson et al., 1999). In rodents, different components of the HPA axis start developing during mid-gestation and the projections mature during the early postnatal period, thus many stress paradigms have been implemented during these periods and shown significant impacts on the offspring HPA axis programming (Weinstock, 2008).

1.4.1 Prenatal chronic variable stress effects on HPA axis

The chronic variable stress (CVS) paradigm was first developed to model daily stressors experienced by humans and to induce depressive-like behaviours in rodents (Hill et al., 2012). It requires the exposure to at least 3 different stressors in an unpredictable manner to prevent habituation to the stress and induce a more robust HPA axis activation (Hill et al., 2012; Koenig et al., 2005). Researchers have investigated different variations of this paradigm as a prenatal stress protocol reporting emotional, cognitive, and social behavioural abnormalities in adult offspring (Emack and Matthews, 2011; Koenig et al., 2005; Mueller and Bale, 2008, 2007). For example, prenatal CVS offspring exhibited hyperactivity, increased aggression, increased depressive-like behaviour, and decreased sensorimotor gating (Koenig et al., 2005; Lee et al., 2007; Mueller and Bale, 2008; Wilson and Terry, 2013). In addition, adult offspring showed decreased object recognition memory and altered spatial learning (Mueller and Bale, 2007; Wilson and Terry, 2013).

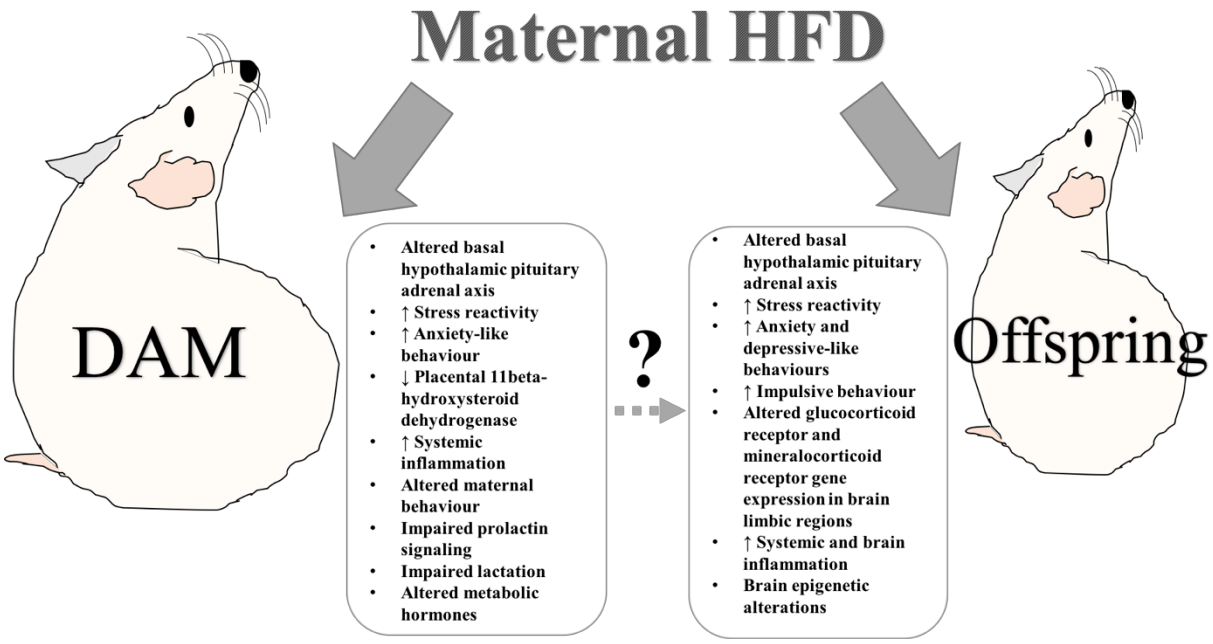

Figure 1.3 Summary of reported effects of maternal high-fat diet. High-fat diet consumption in the dams causes a number of effects in the dams and the offspring. The altered behaviour and physiology in the dams may directly or indirectly influence behavioural and physiological outcomes in the offspring.

In addition, dysregulation in the HPA axis was reported in offspring exposed to prenatal CVS. Some reports have indicated decreased basal Cort levels at birth, while others showed increased stress reactivity in adult (Koenig et al., 2005; Mueller and Bale, 2008; Tamashiro et al., 2009). These phenotypic alterations were accompanied by differential gene expression and DNA methylation. Prenatal CVS was associated with increased Crh mRNA levels in the amygdala and decreased Nr3c1 levels in the hippocampus in adult mice, which was linked to decreased and increased DNA methylation in Crh and Nr3c1 promoters, respectively (Mueller and Bale, 2008). Others demonstrated decreased expression of BDNF in the hippocampus and the amygdala, which was associated with increased DNA methylation in two different promoters of BDNF (Boersma et al., 2013a).

1.5 Thesis objective and aims

Stress is associated with increased preference of palatable food and obesity (Pecoraro et al., 2004; Warne, 2009). On the other hand, obesity is characterized by increased stress reactivity (Sharma and Fulton, 2013). As reviewed above, maternal obesity could render the mother more vulnerable to stress. Adult female virgin rats consuming HFD show increased basal Cort and reduced Nr3c1 expression in the hippocampus (Ressler et al., 2015; Sivanathan et al., 2015). During pregnancy, female mice also show elevated basal Cort levels in addition to decreased 11β-HSD2, which could allow higher Cort exposure to the growing fetuses (Bellisario et al., 2015; Rodriguez et al., 2012). Implemented separately, both models of maternal HFD consumption and prenatal CVS have been demonstrated to impact the adult offspring HPA axis phenotype. Behaviourally, adult offspring from both models exhibit increased emotionality, including anxiety-like behaviour and depressive-like behaviour (Lin et al., 2015; Mueller and Bale, 2008; Sasaki et al., 2013). In addition, these offspring show increased Cort response to stress (Koenig et al., 2005; Mueller and Bale, 2008; Sasaki et al., 2013). These behavioural and physiological alterations were accompanied by changes in neural gene expression and underlying DNA methylation. Adult offspring exposed to perinatal HFD show an increased CRH levels in the PVN (Chen et al., 2009), increased Nr3c1 and MR (Nr3c2) levels in the amygdala (Sasaki et al., 2013), and decreased levels of BDNF in the hippocampus (Tozuka et al., 2010). Prenatal CVS also induced an increase in CRH levels in the hypothalamus, while decreased Nr3c1 levels in hippocampus in adult offspring (Mueller and Bale, 2008). In addition, adult offspring exposed

to prenatal CVS showed decreased BDNF levels in the hippocampus (Boersma et al., 2013a). Epidemiological studies report an association between low economic status, stress, obesity, and mental health (Rosmond et al., 2000). Thus, using both paradigms as altered early environments is important to help us understand the relationship between both systems and how they influence mental health outcome in a relevant model. Given the interaction of stress and diet and their suggested effects on early programming of the HPA axis, it is very compelling to study how prenatal stress against maternal obesity affect the phenotype of the offspring.

The main **objective** of this thesis is to investigate the effects of early life experience of maternal obesity in the form of maternal HFD consumption and prenatal stress in the form of chronic variable stress on programming the HPA axis during postnatal development in the rat. The general **hypothesis is** that prenatal stress on maternal obesity background will amplify the effects of maternal obesity alone on offspring HPA axis related behaviour, physiology, and gene expression. The following outlines the specific objectives, aims, hypotheses, and approaches that were examined in this thesis:

Chapter 2: Objective 1: Examining perinatal HFD exposures and prenatal CVS effects on maternal phenotype and HPA axis programming in adult offspring

Aim 1.1: Characterizing maternal response to the diet and stress manipulations

Offspring are exposed to their early environment primarily through the mother. Therefore, in order to understand how the early environment affects the offspring, I wanted to examine the effects of HFD and CVS on the dams. Maternal measures that have been reported to influence offspring phenotype were assessed, including maternal weight and caloric intake, maternal HPA axis response, maternal care behaviour, and maternal anxiety- and depressive-like behaviours (Bosch et al., 2007; Smith et al., 2004). This aim assessed the following hypotheses:

Hypothesis and approach 1.1.1: Perinatal HFD will induce higher maternal weights and caloric intake throughout the perinatal period, while CVS would not affect maternal weight and food intake.

Earlier studies have demonstrated that placing females on HFD for 4 weeks prior to gestation will ensure higher weights of the dams at conception and throughout gestation, modeling maternal obesity (Bilbo and Tsang, 2010; Sasaki et al., 2013). In addition, the CVS paradigm has

been previously used as a prenatal stressor to HFD dams, which did not alter maternal caloric intake, but decreased gestational weight gain (Tamashiro et al., 2009). One advantage of using prenatal CVS is its limited effects on maternal weight and caloric intake, separating the effects of the stress from what could be intrauterine growth restriction that have been linked to other prenatal stress paradigms (Lesage et al., 2004; Tamashiro et al., 2009). In order to assess these measures, weekly dam and food weights were taken throughout the perinatal period.

Hypothesis and approach 1.1.2: Perinatal HFD and prenatal CVS will increase HPA axis reactivity in dams.

Increased maternal Cort levels is shown to contribute the program the offspring outcomes (Matthews, 2002). In addition, reports have shown that both HFD and CVS separately increase maternal Cort levels either basally or in response to a stressor (Bellisario et al., 2014; Rodriguez et al., 2012). In order to assess the HPA axis activity of dams, their response to a 20-min restraint stress during gestation was examined.

Hypothesis and approach 1.1.3: Perinatal HFD and prenatal CVS will alter maternal care behaviour.

Natural variation in maternal care behaviour indicated by high licking/grooming vs. low licking/grooming is known to impact offspring HPA axis development and behaviour, where litters from high maternal care having lower stress reactivity behaviourally and physiologically, which are governed by epigenetic changes in Nr3c1 expression in the hippocampus increasing the sensitivity to Cort negative feedback signaling (Liu et al., 1997; Weaver et al., 2004). Previous studies have demonstrated that HFD increases maternal behaviour in dams while prenatal stress in some reports leads to a decrease in maternal behaviour (Bertino, 1982; Purcell et al., 2011). I used two different protocols to examine maternal behaviours; 1) home cage maternal behaviour was recoded 1 hour 6 times a day (light phase: 10 am, 1 pm, and 5 pm; dark phase: 9 pm, 1 am, and 5 am) from postnatal day (PND) 1-6 (Champagne, 2003). 2) maternal retrieval on PND7 after 10 min maternal separation.

Hypothesis and approach 1.1.4: Perinatal HFD and prenatal CVS will increase maternal emotionality.

Altered HPA axis is associated with increased anxiety-like and depressive-like behaviours. In addition, both manipulations have been shown to increase anxiety-like and depressive-like behaviours (Hill et al., 2012; Sharma and Fulton, 2013; Sivanathan et al., 2015). Anxiety-like and depressive-like behaviours in dams were measured postpartum using open field test and

sucrose preference test, respectively.

Aim 1.2: Examine the effects of perinatal HFD and prenatal stress on anxiety-like behaviours in adult offspring.

Early life HFD exposure is shown to increase anxiety-like behaviour in adult offspring (Bilbo and Tsang, 2010; Rodriguez et al., 2012). Prenatal CVS is reported to induce depressive-like behaviour and decrease sociality in adult offspring (Mueller and Bale, 2008).

Hypothesis and approach 1.2.1: Prenatal CVS will exacerbate the effects of perinatal HFD on anxiety-like behaviours in adult offspring.

I examined adult emotional behaviour, using tests validated to measure anxiety-like behaviours in rodents, to determine if this paradigm would lead to programming of behaviour later in life. Different types of anxiety tests were used, including elevated plus maze and open field, which measure exploratory behaviour in a novel environment, novelty induced hypophagia to measure consummatory behaviour, and marble burying test. The use of different tests is implemented because it has been reported that these tests assess different aspects of anxiety in rodents (Ramos, 2008; Ramos and Mormède, 1997).

Aim 1.3: Examine the effects of prenatal stress and maternal HFD on the HPA axis physiology in adult offspring.

Anxiety-like behaviours are associated with a heightened HPA axis either basally or in response to stress. In addition, both perinatal HFD and prenatal CVS are shown to increase the HPA axis response measured by Cort levels in response to restraint stress (Koenig et al., 2005; Sasaki et al., 2013).

Hypothesis and approaches 1.3.1: Prenatal CVS will exacerbate the effects of perinatal HFD on HPA axis response to stress in adult offspring.

In order to test HPA axis response, a 20-min restraint challenge was performed on adult offspring exposed to perinatal HFD and CVS, and Cort levels were measured at baseline, stress, and recovery.

Chapter 3: Objective 2: Examining perinatal HFD exposures and prenatal CVS effects on

the HPA axis programming in neonatal offspring

The neonatal rodents have a unique HPA axis during the first two postnatal weeks are characterized as hyporesponsive to stress during the first two weeks of life, where it does not respond to stressors that known to activate the adult animals HPA axis (Vázquez and Akil, 1993). The early postnatal life is an essential period in brain development and sensitive to the surrounding environment. A number of studies examining the effects of perinatal HFD exposure timing on the metabolic and behavioural outcome of offspring have reported that the lactation period is important in mediating the consequences of the diet on the offspring (Abuaish and McGowan, 2017). I am using a HFD feeding paradigm that includes the lactation period, during which the offspring will be directly exposed to the diet through the mother's milk. Earlier work has shown that neonatal offspring exposed to HFD during this period had increased levels of insulin and leptin (Tamashiro et al., 2009). These hormones in addition to their metabolic homeostasis regulation in the body through the brain, act as growth factors in the brain, which could impact the maturation of different neurocircuits, including the HPA axis (Spencer, 2013; Tamashiro et al., 2009). This chapter will explore the developmental trajectory of the HPA axis during the hyporesponsive period (PND7) and the emergence from the hyporesponsive period (PND13) in response to perinatal HFD and prenatal CVS using the following aims:

Aim 2.1: Examine the ontogeny of stress behaviours (ultrasonic vocalization and freezing) in neonates in response to prenatal stress and perinatal HFD at PND7 and PND13.

Rodent pups emit USVs at 40 kHz in response to social isolation and is shown to be associated with some activation of the HPA axis (Hofer, 1996; Takahashi, 1992). This behaviour acts as a survival tool as it elicits maternal behaviour in the dams (Brouette-Lahlou et al., 1992; Brunelli et al., 1994; D'Amato et al., 2005). These "distress calls" has been used as an index of pup anxiety indicated by its attenuation by anxiolytic drugs and enhancement by anxiogenic ones (Insel and Harbaugh, 1989). Additionally, animals bred for high USVs during infancy (PND10) showed increased anxiety-like behaviours in adulthood compared to ones bred for low USVs (Brunelli, 2005). Moreover, USVs are modulated by CRH, the central mediator of the HPA axis (Insel and Harbaugh, 1989; Ise et al., 2008). The above highlight the relevance of USVs as a neonatal behavioural measure to examine when studying HPA axis during early postnatal life. Rat neonates exhibit behavioural inhibition near the end of the hyporesponsive period (PND10),

which includes absence of movement and ultrasonic vocalizations (USVs), under threatening conditions such as exposure to adult male rat or its cues (Takahashi, 1992). It has been shown that this inhibition is mediated through Cort and its action in the hippocampus and amygdala (Moriceau et al., 2004; Takahashi, 1995). This aim will assess the following hypotheses:

Hypothesis and approaches 2.1.1: Perinatal HFD and prenatal CVS will lead to early behavioural inhibition to male bedding odor at PND7

Hypothesis and approaches 2.1.2: Perinatal HFD and prenatal CVS will lead to increased anxiety-like behaviour at PND13

Pups at PND7 and PND13 were exposed to clean or adult male bedding in isolation from their litter and their USV and freezing were measured as an index of anxiety-like behaviour and behavioural inhibition in response to male bedding.

Aim 2.2: Examine the ontogeny of stress physiology (CORT and ACTH) in neonates in response to prenatal stress and perinatal HFD

There are no studies assessing the effect of perinatal HFD effects on the neonatal HPA response to stress, while there are some reports on the effect of prenatal stress on HPA axis reactivity in neonates, with prenatally stressed pups exhibiting increased stress reactivity (Henry et al., 1994; Takahashi et al., 1990). In this aim I examined the response of the HPA axis during and emerging from the hyporesponsive period and to assess the following hypotheses:

Hypothesis and approaches 2.2.1: Perinatal HFD and prenatal CVS will lead to early stress response to male bedding odor during the stress hyporesponsive period at PND7

Hypothesis and approaches 2.2.2: Perinatal HFD and prenatal CVS will lead to an augmented stress response to male bedding odor when pups are emerging out of the hyporesponsive period at PND13

Trunk blood was collected from baseline condition and after male bedding exposure to measure Cort and ACTH levels.

Aim 2.3: Examine differential expression of HPA axis-related genes in PVN and ventral hippocampus in response to perinatal HFD

Results obtained from the two earlier aims indicated that perinatal HFD had stronger impacts on HPA axis physiology and related behaviours. Therefore, for this aim I focused on examining expression patterns of key HPA axis genes in the PVN and the ventral hippocampus, two regions that mediate the activation and inhibition of the HPA axis, repectively, at PND7 and PND13 brains.

Hypothesis and approaches 2.3.1: HFD PND7 pups will have increased CRH expression and decreased Nr3c1 expression in the PVN

CRH regulates the HPA axis activity and plays a role in USV. Studies have shown that CRH levels has a biphasic effect on USV, where increases from low endogenous levels would initiate USV, while higher CRH levels inhibit USV production (Ise et al., 2008; Tamborski Harvey and Hennessy, 1995). Therefore, in order to support the findings obtained from the earlier aims CRH transcript levels were measured in the PVN using real time polymerase chain reaction (qPCR). Therefore, I assessed its transcript levels in the PVN. Finally, I assessed Nr3c1 transcript levels as it has been shown that it regulates CRH in the PVN and inhibit the HPA axis activity (Herman et al., 2016).

Hypothesis and approaches 2.3.2: HFD PND13 pups will have decreased Nr3c1 expression in ventral hippocampus

Based on Cort and ACHT finding obtained from the second aim, I assessed the transcript level of Nr3c1 in the ventral hippocampus, where it executes its function in inhibiting the HPA axis.

Chapter 4: Objective 3: Perinatal HFD effects on USV characterization

USVs are characteristic of neonatal development and exhibit specific developmental patterns, where USVs are shown to decrease with age starting at least from PND7 (Brudzynski et al., 1999; Tonkiss et al., 2003). The distribution of USV call numbers and other acoustic parameters across early PNDs has also been established (Elsner et al., 1990; Tonkiss et al., 2003). Standard acoustic parameters such as call rates, call durations and peak frequencies, are mainly examined in the literature, however, very few studies have examined USV waveforms (Lavooy and Hahn, 2003). USVs exhibit qualitative dimensions that has been previously documented to follow a developmental pattern with proposed links to neurological function. An earlier study has

demonstrated that calls with two or three rapid changes in sound frequency, which is termed frequency sweeps, increased in older pups (Brudzynski et al., 1999). These call types described as sharing features of a siren, have been proposed to be more efficient in capturing the attention of the dam to elicit maternal care (Brudzynski, 2005). Moreover, call types featuring abrupt discontinuous frequency changes, which are termed frequency shifts, have been linked to indicate loss of neurobehavioural integrity in human infants and rat pups prenatally exposed to cocaine (Zeskind et al., 2011). This chapter will focus on only perinatal HFD effects on the developmental trajectory of neonates and their relationship to maternal behaviour by assessing the following aims:

Aim 3.1: Examine the ontogeny of USV acoustic parameters in neonates in response to perinatal HFD

Acoustic parameters of USVs, including call counts, call frequency, and amplitude are used as indicators of developmental trajectory since they follow a reported developmental patterns.

Hypothesis and approaches 3.1.1: HFD neonates will have an altered developmental pattern of USV acoustic parameter

USVs were recorded from PND7 and PND13 pups during maternal isolation and USV counts, frequency and amplitude were measured and analysed for age and diet effects.

Aim 3.2: Characterize USV categories during neonatal development and the effects of perinatal HFD

A couple of studies have characterized USV categories in a developmental pattern and indicated that siren calls increase with age and characterizing them as an efficient call type in recruiting the dam (Brudzynski et al., 1999; Tonkiss et al., 2003).

Hypothesis and approaches 3.2.1: HFD neonates will have an altered developmental pattern of USV categories

USVs were recorded from PND7 and PND13 pups during maternal isolation and USV categories were characterized and examined for age and diet effects.

Aim 3.3: explore how maternal behaviour relate to the USV characteristics

One of the main functions of USVs is to communicate with the mother (Wohr and Schwarting, 2008). One study has reported that dams respond to different acoustic parameters leading to differential pup retrieval (Bowers et al., 2013). Mice dams on HFD were reported to have an impaired maternal pup retrieval (Bellisario et al., 2015).

Hypothesis and approaches 3.3.1: HFD dams will not effectively retrieve their pups at PND7 due to altered pup USVs

Dams were tested using a pup retrieval test after they were separated from their litter for 10 min and latency to retrieve, total number of pups retrieved, and proportion of dams successfully retrieved all pups during the 15 min test were measured.

Chapter 5: Objective 4: Examine perinatal HFD effects on offspring epigenetic signature in neonatal period and adulthood

Pups exposed to perinatal HFD manifest metabolic phenotype that are absent from adult offspring, including higher weights, leptin and insulin levels (Tamashiro et al., 2009). This metabolic milieu might act on the active epigenome to influence the trajectory of development and later epigenetic signature in adult animals. Few studies have reported alteration of the epigenome in maternal HFD adult offspring including target genes and global DNA methylation (Carlin et al., 2013; Marco et al., 2014; Vucetic et al., 2012). However, there are no studies examining the effect of perinatal HFD on the epigenomic signature in the developing rats, which are still under a direct influence of the diet through the mother's milk. Therefore, with collaboration with other members in the lab, we explored the effect of perinatal HFD on the epigenome of the offspring. This chapter will focus on the epigenetic differences in neonatal pups and adult offspring in response to perinatal HFD. Based on an earlier study by our lab, female adult offspring showed stronger behavioural and physiological phenotypic alteration to perinatal HFD in comparison to their male counterparts (Sasaki et al., 2013). In addition, females showed more pronounced differences in gene expression in the amygdala compared to the hippocampus (Sasaki et al., 2013). Therefore, we used female offspring amygdala to examine the underlying epigenetic differences that might be associated with the phenotypic alteration observed in the earlier study.

Aim 4.1: Determine differentially methylated regions (DMRs) in response to perinatal HFD

Hypothesis and approaches 4.1.1: Perinatal HFD will induce significant differential DNA methylation in neonatal and adult offspring

In order to assess the effects of perinatal HFD on offspring genome-wide methylation, we used reduced representation bisulfate sequencing (RRBS) on DNA samples obtained from PND7 and PND90 female offspring amygdala. Then, differentially methylated regions were determined bioinformatically using methylPipe R package, which was conducted by Dr. de Vega.

Aim 4.2: Examine the biological process/pathway enriched in gene sets determined from the DMR analysis

Hypothesis and approaches 4.1.2: Differentially methylated genes will be enriched in biological processes and pathways related to neurodevelopment

In order to contextualize the results of the differential methylation biologically, I preformed gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the gene set obtained from the DMR analysis.

Chapter 2

Perinatal HFD and prenatal CVS effects on the HPA axis programming in adult offspring

2 Perinatal HFD and prenatal CVS effects on the HPA axis programming in adult offspring

2.1 Introduction:

Mothers are the primary channel through which early environmental factors could influence the development of the offspring, which could be mediated by changes in the mothers' own physiology and behaviour. During gestation and lactation mothers exhibit an altered HPA axis reactivity characterized by a reduced drive and attenuation in its response to stress (Brunton et al., 2008). Along with alterations in HPA axis physiology, reduced anxiety in rats and increased calmness in humans have been reported during the lactational period (Hillerer et al., 2012). Different prenatal stress paradigms have demonstrated a heightened HPA axis of the pregnant dams (St-Cyr et al., 2018; Tamashiro et al., 2009). Perinatal HFD also has been reported to increase Cort levels during pregnancy (Bellisario et al., 2015).

Interaction between the altered maternal physiology due to motherhood and environmental factors could render the mother susceptible to develop mood and anxiety disorders. For example, perinatal stress has been associated with depressive- or anxiety-like behaviours postpartum (Brummelte et al., 2006; Maniam and Morris, 2010; Smith et al., 2004). A recent study has also reported that perinatal HFD is associated with postpartum anxiety-like behaviours and alteration in HPA axis (Perani et al., 2015). Animal models and human studies of maternal perinatal mood and anxiety disorders have been reported to program offspring behaviour and HPA axis later in life (Reviewed in Hillerer et al., 2012).

Many early life stress paradigms focus their investigation on the outcome in the offspring with little attention paid to the changes that take place in the mothers. One of the aims of this thesis is to characterize the HPA axis physiological and behavioural outcomes in dams in response to perinatal HFD consumption and prenatal CVS exposure. In this chapter, I examined dams' Cort response to restraint stress during gestation to evaluate the effect of CVS and HFD consumption on HPA axis activity. In addition, I examined the effects of prenatal CVS and perinatal HFD on maternal care and emotionality behaviours, including depressive-like and anxiety-like behaviours

in dams postpartum. Finally, I examined the effects of perinatal HFD and prenatal CVS on adult offspring anxiety-like behaviours and their Cort response to stress.

2.2 Methods:

2.2.1 Animals and diet:

Adult male and female Long Evans rats (7 week) were purchased from Charles River Canada (St. Constant, QC), housed in same-sex pairs until mating and maintained on a 12:12-h light–dark cycle (lights on 7:00 am–7:00 pm) with *ad libitum* access to food and water. Females (n=36) were either maintained on chow diet (CHD; Purina Lab Diets (St. Louis, MO: cat. no. 5001) and contained 28.5% protein, 13.5% fat, and 58% carbohydrate. (n=17)), or high fat diet (HFD; Research Diets, Inc. (New Brunswick, NJ: cat. no. D12492), and contained (by kcal): 20% protein, 60% fat, 20% carbohydrate. (n=19)) for 4 weeks prior to mating. Experimental protocols were approved by the Local Animal Care Committee at the University of Toronto, Scarborough, and were in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 Prenatal chronic variable stress (CVS):

Prior to mating females were weighed once a week to monitor their weight gain on the two different diets. Females were paired with males for a week and sperm plugs were checked twice a day (9am and 5pm) to determine pregnancy. Later females were separated from males, singly housed and weighted every two days to confirm their pregnancy. On the CVS paradigm the pregnant females were exposed to 1-3 stressors a day for 8 days starting on gestation day 13 (Table 2.1). This gestation period has been reported to be the developmental period of the HPA (Xiong and Zhang, 2013). Females were transported to a procedure room (CHD, n=9; HFD, n=11) where they were exposed to the different stressors daily (Table 2.1). Control non-stressed animals (CHD, n=8; HFD, n=8) were kept at the main housing room undisturbed.

2.2.3 Stress reactivity in dams during gestation

I assessed maternal stress reactivity using 20 min restraint challenge in two different experiments. The first experiment examined the stress response of CVS dams on the first day and the last day of the CVS paradigm. This allowed to examine HPA axis reactivity in the same

animals and the effects of diet and CVS (CHD n= 8, HFD n=7). This design, however, did not allow the differentiation between the CVS effect and the effect of day of gestation. Therefore, I carried out a second experiment with a separate cohort, 14 control females (CHD= 6, HFD=8) that did not undergo the CVS paradigm and 16 females (CHD n=7, HFD n=9) that underwent the CVS paradigm to assess females' HPA axis reactivity on the last day of the CVS protocol, gestation day (GD) 20, controlling for GD effect.

A week prior to mating, dams were habituated daily to the blood collection procedure by handling them for 2 min in a loosely fit towel and massaging their tails. On the day of the challenge, animals were transported to the procedure room 2 hours before the test and then handled in a loosely fit towel to make a small incision in the tail to collect blood then immediately the animals were put in a Plexiglass restrainer for 20 min. After the 20 min restraint, another tail bleed was performed and animals returned to their home cages. An hour later one last blood collection was performed. Dams were sacrificed on the same day.

2.2.4 Maternal behaviour recording:

Once litters were born, the dams were moved into clean cages with paper towel strips as nesting material. Pups were weighed and culled to 12 pups/ litter (6 males and 6 females) when possible. Cages were moved into a separate room for maternal behaviour recording and kept there until weaning. Maternal behaviour was recoded 1 hour 6 times a day (light phase: 10 am, 1 pm, and 5 pm; dark phase: 9 pm, 1 am, and 5 am) from postnatal day (PND) 1-6 (Champagne, 2003). Within each observation period the behaviour of each mother was scored every 3 min (20 observations/period X 6 periods per day = 120 observations/mother/day). Behaviours were coded using Observer 10.0 software and proportions of behaviours were calculated. In addition, proportions of scored behaviours were also calculated for the full 6 days. These behaviours were total licking (anogenital (AG) licking and body (BD) licking), total nursing (low and high arched

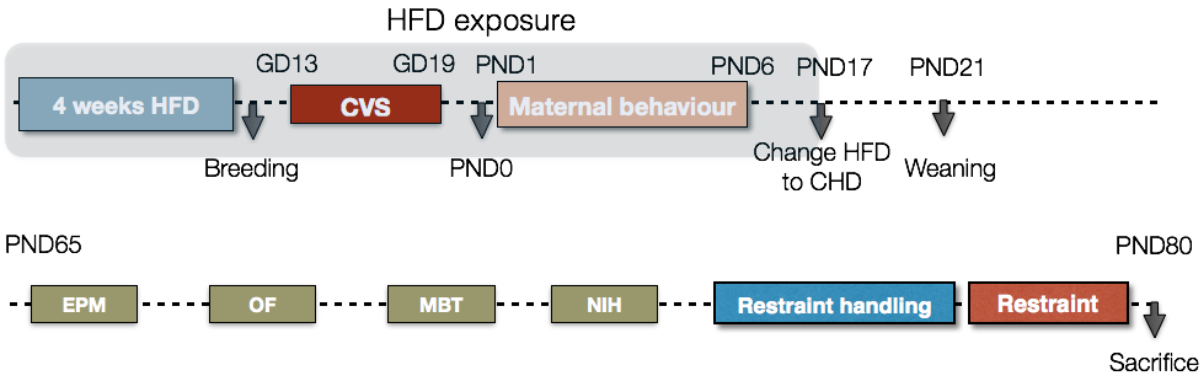

Figure 2.1 Timeline of study.

Table 2.1 Schedule of the chronic variable stress. A 20-min restraint challenge (red) was carried out on the first and last day of the stress to assess HPA axis reactivity

	Time		
Gestation Day	**Morning**	**Noon**	**Afternoon**
13	Restraint-20 min	Swim- 10 min	Restraint- 60 min
14	Wet bedding- 12h (450ml)		
15	Swim- 10 min	Restraint- 60 min	Swim- 10 min
16		Predator odour- 30 min	Overnight illumination
17	Novel object (10 marbles)- 12h		
18	Swim- 10 min	Restraint- 60 min	Swim- 10 min
19		Platform (5 min)	White Noise/cage tilt over-night
20	Restraint-20 min	Swim- 10 min	Restraint- 60 min

back nursing and supine nursing), dam away from nest, and total care (total licking, total nursing, and pup contact (retrieval and mouthing)). Due to some technical issues some data were lost including all observations on PND1 from two CHD/Control litters, which were omitted from the repeated measure ANOVA analysis, and 3-5 observations across different PND from 12 different litters. Proportions were still calculated and included into the analysis.

Pups were weighed on PND10 and PND21. By PND17 pups start consuming food pellets in the cage, so to control for effect of the diet during lactation period, HFD litters were switched onto CHD. On PND21, pups were weaned on CHD and housed in same-sex pairs and weighed once a week.

2.2.5 Maternal emotionality behaviour testing:

In a separate cohort of animals (CHD/Control n=10, CHD/CVS n=8, HFD/Control n=8, HFD/CVS n=7) maternal anxiety- and depressive-like behaviours were examined.

2.2.5.1 Open field (OF)

About ten days after weaning pups on PND 13, the dams were tested for anxiety-like behaviour using an open field test. On the day of testing animals were moved into a testing room where they were habituated to the environment for 30 min. After, animals were placed in 1m X 1m open arena and were allowed to explore for 15 min while recoding their behaviour using EthoVision 12.0 software. The number of entries into and time spent in the center and border of the arena and the distance travelled and velocity travelled at were measured for the full 15 min test.

2.2.5.2 Sucrose preference test (SPT)

Two days after the open field test, dams were tested for anhedonia, lack of interest in rewarding stimuli, a feature of affective disorder, using a two-bottle choice paradigm. Animals were first habituated to the presence of two pre-weighed bottles of tap water for 48 hours in the home cage and the position of the bottles were switched after 24 hours to control for side preference. After the habituation stage, two pre-weighed bottles of tap water or 2% sucrose solution were presented to the animals in their home cage for 48 hours and bottles were switched in position in

24 hours to control for side preference. The bottles were weighed daily to measure the average percentage of sucrose consumption on both days. On the last day of the test, animals were water deprived for 5 hours (10 am-3 pm) and then were given access to both tap and 2% sucrose water for an hour after which the bottles were weighed. % sucrose preference = sucrose consumption/ total liquid consumption (sucrose + water)X100.

2.2.6 Offspring behaviour testing:

About 2/litter/sex adult animals (PND65) (CHD/Control: male= 14, female= 14; CHD/CVS: male= 14, female= 14; HFD/Control: male= 14, female= 14; HFD/CVS: male= 15, female= 15) were tested for anxiety-like behaviours.

2.2.6.1 Elevated plus maze (EPM):

Animals were placed on an 80 cm elevated apparatus consisting of four arms (45 X 10 cm) two of which were surrounded by walls “closed arms” while the other two were not “open arms” and a center platform (10 X 12 cm). In a dimly lit room (33.7 lux), animals were placed in the centre platform and allowed to explore the apparatus in a 5-min trial. The number of entries and the time spent in either arm were recorded along with the distance and speed at which they travelled using EthoVision 12.0 software. Anxiety index was also calculated = 1- ((time spent on the open arm/total time)+(number of entries into the open arm/total entries)/2) (Mayer et al., 2014).

2.2.6.2 Open field (OF):

Animals were placed into an opaque arena (40.3 X 40.3 cm) in a dimly lit room (33.7 lux) facing one of the corners. The animals were left to explore the arena undisturbed for 15 min while recoding using EthoVision 12.0 software. The number of entries into and time spent in the centre of the arena (20.15 X 20.15 cm), the distance travelled and velocity travelled at, and entry into and time spent in the center and border of the arena were measured.

2.2.6.3 Marble burying test (MBT):

Rodents exhibit a burying behaviour in response to unfamiliar objects such as glass marbles. This behaviour has been shown to be modified in response to anxiolytic drugs and suggested as a measure of anxiety-like behaviour and obsessive-compulsive-like behaviour (Schneider and

Popik, 2007; Kedia and Chattarji, 2014). Rats were transferred into new cages with 4 cm of bedding material and 20 marbles arranged a 4 × 5 grid on the surface of the bedding and left undisturbed for 30 min trial while being recorded. Number of marbles buried was counted at the end of the test.

2.2.6.4 Novelty induced hypophagia (NIH)

Hyponeophagia is the reduction of feeding in the animals in response to exposure to a novel environment. It is another anxiety test where the animal is conflicted between approaching desirable food and avoiding the new environment, which has been previously shown to be modified by anxiolytic drugs (Dulawa and Hen, 2005). With minor modifications, the test was carried out as described previously by Nam et al., 2014. Animals were food deprived for 24 hr before testing. On the day of the test, animals were transferred into the testing room and placed into an open field with a pre-weighed chow pellets placed in the center of the arena. The latencies to investigate the food (sniff or touch) and eat the food were recorded in a 20 min trial using EthoVision system. After the trial was over the food was weighed and animals transferred into the home cage with food and 24-hour food consumption was recorded as a control for basal food consumption.

2.2.7 Stress reactivity in adult offspring:

A subset of adult offspring (CHD/Control n=7/sex, CHD/CVS n=7/sex, HFD/Control n=8/sex, HFD/CVS n= 7-5/sex) assessed for their stress response to 20 min restraint challenge. Procedure was carried out similar to dams' restraint procedure described above.

2.2.8 Corticosterone radioimmunoassay

Blood collected from the tail bleed was allowed to coagulate for 30 min on ice and then centrifuged at 4°C at 4000 rpm for 20 min and serum was stored at -80°C. Cort levels were measured using Corticosterone Double Antibody RIA Kit (MP biomedicals, Cat. 07120103; sensitivity 7.7 ng/mL, intra-assay coefficient of variation 10.3%, inter-assay coefficient of variation 7.2%). Samples were diluted to 1:200 and run in duplicates following the manufacturer recommendation.

2.2.9 Statistical Analysis

Statistical analysis was carried out using SPSS (IBM). A Shapiro-Wilk test was used to test for normality for data sets with $n < 30$. For data sets with $n > 30$ normality was assumed (Ghasemi and Zahediasl, 2012) and parametric analyses were carried out. Factorial linear mixed models (LMM) (time x diet x stress) were used to analyze data sets that were repeated measures and contained missing values including maternal weights and food intake. A mixed model repeated measure ANOVA for stress and diet was used to analyze maternal care behaviour, maternal sucrose preference test, restraint challenge results in dams and offspring, and offspring preweaning weights. A univariate ANOVA was used to measure the effect at the different time points when an interaction with time is reported. Factorial (2 diet x 2 stress) ANOVA was used to analyze the litter characteristics and (2 diet x2 stress x2 sex) ANOVA for adult offspring weights. Offspring behavioural measures were analyzed separately in the two sexes using (2 diet x2 stress) ANOVA. Tukey's post hoc test was used for pairwise comparison when indicated.

2.3 Results:

2.3.1 Maternal weights and food intake:

All dams increased their weights throughout the experimental weeks (week effects: $F_{(8,285)}= 127.03$, $p\leq0.0001$; Figure 2.2A). Females consuming HFD were heavier than ones consuming CHD (diet effect: $F_{(1,285)}= 127.87$, $p\leq0.0001$). A diet by week interaction ($F_{(8,285)}= 3.43$, $p=0.001$) and diet by stress interaction ($F_{(1,285)}= 5.22$, $p=0.02$) were observed. There was no effect of diet on dams' weights at PND10 ($F_{(1,27)}= 2.98$, $p=0.1$), where all dams weighed the same. I examined the effects of CVS exposure on weight gain during the last week of gestation. I found a main effect of CVS, which was mainly present in HFD females, where HFD/CVS females gained less weight than HFD/Control females ($F_{(1,32)}= 6.28$, $p=0.02$, Tukey's post hoc: HFD/Control vs. HFD/CVS $p=0.03$; Figure 2.2B).

The increase in weight in HFD was associated with higher in food intake in HFD females compared to CHD (diet effect: $F_{(1,177)}= 99.99$, $p\leq0.0001$; Figure 2.2C). Food intake in all females changed across experimental weeks (week effects: $F_{(5,177)}= 6.19$, $p\leq0.0001$). I also observed a diet by week interaction ($F_{(5,177)} = 11.83$, $p\leq0.0001$), which could be explained by the effects of CVS exposure during the last week of gestation on the HFD females. Indeed, examining the food

intake in the CVS group before (week 6) and after (week 7) CVS exposure, revealed a main effect of stress ($F_{(1,34)}$= 8.07, p=0.008), diet ($F_{(1,34)}$= 5.89, p=0.02), and a diet by stress interaction ($F_{(1,34)}$= 4.15, p=0.05). HFD females were consuming more food prior to the CVS paradigm compared to CHD females (Tukey's post hoc: HFD/pre-CVS vs. CHD/pre-CVS p=0.02). However, after CVS exposure, HFD females significantly reduced their food intake (Tukey's post hoc: HFD/pre-CVS vs. HFD/post-CVS p=0.006; Figure 2.2D). Interestingly, only HFD/CVS showed significant positive correlation between food intake and weight gain during the last week of gestation (r=0.73, n=11, p=0.02).

2.3.2 Maternal stress reactivity:

I examined maternal stress reactivity in two different cohorts using a 20-min restraint challenge. In the first cohort, I examined dams' stress reactivity to CVS exposure by testing them on the first and last day of the CVS paradigm. I found that all females responded with Cort concentration changes to the 20-min restraint challenge (Time effect: $F_{(2,26)}$= 80.97, p≤0.0001; Figure 2.3A). Animals' Cort response to restraint challenge differed depending on the GD the animals were tested on (Time x GD: $F_{(2,26)}$= 8.99, p=0.001). This difference was observed at recovery time point (80 min), where females at GD13 had higher Cort concentrations compared to GD20 (GD effect: $F_{(1,26)}$= 9.96, p=0.004; Tukey's post hoc: CHD-GD13 vs. CHD-GD20 p=0.03, HFD-GD13 vs. HFD-GD20 p=0.003, HFD-GD13 vs. CHD-GD20 p≤0.0001). Examining the area under the curve (AUC) of the stress response revealed a main effect of GD ($F_{(1,13)}$ = 7.27, p=0.02; Figure 2.3B). Only the HFD curves showed a significant difference in their area across the gestation days (Bonferroni post hoc p=0.04).

The second cohort examined females' stress reactivity to restraint challenge on GD20 between a set of females that were exposed to the CVS paradigm and control females that were not exposed (Figure 2.3C). Similarly to the first cohort, all females responded with changes in their Cort concentrations to the restraint challenge (Time effect: $F_{(2,52)}$ = 190.86, p≤0.0001). Animals' Cort

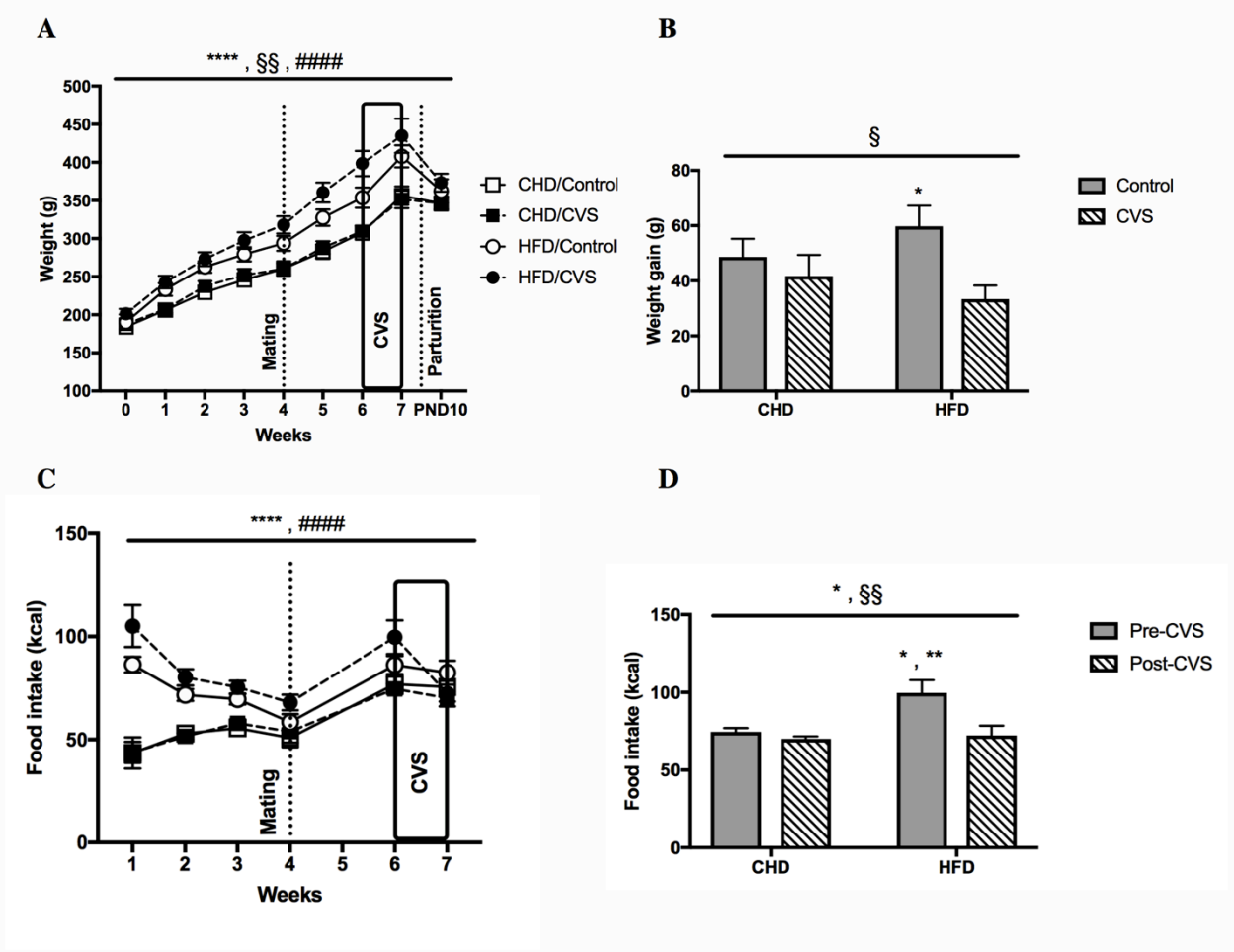

Figure 2.2 Maternal weights and food intake. (A) All dams gained weight throughout the study. HFD dams weighed more than CHD dams. (B) CVS decreased dam weight gain during the last week of gestation. Specifically, HFD/CVS dams gained less weight compared to HFD/Control dams. (C) Dams' food intake changed across the study. HFD dams were consuming more calories compared to CHD dams. (D) CVS decreased food intake in dams. Specifically, HFD/CVS dams ate less calories compared to HFD/Control dams. CHD/Control n=8, CHD/CVS n=9, HFD/Control n=8, HFD/CVS n=11. Data presented are means ± standard error. Weeks effect: ####p≤0.0001. Diet effect: ****p≤0.0001, ***p≤0.001, **p≤0.01. Stress effect: §§p≤0.01, §p≤0.05. Tukey's post hoc: weight gain: HFD/Control vs. HFD/CVS *p≤0.05; Food intake: HFD/pre-CVS vs. CHD/post-CVS *p≤0.05, HFD/pre-CVS vs. HFD/post-CVS and CHD/pre-CVS **p≤0.01.

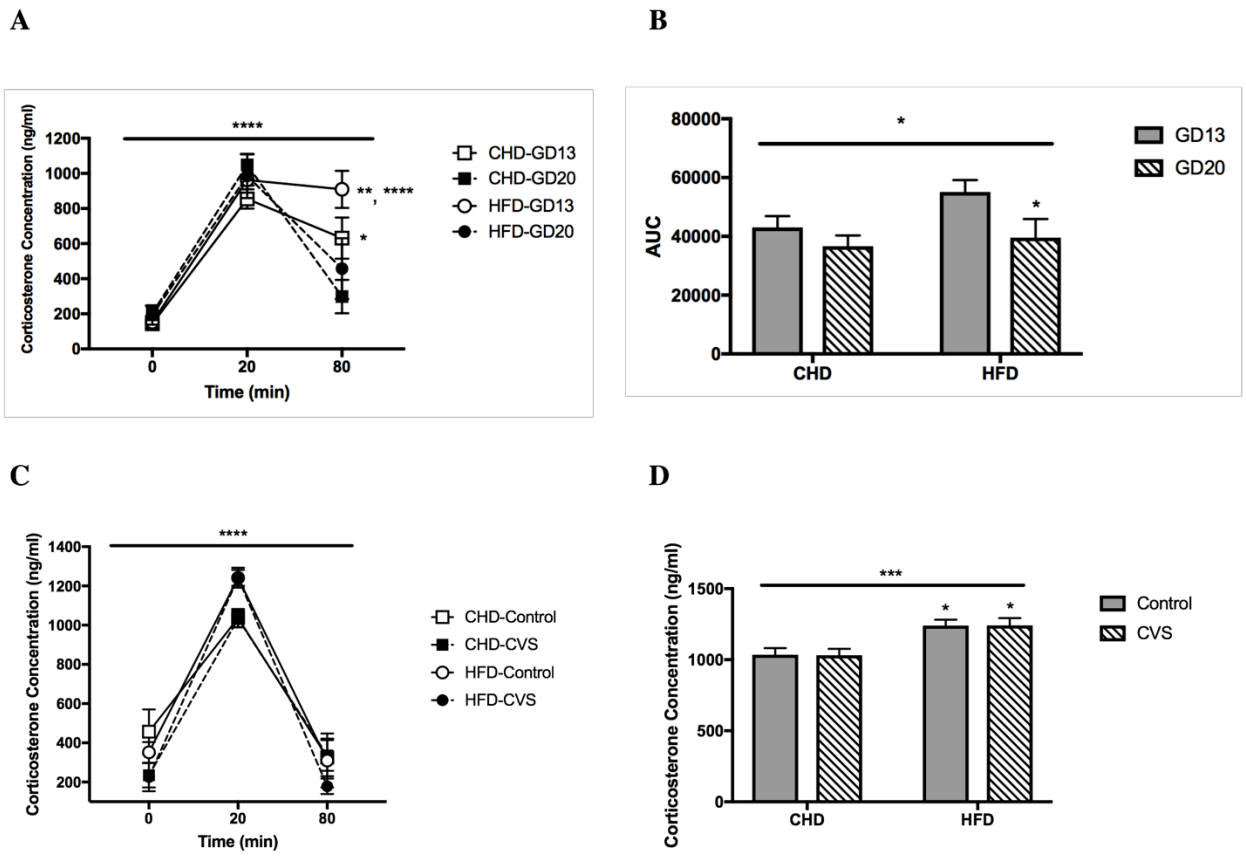

Figure 2.3 Maternal stress reactivity to 20 min restraint challenge. (A-B) Pregnant females were exposed to restraint challenge on the first day of CVS (GD13) and last day of CVS (GD20). (A) On gestation day (GD) 13, females had higher recovery (80 min) Cort concentration compared to GD20. (B) GD20 area under the curve (AUC) of the Cort response was lower than the GD13 AUC, specifically between the HFD females. (C-D) In a separate cohort, pregnant CVS and control females were exposed to a restraint challenge on GD20. (C) There was no main effect of CVS exposure on stress response to restraint challenge of GD20 females. (D) HFD females at GD20 had higher Cort concentration at peak (20min) compared to CHD females. GD13 and GD20 CHD n= 8, HFD n=7. CHD/Control n= 6, CHD/CVS n= 7, HFD/Control n= 8, HFD/CVS n=9. Data presented are means ± standard error. Time effect: ****p≤0.0001, Diet effect: ***p≤0.001, GD effect: *p≤0.05. Tukey's post hoc: (A) CHD-GD13 vs. CHD-GD20 *p≤0.05, HFD-GD13 vs. HFD-GD20 **p≤0.01, HFD-GD13 vs. CHD-GD20 ****p≤0.0001, (B)

HFD-GD13 vs. HFD-GD20 *p≤0.05, (D) HFD/Control and HFD/CVS vs. CHD/Control and CHD/CVS *p≤0.05.

response to restraint challenge differed depending on the diet the animals were exposed to (Time x diet: $F_{(2,52)} = 5.37$, p=0.008). This difference was observed at peak time point (20 min) (Figure 2.3D), where HFD females had higher Cort response to the restraint challenge compared to the CHD females (Diet effect: $F_{(1,26)} = 19.30$, p=0.001; Tukey's post hoc: HFD/Control and HFD/CVS vs. CHD/Control and CHD/CVS p=0.02).

2.3.3 Maternal care behaviour:

Table 2.2 presents the mean percentages of maternal care behaviour observed during the first 6 postnatal days. HFD dams spent more time nursing their pups in general compared to CHD dams ($F_{(1,177)} = 5.05$, p=0.03). Further, they engaged in more blanket nursing posture ($F_{(1,177)} =9.56$, p=0.002) and less supine nursing posture ($F_{(1,177)}= 5.58$, p=0.02) compared to CHD dams. CVS dams also engaged in less supine nursing posture ($F_{(1,177)}= 10.32$, p=0.002). HFD consumption also affected the dams' time spent licking their pups in total ($F_{(1,177)} = 15.2$, p≤0.0001), which was also observed in both type of licking (AG licking: $F_{(1,177)}= 16.48$, p≤0.0001; BD licking: $F_{(1,177)} = 6.09$, p= 0.015), where HFD dams were licking their pups more than CHD dams. In addition, HFD also spent more time building their nests ($F_{(1,177)}= 4.28$, p=0.04) compared to CHD dams. HFD dams spent more time on their nests ($F_{(1,177)}= 4.95$, p=0.03) compared to CHD dams.

I also examined dams' self-directed behaviours such as feeding and grooming. I found that HFD dams spent less time feeding than CHD dams during the first 6 postnatal days ($F_{(1,177)}= 73.84$, p≤0.0001), while CVS dams spent more time feeding than control dams ($F_{(1,177)}= 3.72$, p=0.05). Moreover, HFD dams spent more time grooming themselves compared to CHD dams ($F_{(1,177)} =$ 14.90, p≤0.0001).

2.3.4 Maternal emotionality behaviour:

I did not observe any significant differences between females in the sucrose preference test across the different days of testing (Day x diet: $F_{(2,58)} = 0.703$, p=0.499; day x stress: $F_{(2,58)}= 0.765$, p=0.470; Figure 2.4A). Similarly, there were no differences in the overall average sucrose preference of all days tested between females (Diet: $F_{(1,29)}= 1.63$, p=0.21; stress: $F_{(1,29)} = 0.033$, p=0.85; Figure 2.4B). In addition, the dams did not show any differences in time spent in the

centre of the open field relative to the borders (Diet: $F_{(1,30)} = 2.07$, p=0.16; Stress: $F_{(1,30)} = 0.27$, p=0.60; Figure 2.4C), entries into the center relative to border (Diet: $F_{(1,30)} = 0.86$, p=0.36; Stress: $F_{(1,30)} = 0.65$, p= 0.42; Figure 2.4D), or the overall distance traveled during the test (Diet: $F_{(1,30)} = 0.15$, p= 0.69; Stress: $F_{(1,30)} = 1.85$, p=0.18; Figure 2.4E).

Table 2.2 Maternal care behaviour recorded during the first 6 postnatal days.

Maternal Care	Behaviour % Mean ± SEM				p-value
	Condition				
	CHD/Control	CHD/CVS	HFD/Control	HFD/CVS	
Total Nursing	59.7±2.7	55.4±2.4	62.4±2.6	63.4±2.6	* , ####
AB Nursing	0.3±0.2	0.7±0.2	0.8±0.2	0.7±0.2	NS
Blanket Nursing	53.8±2.6	52±2.4	58.1±2.5	61.7±2.5	** , ####
Supine Nursing	5.6±0.08	2.8±0.8	3.5±0.8	1±0.8	* , §§
Total Licking	5.7±0.6	5.6±0.5	7.4±0.6	8.3±0.6	****
AG Licking	1.9±0.3	1.9±0.3	3±0.3	2.9±0.3	****
BD Licking	3.8±0.4	3.7±0.4	4.4±0.4	5.3±0.4	**
Nesting	2±0.4	2.3±0.4	2.4±0.4	3.5±0.4	* , ####
Hover	1.7±0.3	1.5±0.3	1.5±0.3	1.9±0.3	##
On Nest	61.6±2.7	56.9±2.5	64±2.7	65.3±2.7	* , ####
Self-feeding	9.8±0.7	11.3±0.7	4.3±0.7	5.2±0.7	**** , § , ####
Self-grooming	6.7±0.6	5.4±0.6	8.8±0.6	8.1±0.6	****

AB= Arched back, AG= Anogenital, BD= Body. Diet effect: ****p≤0.0001, **p≤0.01, * p≤0.05. Stress effect: §§p p≤0.01, §p p≤0.05. PND effect: #### p≤0.0001, ## p≤0.01.

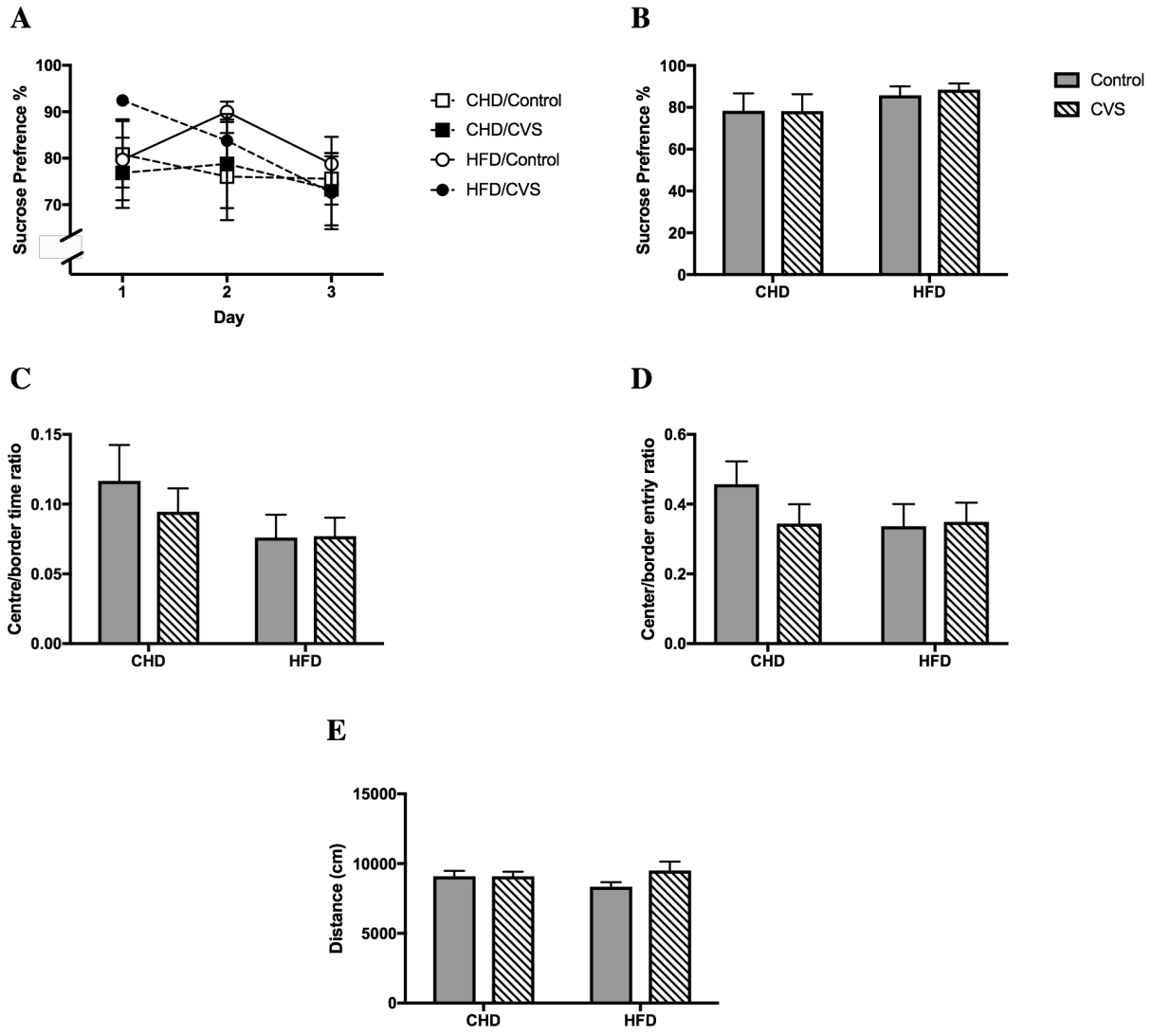

Figure 2.4 Maternal sucrose preference and open filed tests. (A) There were no differences in sucrose preference% among the dams across the three days of testing. (B) There was no difference in average sucrose preference% between dams. There were no significant differences in time spent in (C) or entries into (D) the center of the open field relative to its borders during the full 15 min test. (E) All dams travelled similar distance during the full 15 min test of the open filed. CHD/Control n=10, CHD/CVS n=8, HFD/Control n=8, HFD/CVS n=7.

2.3.5 Litter characteristics and offspring weights:

Prenatal CVS affected the numbers of pups dams gave birth to ($F_{(1,34)} = 8.20$, p=0.007; Table 2.3). HFD/CVS dams had significantly lower litter sizes to HFD/Control dams (Tukey's post hoc p= 0.001). There were no significant effects on the number of still born pups dams gave birth to (Diet: $F_{(1,34)} = 1.04$, p=0.31; Stress: $F_{(1,34)} = 1.62$, p=0.21; Table 2.3). Prenatal CVS also affected the mortality rate of pups by weaning age ($F_{(1,34)} = 4.09$, p=0.05; Table 2.3), with HFD/CVS litters having higher mortality rate compared to all other groups (Tukey's post hoc $p \leq 0.05$). Four HFD/CVS were excluded from further analysis due to the high mortality rate and consequently low litter sizes during the lactation period.

Pups weights increased with age during the pre-weaning postnatal days ($F_{(2,56)} = 1812.36$, $p \leq 0.0001$; Figure 2.5A). Perinatal HFD affected the weight of the pups in an age specific manner, a difference that was observed among the CVS animals, where HFD/CVS pups were heavier than CHD/CVS at PND21 (Diet x PND interaction: $F_{(2,56)} = 3.72$, p=0.03; Tukey's post hoc: p= 0.05; Figure 2.5A). Adult offspring did not differ in weights (Diet: $F_{(1,29.4)} = 1.77$, p= 0.19; Stress: $F_{(1,29.4)} = 0.81$, p=0.37; Figure 2.5B).

2.3.6 Adult offspring behaviour:

2.3.6.1 Elevated plus maze:

In males, perinatal HFD increased the time spent in the open arms relative to the time spent in the closed arms (Diet effect: $F_{(1,50)} = 4.90$, p= 0.03) compared to CHD offspring (Figure 2.6A). There were no treatments effects on the entries in the open arms relative to the closed (Diet: $F_{(1,50)} = 2.68$, p= 0.11; Stress: $F_{(1,50)} = 0.01$, p= 0.91; Figure 2.6B). HFD male offspring showed a decrease in their Anxiety index ($F_{(1,50)} = 5.10$, p= 0.03; Figure 2.6C). There were no differences in

Table 2.3 Litter characteristics during early postnatal days

Litter Measure	Measure Mean ± SEM				Main effects p-Value
	Condition				
	CHD/Control	CHD/CVS	HFD/Control	HFD/CVS	
Litter Size	12.75±0.91	12.78±0.86	15.62±0.91	10.69±0.72***	§§
Still Born	0.125±0.408	0.222±0.385	0.125±0.408	1±0.32	NS
Mortality Rate	1.25±7.76	2.5±7.31	0	28.11±6.08*	§

Stress effect §§p≤0.01, §p≤0.05. Tukey's post hoc: HFD/CVS vs. HFD/Control ***P≤0.001, HFD/CVS vs. all *p≤0.05.

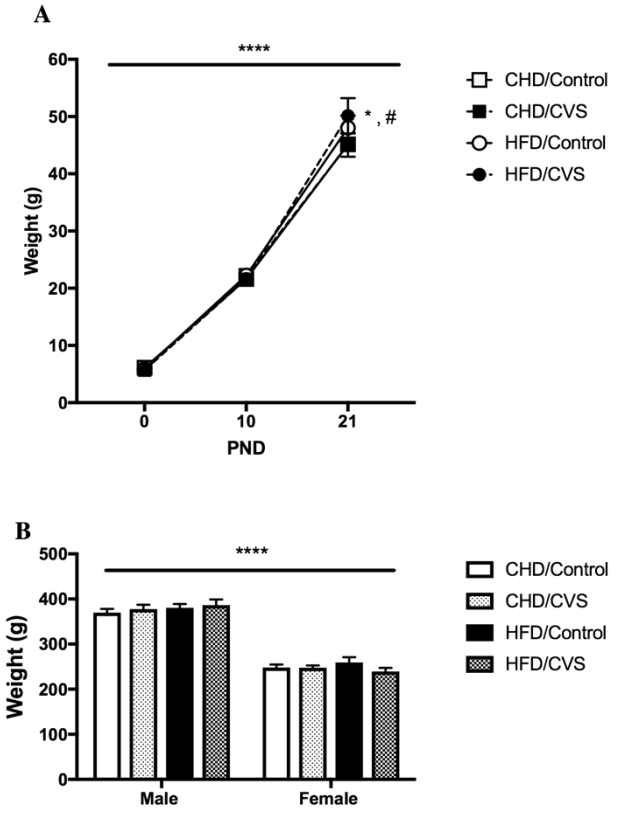

Figure 2.5 Offspring weights during lactation and in adulthood. (A) Offspring gained weight throughout the preweaning period with no effect of diet or stress on their weights. HFD/CVS were heavier than CHD/CVS and CHD/Control on PND21. CHD/Control n= 8, CHD/CVS n= 8, HFD/Control n=8, HFD/CVS n=8. (B) At adulthood, males were heavier than females. There were no effects of diet or stress on adult offspring weights. CHD/Control n=14, CHD/CVS n=12-14, HFD/Control n= 14, HFD/CVS n= 11-12. Time effect ****p≤0.0001. Sex effect

****p≤0.0001. Tukey's post hoc *p≤0.05 HFD/CVS vs. CHD/CVS, #p=0.06 HFD/CVS vs. CHD/Control.

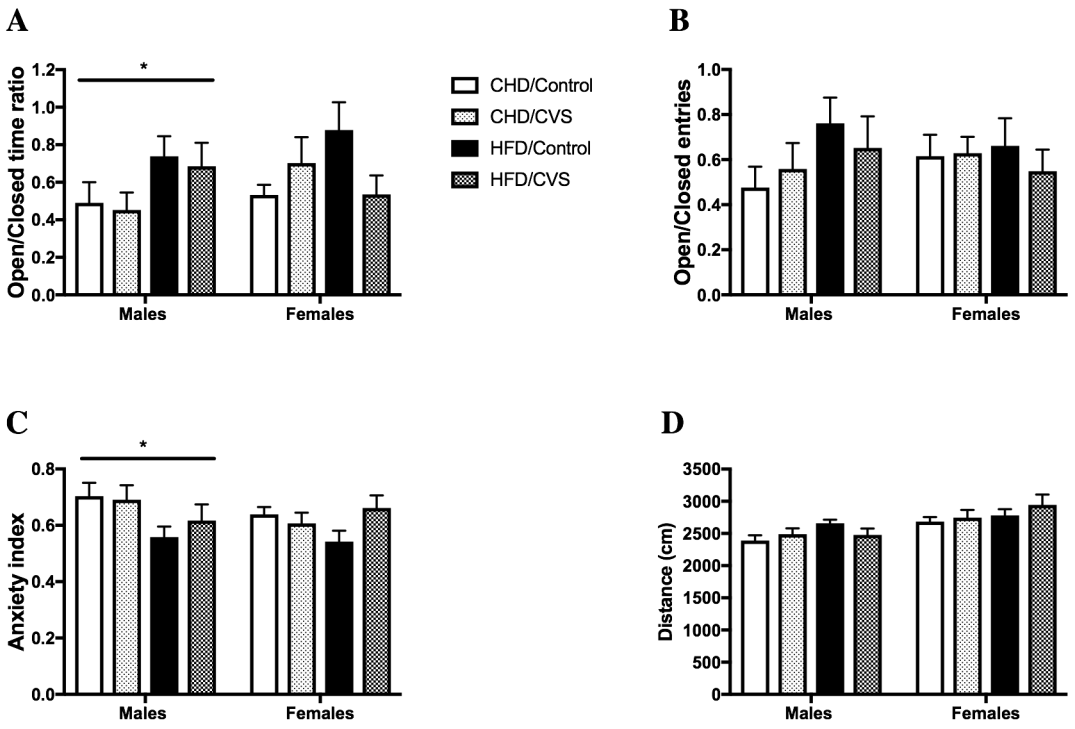

Figure 2.6 Adult offspring behaviour measures in the elevated plus maze (EPM). (A) HFD adult male offspring spent more time in the open arms of the maze relative to the closed arms compared to CHD offspring. There were no significant differences in the time ratio of open to closed arms among adult female offspring. (B) No significant differences were observed in the number of entries into the open arm relative to the closed arms between offspring. (C) HFD adult male offspring had lower anxiety index compared to CHD offspring, while there were no differences observed among female offspring. (D) There were no significant effects of diet or stress on the distance travelled during the test. CHD/Control n= 14, CHD/CVS n= 14, HFD/Control n= 15-16, HFD/CVS n= 11-13. Diet effect *p≤0.05.

the distance travelled by the animals during the test (Diet: $F_{(1,50)} = 2.21$, p= 0.144; Stress: $F_{(1,50)} = 0.17$, p= 0.68; Figure 2.6D).

Females showed a diet by stress interaction effect ($F_{(1,53)} = 4.52$, p= 0.04) on the time spent in the open arms relative to the time spent in the closed arms (Figure 2.6A), where HFD/Control offspring seemed to spend more time in the open arms compared to the other groups, however this did not reach significant difference (Tukey's posy hoc: HFD/Control vs. all groups p>0.05). There were no differences between the experimental groups in the entries in the open arms relative to the closed (Diet: $F_{(1,53)} = 0$, p= 0.99; Stress: $F_{(1,50)} = 0.475$, p= 0.50; Figure 2.6B). The anxiety index was affected by a diet and stress interaction ($F_{(1,53)} = 3.99$, p= 0.05: Figure 2.6C). HFD/Control offspring seemed to have lower anxiety index compared to the other groups, however this did not reach significant difference (Tukey's posy hoc: HFD/Control vs. all groups p>0.05). There were no difference in the distance travelled by the animals during the test (Diet: $F_{(1,53)} = 1.63$, p= 0.21; Stress: $F_{(1,50)} = 1.04$, p= 0.31; Figure 2.6D).

2.3.6.2 Open field:

Prenatally stressed male offspring spent more time in the center of the open field relative to the border (Stress: $F_{(1,50)} = 4.32$, p=0.04; Figure 2.7A). There were no differences in the number of entries into the center of the arena relative to the borders (Diet: $F_{(1,50)}= 0.6$, p=0.42; Stress: $F_{(1,50)} = 1.28$, p=0.26; Figure 2.7B). There were no differences in the activity measured by the distance covered during the test of the animals' either (Diet: $F_{(1,50)}= 1.84$, p=0.18; Stress: $F_{(1,50)} = 0.05$, p=0.82; Figure 2.7C).

Female offspring's time spent in the center of the open filed relative to the borders were affected by a diet by stress interaction ($F_{(1,53)} = 10.46$, p=0.002; Figure 2.7A). Both HFD/Control (Tukey's post hoc p= 0.05) and CHD/CVS (Tukey's post hoc p= 0.02) female offspring spent significantly less time in the center of the open filed relative to the border compared to CHD/Control offspring. Similarly, the number of entries into the center of the open filed relative to the borders were affected by an interaction of diet by stress ($F_{(1,53)} = 4.20$, p=0.045; Figure 2.7B), however no significant differences between the groups were reached. There were no differences in the activity measured by the distance covered during the test (Diet: $F_{(1,53)}= 0.01$, p=0.89; Stress: $F_{(1,53)} = 0.40$, p=0.53; Figure 2.7C).

2.3.6.3 Marble burying and novelty induced hypophagia tests:

There were no significant effects of diet or stress on the number of marbles buried by both male (Diet: $F_{(1,50)} = 0.005$, p= 0.99; Stress: $F_{(1,50)} = 0.32$, p= 0.57; Figure 2.7D) and female offspring (Diet: $F_{(1,53)} = 0.048$, p= 0.82; Stress: $F_{(1,50)} = 2.06$, p= 0.16; Figure 2.7D).

Both male and female offspring's latency to eat during the NIH test was affected by an interaction between maternal diet and prenatal stress (Males: $F_{(1,50)} = 7.56$, p=0.008; Females: $F_{(1,53)} = 5.90$, p=0.02; Figure 2.7E). CHD/CVS female offspring showed reduced latency to eat compared to HFD/CVS female offspring (Tukey's post hoc p=0.04).

2.3.7 Adult offspring stress reactivity:

All offspring of both sexes showed an effect of restraint on their Cort concentration (Males: $F_{(2,46)} = 173.8$, $p \leq 0.0001$; Females: $F_{(2,52)} = 106.76$, $p \leq 0.0001$; Figure 2.8A-B). However, there were no effects of perinatal HFD or prenatal CVS on offspring response to the restraint challenge (Males: Diet: $F_{(1,23)} = 0.32$, p=0.57; Stress: $F_{(1,23)} = 0.42$, p=0.52; Females: Diet: $F_{(1,26)} = 1.61$, p= 0.22; Stress: $F_{(1,26)} = 1.00$, p=0.33).

2.4 Discussion

The aims of this chapter were to examine the maternal and adult offspring stress-related physiological and behavioural responses to perinatal HFD consumption and prenatal CVS. I found that perinatal HFD increased stress reactivity in dams and altered maternal care behaviour. In addition, HFD dams' weight and food intake were affected by exposure to stress during gestation. Offspring on the other hand, showed alterations in their anxiety-like behaviours in a sex specific manner with no changes in their stress response to restraint challenge.

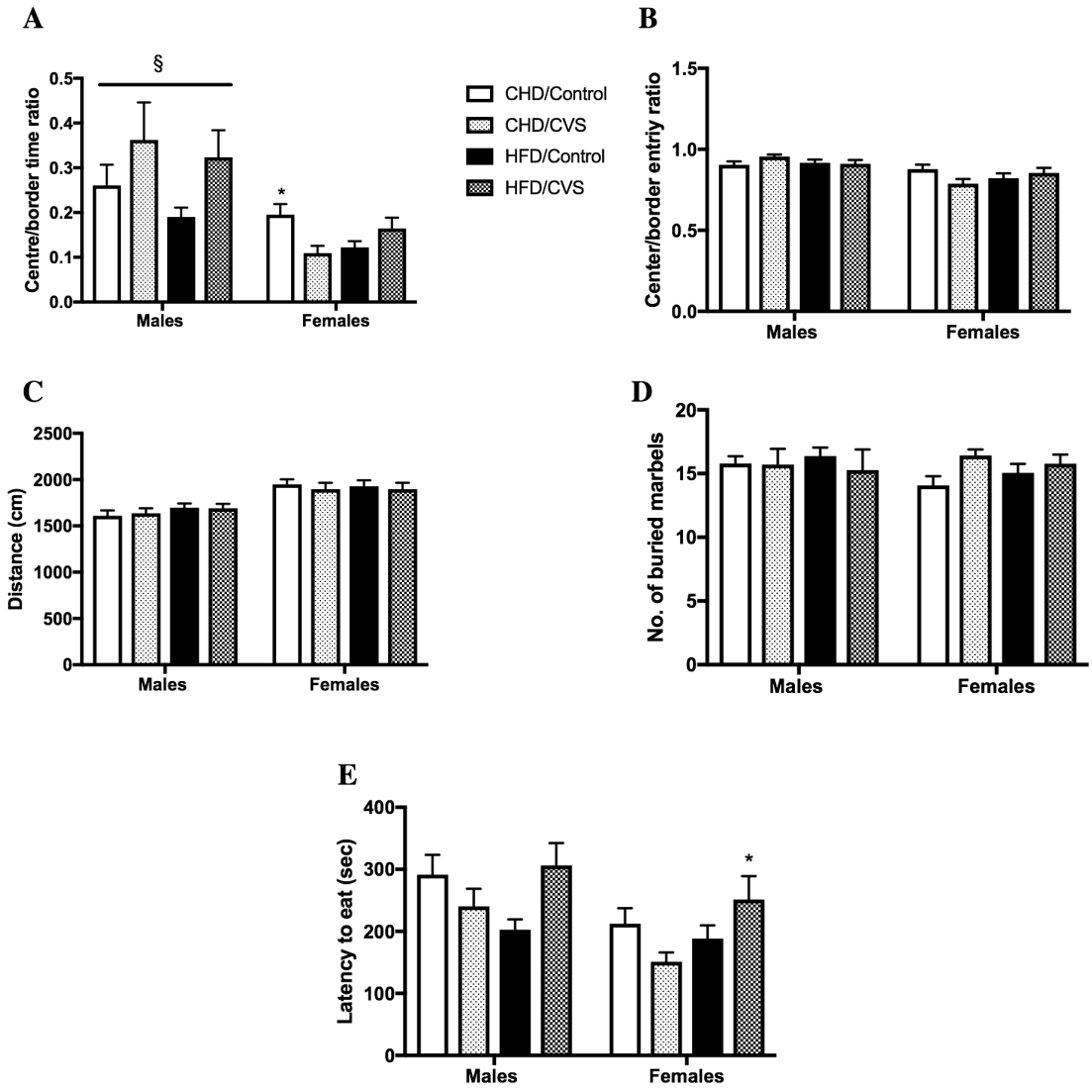

Figure 2.7 Adult offspring behaviours in open field (OF), marble burying (MBT), and novelty induced hypophagia tests (NIH). (A) CVS male offspring spent more time in the center of the open field relative to the border compared to non-CVS offspring. CHD/CVS and HFD/Control female offspring spent significantly less time in the center relative to the border of the open filed compared to CHD/Control female offspring. There were no significant effects of diet or stress on the number of entries into the center of the open files relative to the border (B) or the total distance travelled (C). (D) There were no significant differences in the number of marbles animals buried during the marble burying test. (E) During the NIH test HFD/CVS female offspring had longer latency to eat compared to CHD/CVS female offspring. CHD/Control n= 14, CHD/CVS n= 14, HFD/Control n= 15-16, HFD/CVS n= 11-13. Stress effect §$p \leq 0.05$. Tukey's post hoc: OF: CHD/Control vs. CHD/CVS and HFD/Control *$p \leq 0.05$. NIH: HFD/CVS vs. CHD/CVS *$p \leq 0.05$.

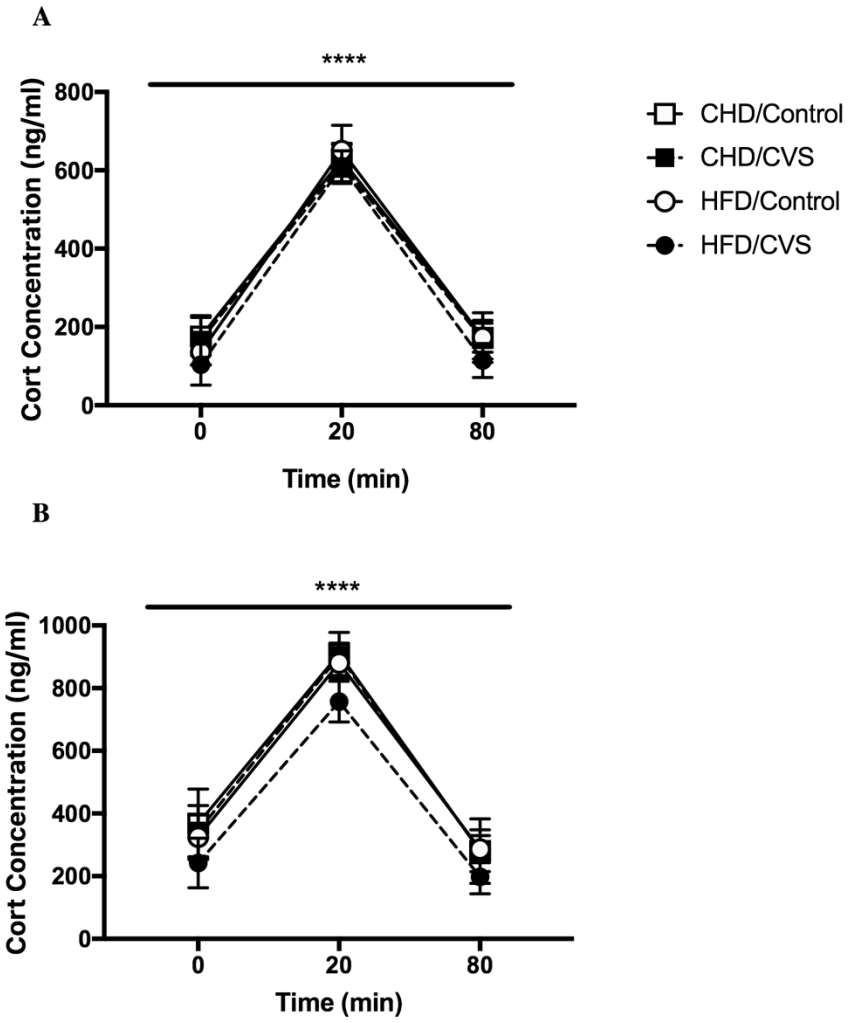

Figure 2.8 Adult offspring stress response to 20 min restrain challenge. All offspring, males (A) and females (B), responded to the restraint challenge by changes in their Cort concentration. There were no significant differences in Cort response among the different groups. CHD/Control n=7/sex, CHD/CVS n=7/sex, HFD/Control n=8/sex, HFD/CVS n= 7-5/sex. Time effect: ****$p \leq 0.0001$.

2.4.1 Maternal physiological response to HFD and CVS

As expected, females consuming HFD were heavier than CHD females prior to and throughout gestation. At PND 10, however, all dams weighed the same. Others have reported that dams on HFD lose weight postpartum (Rolls et al., 1984; Wright et al., 2011). In this chapter, I examined the sensitivity of HFD dams to stress demonstrated by body weight, food intake, and Cort measurements in response to gestational CVS. I found that after a week of CVS exposure, HFD dams had lower body weight gain compared to the HFD control dams. This was accompanied by lower food intake, which was positively correlated to body weight gain specifically in the HFD/CVS dams. One advantage of prenatal CVS is its limited effects on maternal weight gain and caloric intake, which separates the effects of the stress from what could be intrauterine growth restriction that have been linked to other prenatal stress paradigms, such as prenatal restraint stress (Lesage et al., 2004; Tamashiro et al., 2009). The results in here indicate that CVS did not significantly affect CHD dams' weight gain or food intake, similar to other reports (Lesage et al., 2004; Tamashiro et al., 2009). However, CVS significantly affected these measure in the HFD dams. Others using similar experimental design have reported lower weight gain in pregnant HFD dams exposed to CVS, with no effects seen on weight gain in CHD dams (Tamashiro et al., 2009). No changes, however, were reported in caloric intake between animals in that study (Tamashiro et al., 2009). The decrease in caloric intake in this study is in parallel with reports of another study examining the interaction of HFD consumption and chronic stress in rodents. They reported that total caloric intake after a 6-week chronic mild stress paradigm was significantly lower in stressed HFD animals compared to non-stressed HFD animals, an effect that was not observed in the CHD animal groups (Aslani et al., 2015). No weight gain measures were obtained in that study, however animals exposed to stress lost weight independent of their diet (Aslani et al., 2015). Despite the differences in experimental designs across these studies, they all indicate that HFD animals show a unique metabolic response to stress measured in weight and caloric intake alterations. The underlying physiological and molecular pathways governing these alterations in weight gain and caloric intake would be a good avenue for examination in future studies. Cort along with insulin are known to regulate the expression of a

number of genes that involved in food intake such as neuropeptide Y (NPY) and POMC (Reviewed in Yau and Potenza, 2013).

I also measured Cort response to restraint stress in dams during gestation to assess the diet and CVS effects on their HPA axis reactivity. In the first experiment, I observed an effect of day of gestation, where dams had a more reactive HPA axis at GD13, demonstrated by higher Cort levels after 60 min after the restraint. This was also indicated in higher AUC measure of the Cort response at GD13. These results might indicate that after a week of CVS exposure females were showing a decrease in their Cort response. With this experimental design, however, it was difficult to discern whether these differences were a result of the exposure to CVS or the gestation stage. Changes in HPA axis reactivity occur across gestation, with increasing Cort levels towards the end of pregnancy (Stefanski et al., 2005; Takahashi et al., 1998). Interestingly, one study has shown that dams at GD14 continued to have significantly high Cort levels 15 min after the end of a 45-min restraint challenge, while Cort levels in pregnant females at GD19 and 21 were not significantly higher than basal levels at that time point (15 min after the end of stress) (Williams et al., 1999). These findings are similar to the results I find here, where pregnant females at GD13 continued to have high Cort levels even an hour after the end of the stress. However, by GD20, pregnant females show decreased Cort levels similar to baseline an hour after the end of stress. These findings suggest that HPA axis inhibition mechanism is differentially regulated during different phases of gestation in rats. Indeed, an earlier report has shown an increase in glucocorticoid receptors in the hippocampus at GD21 in pregnant females compared to virgin females, indicating an increased HPA axis negative feedback at this stage of pregnancy (Johnstone et al., 2000), however there are no reports of glucocorticoid receptors levels in females in mid gestation (GD13).

The second restraint challenge experiment aimed to test the effect of diet and CVS exclusively and controlling for GD effects by limiting the testing on GD20, last day of the CVS protocol, of stressed and non-stressed females on either diet. Results demonstrated a main effect of diet at the peak level of Cort (20 min), where HFD females independent of CVS had higher Cort levels compared to CHD females. Two previous studies have reported an increase in basal Cort levels during gestation in rats and mice consuming a HFD (Bellisario et al., 2015; Rodriguez et al., 2012). This increase in basal Cort was also accompanied by a decrease in 11β-HSD-2 activity in the placenta, the enzyme that oxidizes Cort to its inactive form (Bellisario et al., 2015). Similar

effects were reported previously in a prenatal stress model (Jensen Peña et al., 2012), suggesting that HFD consumption might act as a stressor during gestation. Together, these results implicate alterations in maternal HPA axis physiology with maternal HFD consumption in programming the offspring stress response.

Unexpectedly, there was no effect of CVS on Cort levels in pregnant females. Earlier studies have reported an increase in basal Cort in serum and amniotic fluid of pregnant females exposed to CVS during gestation (Abdul Aziz et al., 2012; Tamashiro et al., 2009). Yet, it is important to point out that females were still responding to the stress by increasing their Cort levels after 20 min of restraint, indicating that the HPA axis did not habituate to the restraint stress. Consequently, CVS pregnant dams were exposed to almost daily activation of the HPA axis by exposing them to the restraint stress, which we show is activating the HPA axis even after several exposures to it. There is evidence showing that chronic stress facilitates a higher HPA axis response to subsequent acute stressors (Ferland et al., 2014). Restraint was used multiple times during this stress paradigm and animals might be responding in the same manner to the repeated exposure of this stress. Therefore, CVS effects on HPA axis reactivity in pregnant dams could be confirmed in future experiments examining whether CVS animals would respond with a higher Cort levels to a novel acute stress compared to the control animals.

2.4.1.1 Maternal behavioural response to HFD and CVS

In this chapter, I also assessed maternal care, depressive- and anxiety-like behaviours in response to the diet and stress treatments. Maternal care is a well-known modulator of offspring HPA axis phenotypic outcome (Liu et al., 1997). There have been several reports suggesting alteration in maternal care behaviours in response to prenatal stress and maternal HFD. Here, I report HFD effects on a number of maternal behaviours. HFD dams showed an increase in total time spent nursing and licking their pups. The different nursing postures were affected differently by HFD consumption and prenatal stress. HFD dams were engaged in more blanket nursing and less supine nursing compared to CHD, while there were no differences AB nursing. Prenatal CVS reduced the time spent in supine nursing without changing any other measures of maternal care behaviours. Moreover, HFD dams spent more time licking their pups, which was observed in both modes of licking (AG and BD licking). These findings may indicate an enhancement in maternal behaviour in HFD dams as they provide their offspring with more nutrient by increasing their time nursing and engaging less with a passive form of nursing (supine nursing).

However, based on other evidence from the literature suggesting impaired lactation in HFD dams, these findings may indicate compensatory behaviour. For example, prolactin insensitivity has been reported in the mammary glands of HFD mice dams, impairing milk production (Buonfiglio et al., 2016). Others have reported that HFD rat dams showed delayed milk production on PND1, which was associated with mammary gland inflammation (Hernandez et al., 2012). In addition, studies examining maternal behaviour in rats bred for high anxiety-related behaviour (HAB) revealed that they spent more time nursing their pups, compared to rats bred for low anxiety-related behaviour (LAB) (Neumann et al., 2005a). HAB pups separated from their dams daily during the first two weeks of life, consequently receiving less nursing, develop to have lower anxiety-like behaviours in adulthood compared to HAB pups that were not separated from their dams (Neumann et al., 2005b).

In addition, maternal behaviour is known to be modulated by pup and nest temperature. For instance, dams adopt a supine nursing posture and less crouch nursing, while increase pup licking, when the litter temperature is high (Stern and Lonstein, 1996). Others have demonstrated that tactile stimulation decrease pup body temperature or attenuate its increase in warm ambient temperature (Sullivan et al., 1988). Brown adipose tissue (BAT) regulates thermogenesis in neonates and has been shown to be altered in weanling mice exposed to perinatal HFD. For examples, weanlings exposed to perinatal HFD showed higher mass of BAT with lower endocannabinoid receptors, which are known to inhibit BAT thermogenesis, suggested an impaired thermogenic function of BAT of HFD offspring (Almeida et al., 2017). Another study examining lactation HFD effects on thermogenic function of BAT in offspring reported increased BAT mass in weanlings that had an impaired adaptive thermogenesis response to cold exposure (Liang et al., 2016). Future studies should examine the relationship between the alteration of maternal care behaviour observed and thermoregulation of the pups via BAT.

I further examined the effects of perinatal HFD and gestational CVS on depressive- and anxiety-like behaviours in the dams. Postpartum mental health has been linked to offspring health outcomes (Hillerer et al., 2012). Mothers during the lactation period exhibit a hyporesponsive HPA axis with high basal levels of Cort compared to non-mothers animals (Reviewed in Thanh Tu et al., 2005). HFD and prenatal stress have been associated with the induction of postpartum depressive- and/or anxiety-like behaviours in rodents (Hillerer et al., 2011; Leuner et al., 2014; Perani et al., 2015). One study reported increased postpartum anxiety-like behaviours in HFD

dams and an exaggerated Cort response to stress (Perani et al., 2015). In this chapter, I tested the dams on sucrose preference test to assess depressive-like behaviour and OFT to assess anxiety-like behaviour 10 days after euthanizing PND13 pups. I did not observe any differences between the groups in any of the measures of the tests. I believe removing all of the pups on PND13, which were euthanized to collect tissue, was a major caveat. Upon removal of all pups, mainly HFD dams developed engorged mammary glands that did not resolve for a number of days. It is not clear if this might have influence the behavioural outcome in these animals, however this HFD specific phenotype might be a confounding factor when comparing results across the diet groups. In addition, many studies examining effects of gestational stress assesse postpartum emotional behaviour during the first week postpartum (Hillerer et al., 2011; Leuner et al., 2014), the period exhibiting the hyporesponsive HPA axis (Deschamps et al., 2003). Therefore, I would alter this expriemental paradigm in future studies to test the dams during the first postpartum week (PND8) similar to other work examining maternal emotional behaviour.

2.4.2 Offspring anxiety-like behaviours and stress physiology

Another indication of higher stress sensitivity in HFD dams was the reduced litter size in HFD/CVS compared to HFD/Control. Also, HFD/CVS litters had higher pup mortality rate prior to weaning. In addition, two stressed HFD dams failed to give birth to viable pups, with one giving birth to 4 still born pups and the other going into labor but never gave birth. This increased sensitivity of HFD dams was also witnessed in another laboratory when unsuspected disturbances in the animal facility due to construction led to a decrease in litter size and increased numbers of stillborn pups (Grissom and Reyes, 2013).

Offspring did not differ in weight at birth or at PND10. HFD effect on weight was observed at weaning, where HFD pups tended to be heavier than CHD pups and this effect was not observed at adulthood due CHD exposure during development similar to earlier reports (Sasaki et al., 2013). Unlike earlier reports of HFD exposure during early development (Bilbo and Tsang, 2010; Sasaki et al., 2013; Tamashiro et al., 2009), I failed to find increase in body weights in neonates. Weighing the pups once weekly would allow better detection the onset of this effect.

Next, I examined the effects of perinatal HFD and prenatal CVS on the programming of anxiety-like behaviours in adult animals using a number of tests that measure unconditioned spontaneous behaviours such as exploratory, consummatory, and social behaviours in the face of a novel

environment, a stressor (Treit, 1985). As these behaviours are associated with physiological response to a stressor, they shed a light on the functioning of the HPA axis. I found that male and female offspring anxiety-like behaviours were effected differently by perinatal HFD and prenatal CVS. Males exposed to HFD showed increased open arm exploration duration in the EPM test and reduced anxiety index. On the other hand, males exposed to prenatal stress showed increased center exploration in the OF test both indicating decreased anxiety-like behaviour. Female offspring from CHD/CVS and HFD/Control litters had an increase in anxiety-like behaviour in OF test compared to the control offspring measured by decrease in time spent exploring the center of OF. Prenatally stressed HFD females, on the other hand, had a comparable time spent in the centre to CHD/Control, suggesting that perinatal HFD and prenatal CVS combined normalize the effects of either treatment alone, which was not in line with the my hypothesis of an exaggerated effects of both early life treatments. In summary, offspring reacted to early life manipulation whether stress or/and diet by a decrease in emotionality shown in males, while females were more reactive to these tests. My findings contradict what have been reported in the literature about prenatal CVS and anxiety-like behaviour in adult offspring. A number of behavioural alteration has been reported in in adult offspring exposed to prenatal CVS, including reduced activity (Emack et al., 2008), decreased sociality (Lee et al., 2007), increased depressive-like behaviours (Mueller and Bale, 2008), reduced cognition (Abdul Aziz et al., 2012), and a decreased sensorimotor gating (Koenig et al., 2005). However, these studies have reported a consistent lack of effect of prenatal CVS on anxiety-like behaviours in offspring of both sexes in either OF or EPM tests (Abdul Aziz et al., 2012; Emack et al., 2008; Hougaard et al., 2005; Jones et al., 2013; Koenig et al., 2005; Mueller and Bale, 2008; Richardson et al., 2006). Only one study has reported a decrease in anxiety-like behaviour in prenatal CVS offspring indicated by an increase in time spent on the open arm of the EPM (Jones et al., 2010). The female behaviour in HFD offspring is consistent with the earlier report from our lab and others, however male behaviour was not (Bilbo and Tsang, 2010; Glendining et al., 2018; Saben et al., 2014; Sasaki et al., 2013). Sasaki et al. (2013) showed that HFD males spent less time in the center of the OF relative to the border compared to the CHD males during the full 15 min test, while the OF test here only shows a prenatal stress in males and HFD/control were not significantly different than CHD/Control males at the 5min time bin or the full 15 min (data not shown). The full 15 min results are not shown because the effects on the females are not evident at that point anymore. Further, while Sasaki et al. (2013) showed lower ratio of entries into the

open arm of the EPM relative to the closed arm in only HFD female and not male offspring, I did not observe any significant differences between groups in either sex. The differences observed in this study were in the duration spent in the open arm relative to the closed arms, a measure that was not reported by Sasaki (2013). Others have reported increased anxiety-like behaviours in OF (reduced distance in centre; Kang et al., 2014) and EPM (reduced time on open arms; Glendining et al., 2018) in female offspring and not males. Another study reported increased anxiety-like behaviour only in males indicated by reduced time spent on the open arms of the EPM (Bilbo and Tsang, 2010). These findings in general indicate increased anxiety-like behaviour in adult offspring after perinatal HFD exposure. My results only support these findings in the female offspring indicated by the OF results.

In addition, I did not observe any differences in the Cort response to restraint stress among the different groups in neither males nor females. Perinatal HFD was reported to reduce basal Cort and increase Cort response to restraint stress in adult offspring (Lin et al., 2015; Sasaki et al., 2013). In addition, studies have reported an increase in stress reactivity in prenatal CVS adult offspring indicated by increased Cort response to restraint stress (Koenig et al., 2005; Mueller and Bale, 2008). ACTH is another stress hormone that could be measured to draw a final conclusion on the physiological effects of this paradigm.

One possible explanation for the lack of replication between this study and earlier work done in our laboratory could be switching the dams from the HFD to CHD at PND17 to ensure that the offspring did not consume the diet themselves and the effects of the diet were mediated through the mothers only (Tozuka et al., 2009). Withdrawal from HFD after 6 weeks consumption by switching adult mice onto a low fat diet has been reported to activate the HPA axis and induced anxiety-like and depressive-like behaviours (Sharma et al., 2013). Dams exposed to HFD prior to gestation and throughout gestation and lactation were reported to have an atypical HPA axis during lactation, characterized by basal hypocotricism and an exaggerated Cort response to stress (Perani et al., 2015). With HFD dams having a reactive HPA axis during a period of a naturally blunted stress response due to lactation, the diet switch might act as a stressor to both dams and offspring during the postpartum period, which could be associated with the adult offspring phenotype observed in this study.

2.4.3 Conclusion

The first aim of this chapter was first to characterize the maternal response to the diet and stress manipulations used in this thesis, including physiological and behavioural responses. I found that as predicted, HFD dams were more sensitive to stress evident by a higher Cort, reduced body weight gain, and food intake during gestation in response to stress. Maternal care behaviour was affected by HFD consumption, as HFD dams engaged in more nursing and licking of their pups in support of earlier findings (Purcell et al., 2011; Rincel et al., 2016). I observed no differences in depressive- or anxiety-like behaviours between dams postpartum. The second aim of in this chapter was to examine the effects of perinatal HFD and prenatal CVS on adult offspring HPA axis stress response and anxiety-like behaviour. Adult offspring showed a sex dimorphic response to the early life environmental stressors, where males showed decreased anxiety-like behaviours, while females showed increased anxiety-like behaviour, with no change in Cort response to stress in both sexes. Unexpectedly, I was not able to replicate findings reported in earlier studies of perinatal HFD and prenatal CVS. In addition, I was not able to support my hypothesis of additive effect of perinatal HFD and prenatal CVS on offspring phenotype. Despite limitations discussed above, these findings highlight the importance of examining maternal outcomes in order to understand how different models of early life stress manipulations could affect the offspring phenotype.

Chapter 3

Perinatal HFD and prenatal CVS effects on the HPA axis programming in neonates

Parts of this chapter are adapted from:

Abuaish, S., Spinieli, R.L., McGowan, P.O., 2018. Perinatal high fat diet induces early activation of endocrine stress responsivity and anxiety-like behaviour in neonates. Psychoneuroendocrinology 98, 11–21. doi:10.1016/j.psyneuen.2018.08.003

3 Perinatal HFD and prenatal CVS effects on the HPA axis programming in neonates

3.1 Introduction

Neonatal rodents unlike adults have a unique HPA axis during the first two postnatal weeks that is hyporesponsive to stress. During this period, only a few stimuli, such as ether and endotoxin exposure, can lead neonates to mount a Cort response (Shanks and Meaney, 1994; Walker et al., 1991). Generally, the hyporesponsive period is considered to start on PND2 and ends by PND14 (Vázquez and Akil, 1993). However, a gradual HPA response has been observed as early as PND10, while an adult-like response is established by weaning age (PND21) (Vázquez and Akil, 1993). Studies examining development during this period have shown that hyporesponsive neonates begin to exhibit HPA axis activation following 24-hour maternal deprivation (Suchecki et al., 1995). This led to identifying maternal factors that are believed to mediate this hyporesponsivness including feeding, tactile stimulation, and maternal contact (Levine, 2002). Further, in conjunction with maternal factors, neonates exhibit immature adrenal response to ACTH and high suppression of HPA axis activity mediated through low corticosteroid-binding globulin (CBG), high free Cort, and GR at adult levels in the pituitary (Henning, 1978; Sakly and Koch, 1981; Schmidt, 2010).

Neonatal rats exhibit behaviours that have been associated with HPA axis activity. For example, neonatal rats exhibit behavioural inhibition near the end of the hyporesponsive period (PND10), which is characterized by the absence of movement and USVs under threatening conditions such as exposure to adult male rat or its cues (Takahashi, 1992). It has been shown that this inhibition could be mediated through Cort actions in the hippocampus and amygdala (Moriceau et al., 2004; Takahashi, 1995). Neonatal rats injected with Cort exhibited behavioural inhibition, which was associated with activation of the basolateral nucleus of the amygdala (Moriceau et al., 2004). However, hippocampal lesions prevented behavioural inhibition in neonatal rats after Cort injection (Takahashi, 1995). Social isolation induces neonatal USV at 40 kHz and activation of the HPA axis (Hofer et al., 2002; Lorey K Takahashi, 1992). USVs act as a survival tool as it elicits maternal search, pup retrieval, and nest building behaviours (Brouette-Lahlou et al., 1992;

Brunelli et al., 1994; D'Amato et al., 2005). In addition, these "distress calls" have been used as an index of pup anxiety, as anxiolytic and anxiogenic drugs either attenuate or enhance USVs, respectively (Insel and Harbaugh, 1989). Further, animals bred for high USVs during infancy (PND10) showed increased anxiety-like behaviours in adulthood compared to ones bred for low USVs (Zimmerberg et al., 2005). Moreover, USVs are modulated by CRH, the central mediator of the HPA axis (Insel and Harbaugh, 1989).

Given the importance of the early postnatal life in shaping the individual's health into adulthood and the limited reports on HFD effects on HPA axis function in the early postnatal life, the aim of this study was to examine the effects of perinatal HFD exposure on the development and functionality of the HPA axis in neonatal offspring during and as they emerge from the stress hyporesponsive period. Previous studies have demonstrated that early activation of the neonatal HPA axis as a result of maternal separation during the hyporesponsive period is associated with increased HPA axis activity in neonates and weanlings (Mccormick et al., 1998; McCormick et al., 2002). I hypothesized that rapid development induced by perinatal HFD and prenatal CVS would lead to early activation of the HPA axis during the hyporesponsive period, potentiating heightened responses to stress the beyond that period.

3.2 Methods:

3.2.1 Animals and diets:

Adult female Long Evans rats (7 week) were purchased from Charles River and housed in same sex pairs until mating and maintained on a 12:12-h light– dark cycle (lights on 7:00 am–7:00 pm) with *ad libitum* access to food and water. Females (n=37) were either maintained on chow diet (CHD; Purina Lab Diets (St. Louis, MO: cat. no. 5001) and contained 28.5% protein, 13.5% fat, and 58% carbohydrate (n=20), or high fat diet (HFD; Research Diets, Inc. (New Brunswick, NJ: cat. no. D12492), and contained (by kcal): 20% protein, 60% fat, 20% carbohydrate. (n=17)) for 3 weeks prior to mating. Prior to mating females and their food were weighed once a week to monitor weight gain and food intake on the two different diets. Females were paired with males and sperm plugs were checked twice a day to determine the onset of pregnancy. All females were housed for a week with males regardless of when the plug was observed to increase the chances of pregnancy, as the presence of vaginal plugs does not always guarantee pregnancy. The presence of vaginal plugs was noted and this information was combined with the monitoring

of maternal body weight to confirm pregnancy.

Later females were separated from males and singly housed throughout their pregnancy. Once litters were born, the dams were moved into clean cages with paper towel strips used as nesting material. Pups were weighed and culled to 12 pups/ litter (6 males and 6 females) when possible. Cages were moved into a separate room for maternal behaviour recording and kept there until PND13. Dams and pups were weighed and nose-anal lengths of pups were measured on PND0, PND7 and PND13. Lee index, used as an index of obesity in rodents and has been shown to correlate to carcass fat percentage, calculated as

$\sqrt{bodyweight(g)}/nose\text{-}anal\ length\ (mm)\times 1000$ (Simson and Gold, 1982).

During the weighing procedure on PND13, litters were examined for eye opening, a measure of maturation. One CHD dam only gave birth to 7 pups, 2 of which were euthanized on PND7, leaving only 5 pups, which is half the litter size of other litters, therefore it was excluded from further analysis on PND13. Small litters have been used as a paradigm of overnutrition inducing obesity in early life (Spencer and Tilbrook, 2009), therefore we did not keep the litter with the 5 pups for any confounding effects that might have on body weight and maternal care provided to the pups.

Experimental protocols were approved by the Local Animal Care Committee at the University of Toronto, Scarborough, and were in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2 Prenatal chronic variable stress (CVS):

Females were transported to a procedure room (CHD, n=9; HFD, n=8) where they were exposed to the different stressors daily, see chapter 2 section 2.2.2. I modified the CVS protocol by replacing the platform stress by an exposure to an elevated plus maze, as few animals were falling off the platform causing detrimental effects on the litters born. Control non-stressed animals (CHD, n=11; HFD, n=9) were kept at the main housing room undisturbed.

3.2.3 Maternal behaviour

See chapter 2 section 2.2.4.

3.2.4 Pup testing apparatus description

Two modified operant conditioning chambers inside sound attenuating boxes were set up with either clean bedding (CB) or soiled bedding (MB) from a sexually experienced unfamiliar adult male rat in the collecting tray of the chambers. MB was obtained from males that were not the sires mated with the dam of the pup tested and they were housed in a separate room from the room housing the dams and pups. The electrical grid was lined with vinyl shelf liners to prevent the pup from sliding through the electrical grid. Condenser microphones Avisoft Bioacoustics CM16/CMPA were suspended 15cm above the center of the chambers. Cameras were mounted on the inside of the boxes to record pup behaviour during the test

3.2.5 Pup isolation testing procedures and tissue collection

Pup testing was carried out on PND 7 and PND 13 similar to (Hofer et al., 2002). At PND 7, two males and females of similar weights were transferred into a mouse cage with bedding from their home cage and then transferred to an adjacent testing room where they were placed onto a heating pad set at 34°C. The pups were allowed to habituate for 10 min prior to placing them in the isolation chambers. After habituation, two pups of the same sex were placed in the center of either the CB or MB isolation chambers at room temperature for 10 min, where their USVs and behaviour were recorded. After the test, the pup from the MB isolation chamber was removed and euthanized in an adjacent room by decapitation. Trunk blood and brain were collected for later analysis. The pup from the CB isolation chamber was marked by an ear punch and returned back to their litters. The chambers were wiped clean with Virox between trials.

A separate cohort of animals (CHD/Control n=7, HFD/Control n=8) were used to collect baseline measures in PND7 pups. One pup per sex was rapidly removed from the home cage and immediately decapitated in an adjacent room and trunk blood and brain were collected.

At PND 13, three male and female pups were transferred into a mouse cage with bedding from their home cage and then transferred to an adjacent testing room where they were placed onto a heating pad set at 34°C. The pups were allowed to habituate for 10 min prior to placing them in the isolation champers. After habituation, two pups of the same sex were placed in the center of either the CB or MB isolation chambers for 10 min where their USVs and behaviour were recorded. After the test both pups were euthanized and trunk blood was collected. Lastly, the third set of pups of the same sex (1pup/sex) were individually placed in the MB isolation

chamber without recording their USV and behaviour for 10 min after which they were marked and put back with their dam for an hour. Pups were then removed and euthanized to collect trunk blood. The chambers were wiped clean with Virox between trials. For baseline measurements, one pup per sex was rapidly removed from the home cage and immediately decapitated in an adjacent room and trunk blood and brain were collected.

3.2.6 Pup ultrasonic vocalization recording and analysis

The condenser microphones Avisoft Bioacoustics CM16/CMPA are sensitive to frequencies between 10 kHz-200 kHz and were connected to Avisoft UltraSoundGate 416 USB Audio device, which was connected to a personal computer. Settings included sampling rate at 166.66 kHz, format 16 bit, and high-pass filter setted at 20 kHz. Acoustic analysis was performed using Avisoft SASLab Pro where a fast Fourier transform was done (512 FFT length, 100% frame, Hamming window and 50% time window overlap) with 20 ms hold time. Calls were also inspected manually to ensure that all USVs detected using the software's automatic processing algorithms were valid calls. All false signals were removed before statistical analysis was performed. The first 5 min was analyzed for call numbers, mean call duration, and peak frequency. Hofer et al., 2002 identified the first 2-6 min as informative of aversive and anxiety-like behaviours associated with altered USV production.

3.2.7 Pup behaviour analysis during the isolation testing

On both days, pup behaviours were recorded and later analyzed in 5 min time bins using The Observer XT 10 software (Noldus). Earlier studies have indicated that neonatal rats do not exhibit the conventional freezing behaviour observed in adult rats, which includes piloerection and crouching position, therefore here immobility/freezing is defined as cessation of movement (Moriceau et al., 2004).

3.2.8 Corticosterone and ACTH radioimmunoassay after dams' restraint challenge and male bedding exposure in pups

Blood from pups and dams was allowed to coagulate for 30 min on ice and then centrifuged at 4°C at 4000 rpm for 20 min and serum was stored at -80°C. Cort levels in both dams and pups were measured using Corticosterone Double Antibody RIA Kit (MP biomedicals, Cat. 07120103; sensitivity 7.7 ng/mL, intra-assay coefficient of variation 10.3%, inter-assay

coefficient of variation 7.2%). For neonatal animals specifically, two additional low concentration standards were created to acquire accurate measures for low Cort levels expected in neonatal rats. Duplicate samples were diluted 1:100 instead of the manufacturer recommended 1:200 dilution in order to detect low Cort in the samples. Dam samples were diluted following the manufacturer recommendation of 1:200.

ACTH levels were determined only in pups using hACTH Double Antibody RIA Kit (MP biomedicals, Cat. 07106102; sensitivity 5.7 pg/mL, intra-assay coefficient of variation 6.8%, inter-assay coefficient of variation 10.7%). Pooled samples of equal amounts from littermates of both sexes were used to obtain the required 100ul/sample. Samples were run in duplicates. One additional low concentration standard was created to acquire accurate measures for the low ACTH levels expected in neonatal rats.

3.2.9 Brain preparation and RNA extraction

Brains were flash frozen in isobutene and stored at -80°C. Brains obtained from baseline animals from both PNDs were later cryosectioned into 50 μM sections using Research Cryostat Leica CM3050 S (Leica Biosystems) and brain regions were then microdisected based on stereotaxic coordinates (Paxinos et al., 1991) (PVN: bregma: -0.20mm to -0.80mm), ventral hippocampus: bregma: -2.00mm to -2.80mm)).

RNA was extracted using MasterPure™ Complete DNA and RNA Purification Kit (Lucigen, Cat. MC85200), according to manufactures instructions. Quantification and purity of the RNA were assessed with a spectrophotometer (Nanodrop ND-2000C, Thermo Scientific). RNA (PVN: 0.5μg, ventral hippocampus: 1μg) was converted to cDNA using High Capacity cDNA Conversion Kit (Applied BioSystems, Cat. 4368814) according to manufactures instructions.

3.2.10 Gene expression analysis in pup brains by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

The expression patterns of five genes (Table 3.1) were quantified and analyzed using StepOne Plus real-time PCR with Fast SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The quantification was determined using a stand curve generated from 11 serial dilutions of a pool of all offspring cDNA for each brain region and PND

examined. Three housekeeping genes (*ActinB*, *Ubc*, and *Ywhaz*) were measured for the PVN and Hippocampus. The transcripts relative abundance was determined by normalization against *Ywhaz* abundance because it showed the greatest stability among the housekeeping genes examined. Primers (Table 3.1) were designed according to GenBank sequence information at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov).

Table 3.1 qPCR primer sequences

Gene	Forward Primer	Reverse Primer
Ywhaz	5′-TTGAGCAGAAGACGGAAGGT-3’	5′-GAAGCATTGGGGATCAAGAA-3′
Crh	5'-TTCCTGTTGCTGTGAGCTTG-3'	5'-TCACCTTCCACCTTCTGAGG-3'
Nr3c1	PPR52805B, SAbiosciences, Qiagen, Valencia, CA, USA	
Nr3c2	5′-GGCAGCTGCAAAGTCTTCTT-3′	5′-GACAGTTCTTTCGCCGAATC-3′
Gad1	5'-ACTGGGCCTGAAGATCTGTG-3'	5'-CCGTTCTTAGCTGGAAGCAG-3'

Ywhaz: 14-3-3 protein zelta/delta, *Crh*: Corticotropin releasing hormone, *Nr3c1*: Glucocorticoid receptor, *Nr3c2*: Mineralocorticoid receptor, *Gad1*: glutamic acid decarboxylase 1.

3.2.11 Statistical analysis

Statistical analysis was carried out using SPSS (IBM). A Shapiro-Wilk test was used to test for normality for data sets with $n < 30$. When data sets were not normality distributed, extreme outliers were examined using box plots and removed if they were more than 3 times the interquartile range of the data (Ghasemi and Zahediasl, 2012). If the data set did not have outliers and was not normal, square root values were calculated to normalize the data. For data sets with $n > 30$ normality was assumed (Ghasemi and Zahediasl, 2012) and parametric analyses were carried out. Factorial linear mixed models (LMM) (time x diet x stress) were used to analyze data sets that were repeated measures and contained missing values, and to control for dam ID as a random factor when using more than 2 individuals from the same litter (PND13 pup ACTH and Cort responses). Dam ID did not contribute to the variation in pup behaviour and USVs when analyzed initially using LMM, therefore a factorial general linear models (GLM) was used (diet

x stress x treatment). Since there were no significant sex effects in any of the reported measures in this study, sex was removed as a factor from the analysis. Two tailed t-tests were used to analyze the diet effect on relative transcript abundance. Bonferroni post hoc testing was used for pairwise comparisons between diet groups across diet groups and time points.

3.3 Results:

3.3.1 Maternal weights and food intake:

All females gained weight throughout the experiment ($F_{(9,337)}$= 92.82, p≤0.0001; Figure 3.1A). HFD induced an increase in the maternal weights throughout the dietary exposure period ($F_{(1,337)}$= 93.70, p≤0.0001; Figure 3.1A). There was a main effect of CVS on the weights of the dams ($F_{(1,337)}$= 8.61, p=0.004). HFD females' weights differed depending on the week of the study (Diet x week interaction: $F_{(9,337)}$= 3.85, p≤0.0001). Parallel to the results presented in Chapter 2, diet ($F_{(1,32)}$= 4.81, p=0.03) and CVS ($F_{(1,32)}$= 16.7, p=0.0003) affected the weight gain during the last week of gestation. HFD/CVS pregnant dams were significantly gaining less weight compared to CHD/Control (Bonferroni post hoc: p= 0.0007) and HFD/Control (Bonferroni post hoc: p= 0.01). I further examined the weight differences during the postpartum period (Figure 3.1B); prenatally stressed dams weighed less than control dams (CVS effect: $F_{(1,65)}$= 4.11, p=0.05). Examining the weight change of dams during the postpartum period revealed a main effect of diet ($F_{(1,65)}$= 56.72, p≤0.0001) and CVS ($F_{(1,56)}$= 5.28, p=0.02), with HFD losing weight, while CVS dams gaining more weight compared to their respective diet control dams.

The weights differences were accompanied by differences in caloric intake throughout the period of the study ($F_{(6,234)}$= 292.15, p≤0.0001; Figure 3.1C). HFD dams consumed more calories than CHD dams ($F_{(1,234)}$ = 49.67, p≤0.0001). HFD dams differed in their caloric intake depending on the week of testing (Diet x week interaction: $F_{(6,234)}$ = 18.79, p≤0.0001). In addition, I observed diet by stress interaction ($F_{(1,234)}$ = 5.81, p=0.02). In order to understand the interaction effects, I examined the caloric intake during gestation and postpartum separately (Figure 3.1D). During gestation, there was an overall main effect of diet ($F_{(1,66)}$ = 11.6, p=0.001), indicating HFD dams consuming more calories than CHD dams. Moreover, a CVS by week interaction was observed ($F_{(1,66)}$ = 5.90, p=0.02), with a main effect of CVS observed after the exposure to the stress protocol on the last week of gestation ($F_{(1,33)}$ = 6.90, p=0.01). During postpartum weeks, all dams increased their caloric intake (Week effect: $F_{(1,65)}$ = 164.62, p≤0.0001), however HFD dams consumed less

calories (Diet effect: $F_{(1, 65)} = 5.11$, p=0.03), with HFD/Control consuming less calories than CHD/Control during the first postpartum week (Bonferroni post hoc: p= 0.058).

3.3.2 Maternal care behaviour:

Overall, no changes in maternal behaviour were observed between dams during the first 6 days postpartum in nursing behaviour (Diet effect: $F_{(1,33)}= 0.00$, p=0.99; Stress effect: $F_{(1,33)}= 1.72$, p=0.20; Figure 3.2A) or licking/grooming (Diet effect: $F_{(1,33)}= 1.47$, p=0.23; Stress effect: $F_{(1,33)}= 0.46$, p=0.50; Figure 3.2D). During the dark phase of the circadian cycle, however, HFD dams spent more time nursing compared to the CHD dams ($F_{(1,32)}= 4.29$, p=0.05; Figure 3.2C), while CVS dams spent less time nursing compared to non-stressed dams ($F_{(1,32)}= 6.45$, p=0.02; Figure 3.2C) and no differences were observed during the light phase (Figure 3.2B). Dams did not differ in total %licking/grooming (body and anogenital licking combined) during the day and night periods compared to CHD dams (Figure 3.2E-F). Table 3.2 represents a summary of the different maternal care behaviours measures recorded during the first 6 postnatal days.

3.3.3 Litter characteristics and pup body weights

I did not observe any effects of diet or stress on litter size, still born births, or mortality rate in this cohort of animals. As expected, pups gained weight overall during the first two postnatal weeks ($F_{(2, 63.6)}= 2956.42$, p≤0.0001; Figure 3.3A). In addition, a main effect of diet ($F_{(1, 32.76)}=10.70$, p=0.003) and an interaction between diet and PND ($F_{(2, 63.6}=16.30$, p≤0.0001) were observed. At birth, CVS offspring were lighter than control offspring (CVS effect: $F_{(1, 33)}=7.03$, p=0.01). At PND7, HFD offspring tended to be heavier than CHD offspring (Diet effect: $F_{(1, 33)}=3.88$, p=0.057); specifically HFD/CVS were heavier than CHD/CVS (Diet x PND interaction: $F_{(1, 33)}=5.40$, p=0.03; Bonferroni post hoc: p=0.04). At PND13, HFD pups were heavier than CHD pups (Diet effect: $F_{(2,31)}= 13.75$, p=0.0031). As they grew, pups showed an overall decrease in their Lee index (ratio of body weight to length - see Methods; PND effect: $F_{(2,64.39)}= 69.39$, p≤0.0001; Figure 3.3B), however HFD pups showed differential Lee indexes across the first two postnatal weeks compared to CHD pups (Diet effect: $F_{(1,33.26)}= 8.93$, p=0.005; Diet x PND interaction: $F_{(2,64.39)}= 12.04$, p≤0.0001). Examining each PND separately showed an interaction between diet and CVS ($F_{(1,33)}= 4.31$, p=0.05). At PND 7 and 13, HFD offspring showed a higher Lee index compared to CHD offspring (PND7: Diet effect: $F_{(1,33)}= 4.26$, p=0.05; PND13: Diet effect: $F_{(1,33)}= 31.54$,

p≤0.0001).

3.3.4 Pup endocrine stress reactivity

At PND7, exposure to male bedding induces a significant difference in ACTH between HFD and CHD pups ($F_{(1,23)}$= 4.35, p=0.05; Figure 3.3C). In addition, Cort response to male bedding (MB) was affected by diet (non-significant trend: $F_{(1, 25.56)}$= 3.48, p=0.07), CVS ($F_{(1,25.56)}$= 4.61, p=0.04), and an interaction between diet and CVS (Diet x CVS: $F_{(1.25.56)}$= 4.05, p=0.05; Figure 3.3D). Only HFD/Control offspring had a pronounced increase in Cort levels in response to MB exposure (Bonferroni post hoc: **p≤0.01 HFD/Control vs. all).

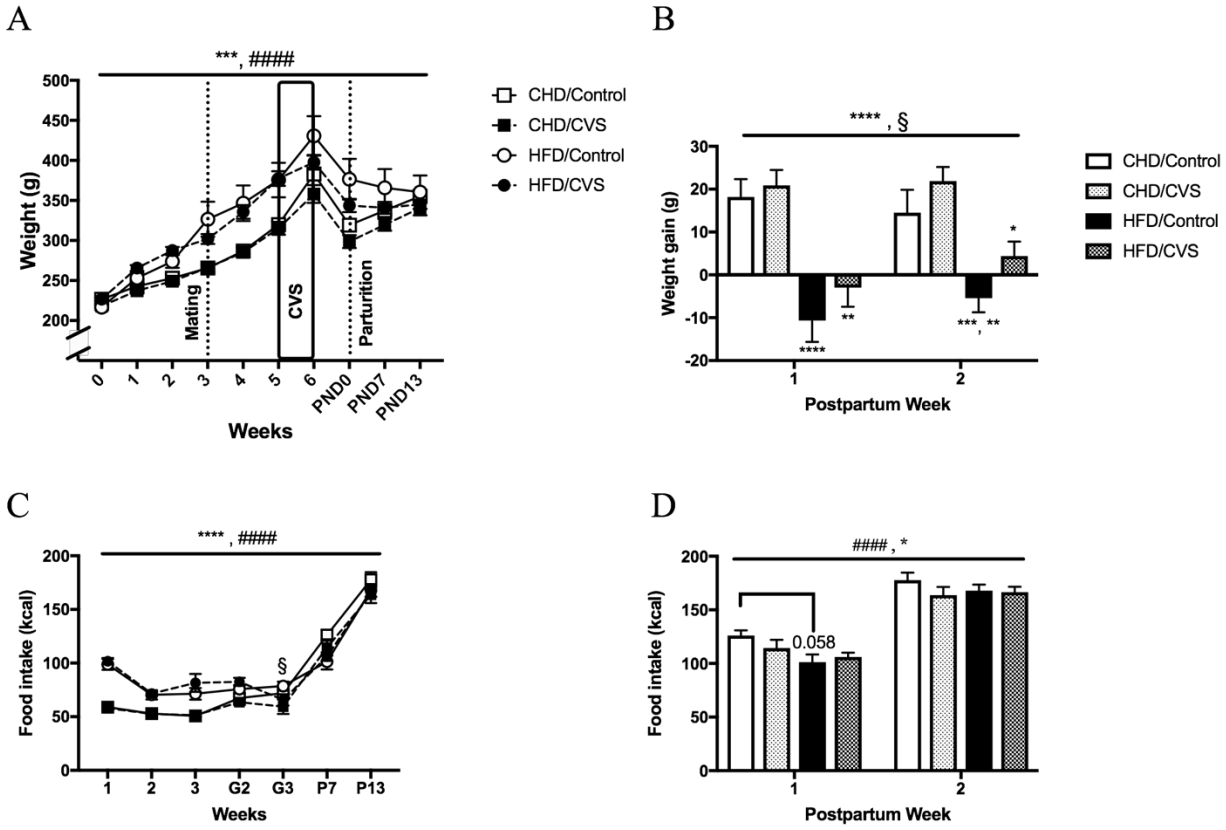

Figure 3.1 Maternal weights and food intake. (A) All dams gained weight throughout the study. HFD dams weighed more than CHD dams. (B) HFD dams were losing weight during the first two postnatal weeks. (C) Dams' food intake changed across the study. HFD dams were consuming more calories compared to CHD dams. CVS decreased food intake by dams during the last week of gestation. (D) HFD dams were consuming less calories during the postpartum weeks compared to CHD dams. CHD/Control n=11, CHD/CVS n=9, HFD/Control n=8, HFD/CVS n=9. Data presented are means ± standard error. Weeks effect: ####p≤0.0001. Diet effect: ****p≤0.0001, *p≤0.05. Stress effect: §p≤0.05. Bonferroni post hoc: weight gain: ****p≤0.0001 HFD/Control vs. CHD dams, ***p≤0.001 HFD/Control vs. CHD/CVS, **p≤0.01 HFD/Control vs. CHD/Control and HFD/CVS vs. CHD dams, *p≤ 0.05 HFD/CVS vs. CHD/CVS.

Table 3.2 Maternal care behaviour recorded during the first 6 postnatal days.

Behaviour	Time	Mean behaviour % ± SEM				P-value
		Condition				
		CHD/Control	CHD/CVS	HFD/Control	HFD/CVS	
BD licking	**Total**	1.8±0.3	1.1±0.3	1.4±0.3	1.9±0.3	# , t
	Day	1.4±0.3	1±0.3	1.3±0.3	1.5±0.3	NS
	Night	2.3±0.3	1.3±0.3	1.4±0.3	2.3±0.3	tt
AG licking	**Total**	4.8±0.5	3.5±0.6	4.6±0.6	5.0±0.6	NS
	Day	4.5±0.5	3.7±0.6	4.5±0.6	4.1±0.6	NS
	Night	5.1±0.7	3.2±0.7	4.7±0.8	5.9±0.8	t
AB nursing	**Total**	6.4±0.9	5.9±1	5.4±1	4.8±1.1	NS
	Day	6.3±1.1	7.2±1.2	5.6±1.2	4.0±1.2	NS
	Night	6.6±1	4.7±1.1	5.3±1.1	5.6±1.1	NS
Supine nursing	**Total**	7.0±1.2	3.0±1.3	3.9±1.3	2.5±1.4	## , §§
	Day	5.7±1	3.8±1.1	1.3±1.1	1.9±1.2	**
	Night	8.7±1.8	2.5±2	6.2±2.1	3.0±2.1	§
Blanket nursing	**Total**	50.8±2.4	51.1±2.6	55.0±2.6	53.7±2.8	####
	Day	49.2±2.8	48.3±3.1	47.4±3.1	51.3±3.3	####
	Night	51.5±2.9	54.5±3.2	64.4±3.4	56.8±3.4	#### , *
Nesting	**Total**	1.8±0.3	1.6±0.3	2.3±0.3	2.1±0.3	#### , *
	Day	1.7±0.3	1.5±0.4	2.5±0.4	1.7±0.4	####
	Night	1.8±0.4	1.8±0.4	2.1±0.4	2.4±0.4	####
On nest	**Total**	68.7±2.2	65.7±2.5	70.5±2.5	65.5±2.6	####
	Day	66.1±3	64.0±3.3	60.9±3.3	62.5±3.5	####
	Night	70.9±2.6	66.5±2.9	81.3±3	69.4±3	#### , *, §§

Hover	**Total**	4.4±0.5	4.5±0.6	6.2±0.6	4.6±0.6	####
	Day	4.8±0.7	4.5±0.8	6.7±0.8	4.7±0.8	####
	Night	4.0±0.6	4.6±0.7	5.3±0.7	4.5±0.7	##
Self-feeding	**Total**	11.9±0.7	13.8±0.8	6.1±0.8	5.9±0.8	#### , ****
	Day	12.3±1.1	14.6±1.2	7.9±1.2	7.8±1.3	#### , ****
	Night	11.5±0.9	13.2±1	4.4±1	3.9±1	### , ****
Self-grooming	**Total**	6.3±0.7	5.9±0.8	12.2±0.8	10.6±0.8	****
	Day	7.2±1	7.1±1.1	15.1±1.1	12.2±1.2	****
	Night	5.5±0.6	5.0±0.7	8.2±0.7	9.2±07	****

PND effect: ####p≤0.0001, ###p≤0.001, ##p≤0.01, #p≤0.05. Diet effect: ****p≤0.0001, **p≤0.01, *p≤0.05. CVS effect: §§p≤0.01, §p≤0.05. Diet x CVS interaction ttp≤0.01, tp≤0.05.

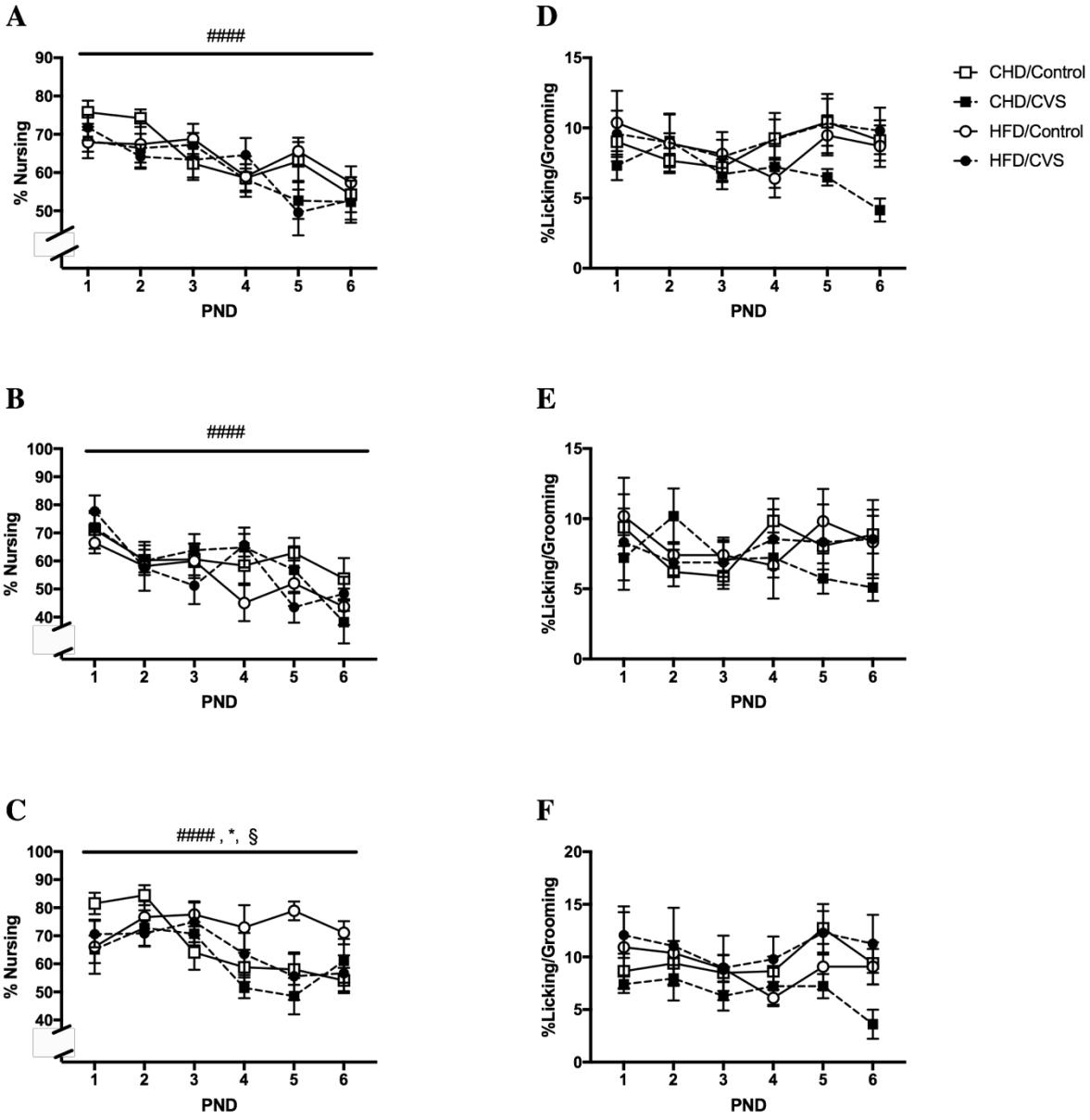

Figure 3.2 Maternal %nursing and %licking/grooming during the first 6 postnatal days. No differences were observed in %nursing during overall recorded (A) or during the light period (B). (C) HFD dams nursed their pups more during the dark periods than CHD, while CVS dams nursed their pups less than control dams. No differences were observed in %licking/grooming during overall recorded times (D), day period (E), or dark period (F). CHD/Control n=11, CHD/CVS n=9, HFD/Control n=8, HFD/CVS n=9. Data presented are means ± standard error. Postnatal day effect: ####$p \leq 0.0001$. Diet effect *$p \leq 0.05$. Stress effect §$p \leq 0.05$.

Overall at PND13, a main effect of time was observed ($F_{(2.51,29)}$= 5.70, p=0.006). HFD pups had higher ACTH levels in response to MB (Diet effect: $F_{(1,30.27)}$= 7.27, p=0.01; Figure 3.3E), a difference most apparent an hour after the termination of the stress between HFD/Control and CHD/Control (Bonferroni post-hoc p=0.01) and CHD/CVS (Bonferroni post-hoc p=0.02). Cort levels changed with time in response to MB (Time effect: $F_{(2,159.67)}$= 14.96, p≤0.0001; Figure 3.3F). Only CHD/Control (Bonferroni post-hoc p=0.03) and HFD/CVS (Bonferroni post-hoc p≤0.0001) had significantly higher Cot levels an hour (70 min) after the end of the stress compared to baseline (0 min). In addition, Cort levels were affected by diet ($F_{(1,32.34)}$= 4.03, p=0.05) and interaction between diet and CVS ($F_{(1,32.27)}$= 5.75, p=0.02). HFD/CVS offspring showed a pronounced Cort response to MB. After 10 min of MB exposure, HFD/CVS showed significantly higher Cort levels compared to HFD/Control (Bonferroni post-hoc p=0.05) and CHD/CVS (Bonferroni post-hoc p=0.01). An hour after termination of stress, HFD/CVS had significantly higher Cort levels compare to CHD/Control (Bonferroni post-hoc p=0.02), CHD/CVS (Bonferroni post-hoc p=0.001), and HFD/Control (Bonferroni post-hoc p=0.002).

3.3.5 3.3.4. Pup ultrasonic vocalizations and behaviour

At PND 7, there was a main effect of isolation environment on the USVs of the pups ($F_{(1,116)}$= 31.98, p≤0.0001), as pups isolated in the MB box vocalized less compared to CB isolation box; more specifically in the CHD/Control (Bonferroni post-hoc p=0.0015) and CHD/CVS (Bonferroni post-hoc p≤0.0001) groups (Figure 3.4A). In addition, there was a diet effect ($F_{(1,116)}$=11.26, p=0.001) with HFD pups vocalizing less overall compared to CHD. An isolation environment by diet interaction effect ($F_{(1,116)}$= 12.86, p≤0.0001) was observed with a main diet effect at the CB isolation environment and not the MB environment ($F_{(1,58)}$= 17.97, p≤0.0001), where HFD pups vocalized less compared to CHD pups. Time spent immobile showed an isolation environment by diet interaction ($F_{(1, 128)}$= 5.40, p=0.02). The diet effect was evident in the MB isolation environment ($F_{(1,64)}$=4.12, p=0.05), where HFD pups spent less time immobile compared to CHD pups (Figure 3.4B).

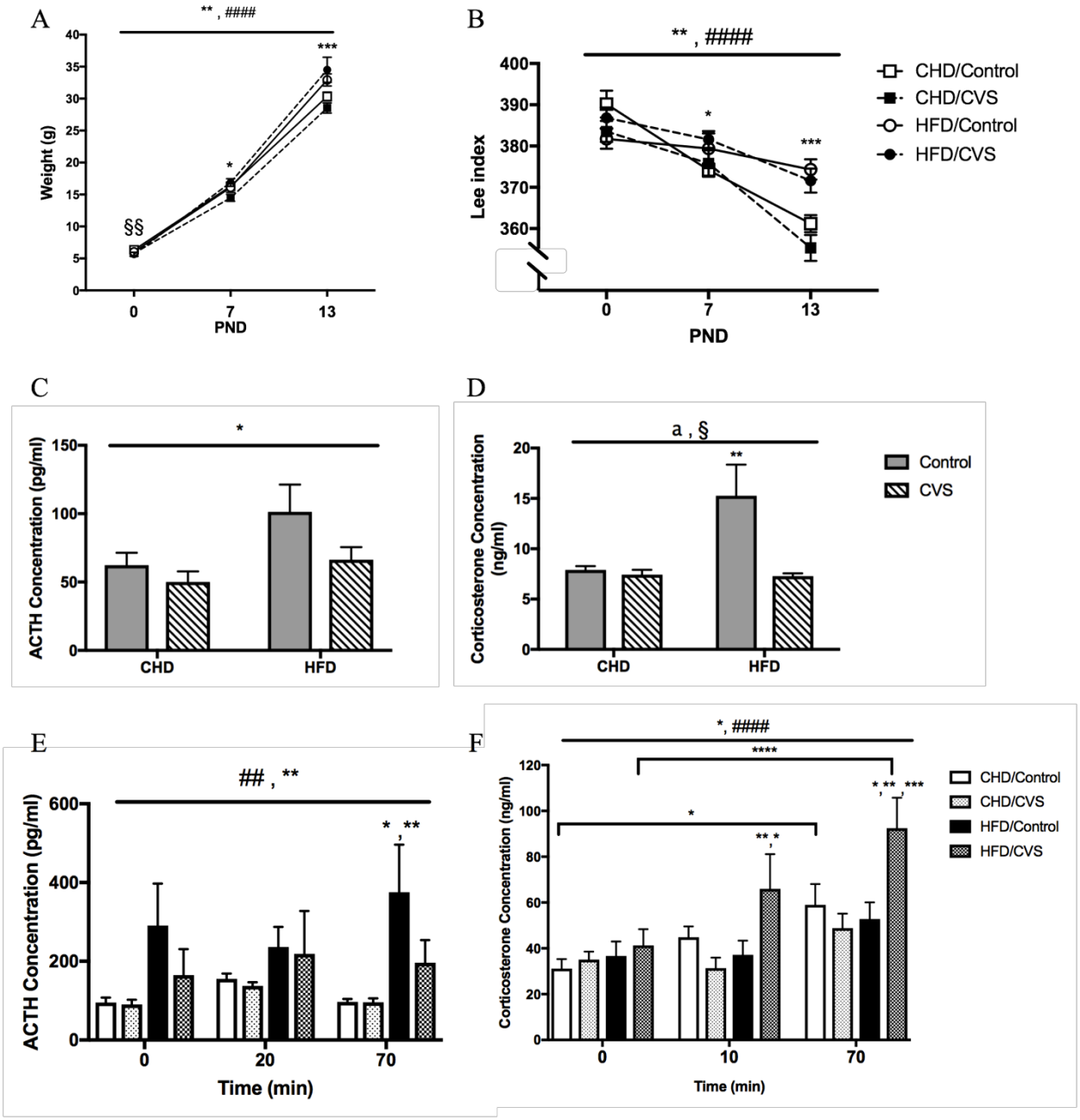

Figure 3.3 Neonatal physiology. Offspring weights: (A) CVS pups were lighter than control pups at birth. HFD pups were heavier than CHD by postnatal day (PND) 13. (B) HFD had lower Lee index at birth but higher as they grew than CHD pups. CHD/Control n=11, CHD/CVS n=9, HFD/Control n=9, HFD/CVS n=8. Offspring stress physiology in response to 10 min soiled adult male bedding (MB) exposure: (C) on PND7, HFD offspring had higher adrenocorticotropic hormone (ACTH) response to MB than CHD offspring. (D) Only HFD/Control pups Cort levels

were higher in response to MB compared to all pups at PND7. ACTH: CHD/Control n=6, CHD/CVS n= 6, HFD/Control n=7, HFD/CVS n=4, Cort: CHD/Control n=17, CHD/CVS n= 15, HFD/Control n=16, HFD/CVS n=12. (E) On PND13, HFD pups had an exaggerated ACTH response to MB, which induced ACTH release in all pups. (F) HFD/CVS offspring showed a pronounced increase in Cort levels in response to MB compared to all pups at the 70 min time point and compared to 0 min time point. CHD/Control pups also showed a Cort response to MB, where Cort levels were higher at 70 min compared to 0 min. 0= baseline, 10= 10 min after MB exposure, 70= 60 min after the termination of MB stress. ACTH: CHD/Control n=9-10, CHD/CVS n= 8-7, HFD/Control n=5-8, HFD/CVS n= 8-6. Cort: CHD/Control n=15-20, CHD/CVS n= 16-14, HFD/Control n=17-18, HFD/CVS n= 15. Data presented are means ± standard error. PND effect: ####$p \leq 0.0001$. Diet effect ***$p \leq 0.001$, **$p \leq 0.01$, * $p \leq 0.05$, a$p=0.07$. CVS effect: §$p \leq 0.05$. Time effect ####$p \leq 0.0001$, ##$p \leq 0.01$. PND7 stress physiology: Bonferroni post hoc **$p \leq 0.01$ HFD/Control vs. all. PND13 stress physiology: ACTH: Bonferroni post hoc **$p \leq 0.001$ ACTH: HFD/Control-70 vs. CHD/Control-70, *$p \leq 0.05$ HFD/Control-70 vs. CHD/CVS-70; Cort: ***$p \leq 0.001$ HFD/CVS-70 vs. CHD/CVS-70, **$p \leq 0.01$ HFD/CVS-10 vs. CHD/CVS and HFD/CVS-70 vs. HFD/Control, *$p \leq 0.05$ HFD/CVS-10 vs. HFD/Control-10 and HFD/CVS-70 vs. CHD/Control-70.

At PND 13, there was a main effect of isolation environment on the USVs of the pups ($F_{(1,132)}$=8.18, p=0.008), where pups isolated in the MB box vocalized less compared to pups isolated in the CB isolation box (Figure 3.4C). An overall effect of diet was observed in the time spent immobile ($F_{(1,131)}$= 6.00, p=0.02), where HFD offspring spent more time immobile during the test (Figure 3.4D).

3.3.6 Pup neural gene expression

Due to the strong main effects of the diet on behaviour and physiology, I focused the molecular work on examining the gene expression in the HFD/Control offspring and CHD/Control. On PND7, HFD pups had higher Crh transcript abundance in the PVN compared to CHD pups ($t_{(2, 21)}$=2.16, p=0.042; Figure 3.5A). Nr3c1 transcript levels tended to be lower on PND7 in HFD pups in the PVN ($t_{(2, 21)}$= 1.79, p=0.087; Figure 3.5B), while Nr3c2 transcript abundance was similar between diet groups (Figure 3.5C). In addition, there were no significant differences in Gad1, Nr3c1, or Nr3c2 transcript abundance between diet groups in the ventral hippocampus (Figure 3.5D-F). On PND13, there were no observable differences in Crh, Nr3c1, Nr3c2 transcript levels between diet groups in the PVN (Figure 3.6A-C). In the ventral hippocampus, HFD pups had higher Gad1 ($t_{(2, 19)}$= 2.32, p=0.03; Figure 3.6D) and lower Nr3c1 ($t_{(2, 18)}$= 3.56, p=0.002; Figure 3.6E) and transcript abundance, while Nr3c2 transcript abundance was similar among pups (Figure 3.6F).

3.4 Discussion

In this chapter I examined the effects of perinatal HFD and prenatal CVS on HPA axis development in neonatal rat offspring. HFD alone induced an early activation of the HPA axis at PND7 altering USVs and Crh transcript levels in the PVN. Prenatal CVS seemed to normalize the effect of the HFD on HPA axis activation. HFD pups independent of CVS exposure showed an augmented HPA axis activation at PND13, shown by a greater ACTH and Cort response, and impaired HPA negative feedback, suggested by lower Nr3c1 transcript levels in the ventral hippocampus in HFD/Control offspring. HFD pups showed increased immobility, indicating increased anxiety-like behaviour. These findings suggest that perinatal HFD interacts with prenatal stress to alter the typical developmental trajectory of the HPA axis, increasing stress-related behavioural responses in early life.

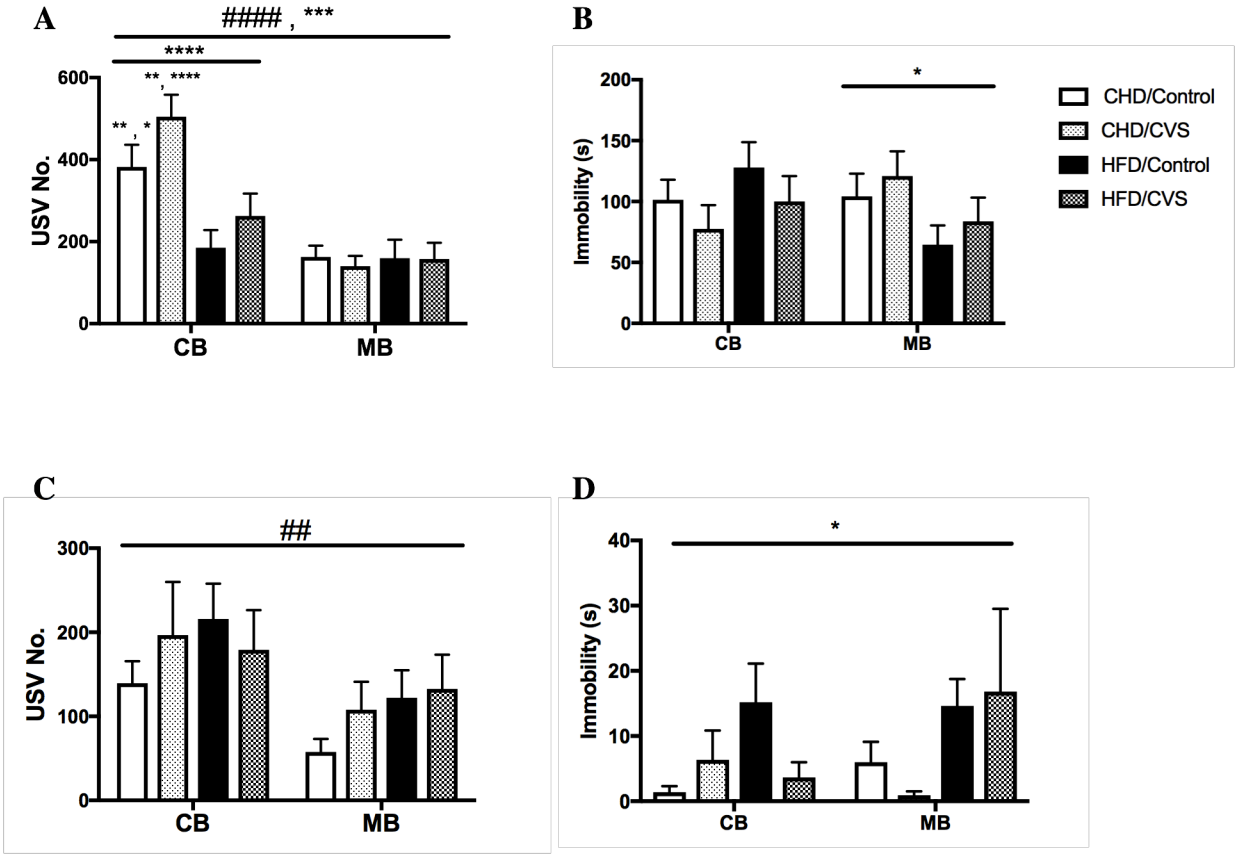

Figure 3.4 Offspring ultrasonic vocalization (USV) counts and immobility during the first 5 min of isolation test with clean (CB) or MB. (A) On PND7, HFD pups vocalized less than CHD pups. When exposed to MB, CHD specifically, vocalized less. (B) HFD pups spent less time immobile when exposed to MB compared to CHD pups. (C) On PND13, all pup vocalized less when exposed to MB compared to CB. (D) HFD pups spent more time immobile compared to CHD pups. CHD/Control n=19-20/treatment, CHD/CVS n= 13-16/ treatment, HFD n=17-18/treatment, HFD/CVS n= 16-16/treatment. Data presented are means ± standard error. MB effect: ####p≤0.0001, ##p≤0.01. Diet effect: ****p≤0.0001, ***p≤0.001, *p≤0.05. Bonferroni post hoc ****p≤0.0001 CB-CHD/CVS vs. all MB groups, CB-CHD/Control, and CB-HFD/Control, **p≤0.01 CB-CHD/CVS vs. CB-HFD/CVS, CB-CHD/Control vs. all MB groups, *p≤0.05 CB-CHD/Control vs. CB-HFD/Control.

3.4.1 HFD and stress effects on maternal physiology and maternal behaviour

As expected, HFD dams in this chapter gained more weight and consumed more calories during the 3-week period prior to gestation and throughout gestation. During the first two postnatal weeks, HFD dams had comparable body weight and caloric intake to CHD dams and consumed fewer calories during the first postnatal week. While CHD dams were gaining weight during this time, HFD dams were losing weight independent of their stress exposure, as previously reported (Rolls et al., 1984; Wright et al., 2011). HFD dams were also consuming less calories during lactation. Interestingly, HFD dams were self-feeding less often during the period of maternal behaviour recording as well. Typically, during late gestation and lactation dams become hyperphagic and accumulate fat to meet the metabolic demands of nursing, a phenomenon mediated in part by prolactin (Woodside, 2007). Recently, a study in mice has reported reduced responsiveness to prolactin signalling in the hypothalamus of HFD dams during lactation (Buonfiglio et al., 2016), which may explain the weight and caloric intake alteration observed in this study.

Overall, combined results from the light and dark periods of maternal recordings showed no HFD effects on measures of maternal care. However, when examined separately, we found that HFD dams spent more time nursing during the dark period, while CVS dams spent less time nursing their pups. These effects seem to be driven mainly by the difference in HFD/Control dams, which spent more time nursing, while HFD/CVS showed comparable results to the CHD dams. Earlier study examining maternal HFD and prenatal CVS reported similar findings (Purcell et al., 2011). However, the increase in nursing was observed in all HFD dams including the ones exposed to the CVS in that study, which is in contrast to what I find in my study. It is worth mentioning that Purcell and colleagues used a different feeding paradigm than the one I used in this study, which exposed dams to the HFD only during the gestation and lactation period and their HFD animals lacked the higher body weight phenotype that is typical of a chronic HFD feeding that I implemented in this study. The differences in the feeding protocols used in the maternal HFD model is one of the sources of discrepancy seen across studies as discussed in chapter one. In this cohort of animals, I was not able replicate some finding from the first cohort,

including increased overall nursing and increased licking in HFD dams. However, given the loss of some maternal behaviour data in Chapter 2 due to technical issues, the finding presented in this chapter are more accurate since it is encompasses a full set of data. The increase in nursing in HFD dams has been associated with increased ingestive behaviour of high caloric milk in HFD pups contributing the metabolic phenotype of these offspring (Purcell et al., 2011).

3.4.2 HFD effects during the HPA hyporesponsive period

The following will discuss the effects of the perinatal HFD alone on neonatal offspring phenotype due to the novelty of the findings and later in a separate section I will discuss the findings of the interaction between the diet and CVS.

The stress hyporesponsive HPA axis may constitute an adaptive mechanism to protect the developing brain from the catabolic actions of Cort (Vázquez and Akil, 1993). Only limited factors have been reported to activate the HPA axis during this period, including maternal deprivation, ether exposure, and endotoxin injection (Shanks and Meaney, 1994; Suchecki et al., 1995; Walker et al., 1991). In this investigation, I report that maternal perinatal HFD alone also leads to early activation of the HPA axis at PND7, when neonates are hyporesponsive to stress. HFD/Control pups showed higher Cort levels after exposure to adult male bedding stress. Although, different maternal prenatal or early postnatal psychosocial stressors are associated with increased Cort after stress during the hyporesponsive period (Avishai-Eliner et al., 2001; Henry et al., 1994; Moriceau et al., 2009), prenatal CVS here did not affect the stress response during this period.

HPA axis activation in HFD pups was associated with a lower USV production in the clean bedding condition. Earlier work showed that Cort injection leads to the suppression of USVs in neonates (Takahashi, 1994). These previous results are consistent with our findings of increased Cort levels and supressed UVSs after isolation among HFD pups. However, CHD pups also supressed their USVs in the presence of adult male bedding in the absence of a Cort response, suggesting that Cort is not the only factor modulating USV production. A recent study reported that PND7 pups avoid adult male odor in a Y-maze, which was associated with olfactory bulb activation (Perry et al., 2016). Neonatal rats have lower odor acuity, which increases with age, indicated by their respiratory response to different dilutions of odorants including adult urine (Alberts and May, 1980). In our study the pups were in closer proximity to the odor compared to

other studies (Takahashi, 1992; Takahashi, 1994). It is possible that USV suppression in neonatal pups may also be modulated by distance from the source of the odorant.

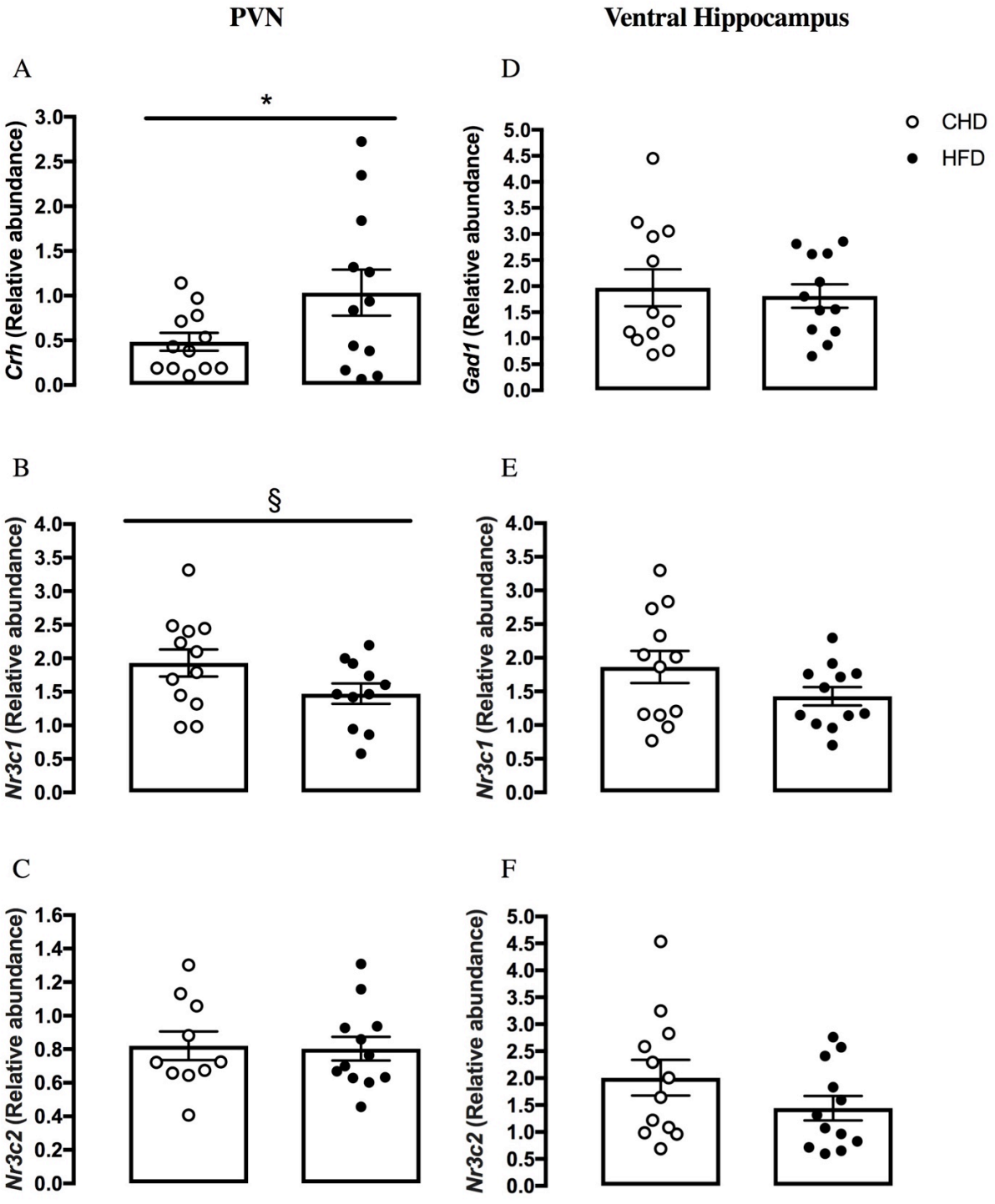

Figure 3.5 Relative transcript abundance in PND7 pups' hypothalamic paraventricular nucleus (PVN) and ventral hippocampus. In the PVN, HFD pups had higher Crh relative transcript abundance (A) and tended to have lower Nr3c1 abundance (B) compared to CHD. No changes were found in Nr3c2 relative transcript abundance (C). In the ventral hippocampus, comparable relative transcript abundance was observed across the diets (D-F). HFD n=11-12, CHD n= 10-12. Data presented are means ± standard error. *p≤0.05, §p=0.087.

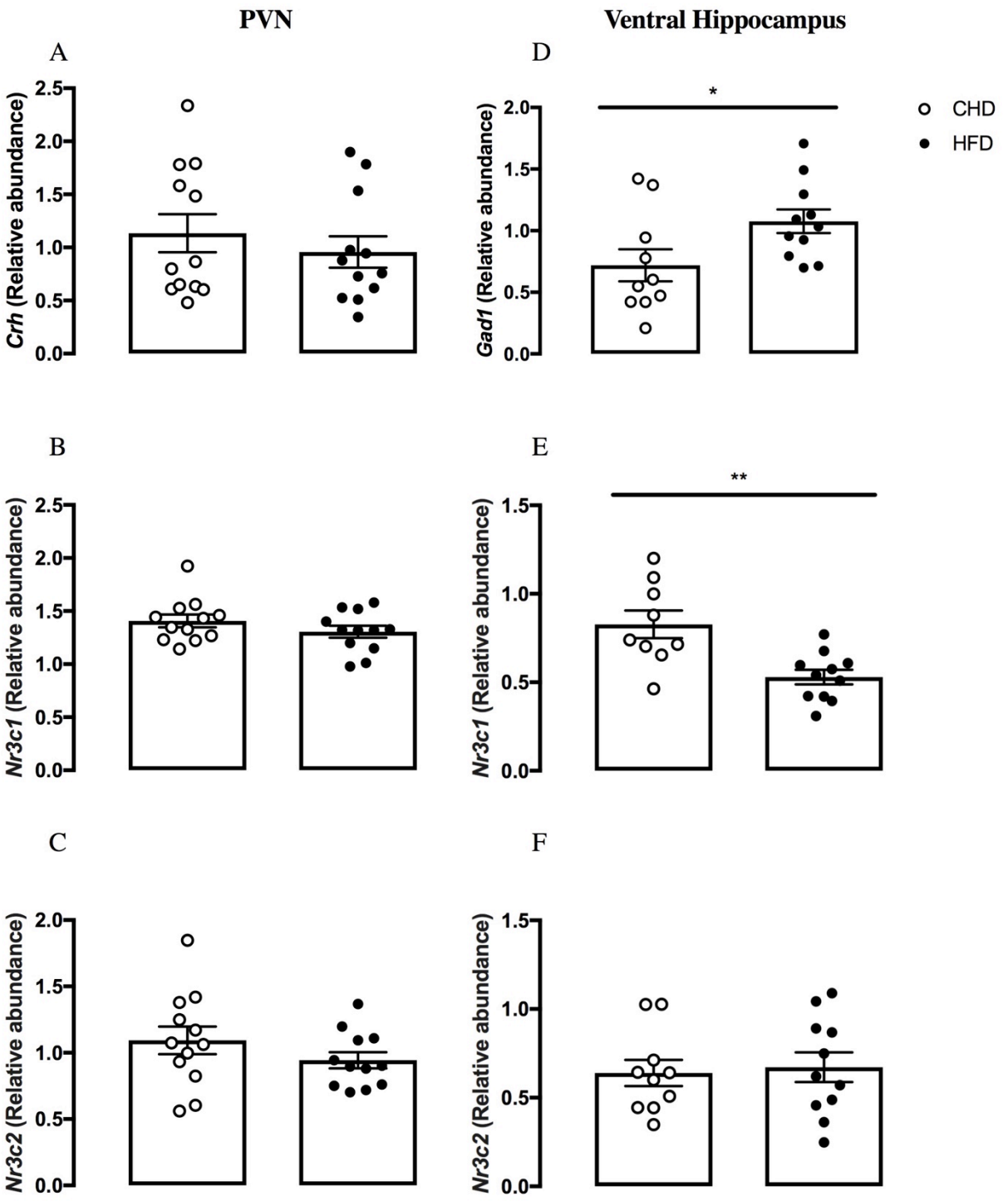

Figure 3.6 Relative transcript abundance in PND13 pups' PVN and ventral hippocampus. In the PVN, comparable relative transcript abundance was observed across the diets (A-C). In the ventral hippocampus, HFD pups had higher Gad1 relative transcript abundance (D) and lower Nr3c1 (F) , while no changes were found in Nr3c2 relative transcript abundance (F). HFD n= 11-12, CHD n=9-12. Data presented are means ± standard error. **p≤0.01 *p≤0.05.

A number of studies have demonstrated an increase of CRH in the PVN in response to stress during early postnatal life (Schmidt, 2010). CRH is known to modulate USV production in an inverted-U-shaped fashion, where a modest increase in endogenous levels of CRH stimulates USV production, while a further increase suppress USVs (Insel and Harbaugh, 1989). HFD pups had higher basal Crh levels compared to CHD pups, which might be associated with the lower USVs. HFD pups also tended to have lower Nr3c1 expression, which has been reported to negatively regulate Crh expression (Malkoski and Dorin, 1999).

A possible explanation for increased basal Crh levels is neuroinflammation. One of the few activators of the HPA axis during the hyporesponsive period is interleukin 1 beta (IL-1B), a cytokine stimulated by endotoxins such as lipopolysaccharide (LPS). IL-1B mediates activation of the HPA axis via CRH, as injection of CRH antagonist blocks the increase in ACTH and Cort (Shanks and Meaney, 1994). Interestingly, an early study examining the neuroinflammatory effects of perinatal HFD reported an increase in basal and LPS induced IL-1B protein levels in the hippocampus of PND20 rat pups (Bilbo and Tsang, 2010). Further, PND1 pups had an increase in inflammatory markers in the hippocampus (Bilbo and Tsang, 2010). Additional work is needed to determine whether neuroinflammatory induction in neonatal HFD offspring primes early activation of the developing HPA axis, possibly via IL-1B actions on CRH.

3.4.3 HFD effects during emergence from the HPA hyporesponsive period

I observed a stress response after male bedding exposure in both CHD and HFD pups at PND13. CHD pups showed both an ACTH and a Cort response to male bedding exposure, however HFD pups only showed an ACTH response without a change in Cort levels. In CHD pups ACTH levels increased after 10 min and decreased after an hour of the stress termination. However, HFD pups showed a continuous increase in ACTH levels even an hour after the end of exposure to the stressor. The lack of inhibition of ACTH in HFD pups could be linked to the lack of Cort response in these animals, possibly due to reduced adrenal sensitivity to ACTH signaling or impaired steroid synthesis. Additional experiments are needed to examine these possibilities.

Male bedding suppressed USVs in both diet groups but did not affect time spent immobile. HFD pups exhibited a non-significant increase in USVs and a significant increase in immobility in both isolation conditions. Selective breeding of rats with high numbers of USVs at PND10 yielded adult rats with increased anxiety-like behaviour, suggesting that high levels of USVs are an index of anxiety (Zimmerberg et al., 2005). By PND10, the production of USVs is no longer influenced by thermal factors, and individual pups maintain their characteristic USV response through PND14 (Brunelli et al., 1997). These findings indicate that HFD pups exhibit increased immobility and decreased exploration when they emerge from the stress hyporesponsive period are thus consistent with the interpretation that the increase in USVs indicates anxiety-like behaviour.

The augmented ACTH levels and anxiety-like behaviours exhibited by HFD pups suggest an alteration in the negative feedback of the HPA axis. Nr3c1 transcript relative abundance facilitates the HPA negative feedback via the PVN and ventral hippocampus (Herman et al., 2016). HFD pups showed lower Nr3c1 transcript abundance in the ventral hippocampus. The hippocampus indirectly inhibits the HPA axis by sending excitatory glutamatergic neurons to inhibitory regions surrounding the PVN (Gunn et al., 2015). The hippocampus is highly populated with GABAergic neurons that regulate the excitatory output of the hippocampus (Gunn et al., 2015). I examined the relative abundance of glutamic acid decarboxylase 1 (Gad1) transcript, a rate limiting factor for GABA synthesis and a marker of GABAergic neurons. HFD pups showed an increase in Gad1 transcript abundance in the ventral hippocampus, which may indicate an increase in the inhibitory regulation of the hippocampus, leading to the inhibition of the excitatory output that control the HPA axis negative feedback (Dent et al., 2007). Similarly, restraint stress in HPA responsive neonates (PND18) was shown to increase levels of Gad1 transcript in the hippocampus (Dent et al., 2007). Thus, HFD may be acting as a stressor in neonates leading to the upregulation of Gad1.

The differential sensitivity between the PVN and hippocampus could be due to distinct temporal dynamics of stress-responsive genes in each brain region during development. Nr3c1 expression in the hippocampus, which is low at birth, increases to adult-like levels by PND9 (Meaney et al., 1985). In contrast, Crh levels in the PVN decrease after PND6 (Dent et al., 2000). Restraint stress induces a robust increase of Crh mRNA in the PVN of hyporesponsive pups (PND6) and not beyond that (PND18) (Dent et al., 2000). In the hippocampus, lower levels of Nr3c1

expression were observed at PND9 subjected to maternal deprivation (Avishai-Eliner et al., 1999). Prenatal stress decreases Nr3c1 binding in the hippocampus by PND21 but not at PND3 (Henry et al., 1994). Crh in the PVN was responsive to augmented maternal care at an earlier age (PND9) compared to hippocampal Nr3c1, which showed a much later response (PND45) (Avishai-Eliner et al., 2001). In this chapter, differential expression of Crh in the PVN is responsive to prenatal and postnatal stress at PND7, while Nr3c1 expression differences start to emerge at PND13, suggesting the distinct developmental patterns of these genes may contribute to the differential responses to perinatal HFD.

3.4.4 HFD and CVS interaction effects on HPA axis development

In this chapter I did not observe any CVS effects on litter sizes or pup mortality rates. I believe that this was linked to replacing the platform stress with the elevated plus maze. Dams in the first cohort tended to fall off the platform, which might have been associated with the effects observed in the litter characteristics. Another differences observed in this cohort relative to the first was the weights of the pups. At birth, CVS offspring weighed less than control offspring, a finding that has been reported previously in a similar experimental design (Tamashiro et al., 2009). HFD pups started to show differences in weight by PND7 and later were significantly heavier than CHD offspring at PND13, which is more in line with earlier reports (Bilbo and Tsang, 2010; Sasaki et al., 2013) and the lack of that difference was puzzling in the first cohort.

Earlier work has shown that prenatal restraint stress activates the HPA axis during the hyporesponsive periods (PND7) (Henry et al., 1994). In addition, others reported that prenatal shock stress augmented the stress response in PND14 offspring, which was associated with lower USV counts (Takahashi et al., 1990). Here, prenatal CVS alone did not have any major effects on the development of the HPA axis or related behaviours. However, CVS exposure on the HFD background had PND specific effects on HPA axis physiology and behaviour. During the hyporesponsive period, prenatal CVS normalized the effects of the perinatal HFD effects alone. For example, HFD/CVS pups had comparable ACTH and Cort levels to the CHD pups. In addition, their USV numbers were not significantly different from CHD/Control pups despite the overall main effect of the HFD, which was driven mainly by HFD/Control and was significantly different from both CHD groups. CVS significantly affected HFD maternal weight gain and food intake. It also normalized the effects of perinatal HFD on time spent nursing. The normalization

of the dam's phenotype could be associated to the phenotype of the pups at least during the first postnatal week. At PND13, HFD/CVS showed a heightened HPA axis to stress shown by the significantly higher Cort levels compared to all other groups. This suggests that prenatal CVS against a perinatal HFD resulted in a synergetic effect on HPA axis response to stress in neonates with a functional HPA axis emerging from the hyporesponsive period. Future examination of gene expression in the brain would shed light on where are the programming effects are taking place in these animals.

3.4.5 Conclusion

In this chapter, perinatal HFD appeared to alter the trajectory of HPA axis development in neonates. Neonates from dams consuming HFD showed evidence of early activation of the HPA axis during the stress hyporesponsive period, with increase in Cort levels, decrease in USVs, and increased Crh transcript abundance in the PVN. In offspring emerging from the stress hyporesponsive period, neonates from HFD dams exhibited aberrant HPA axis function, showing increased ACTH levels and impaired negative feedback, increased anxiety-like behaviours, and reduced Nr3c1 transcript abundance in the ventral hippocampus. CVS alone did not affect the measured outcomes in the offspring, however CVS interacted with perinatal HFD to induce a heightened stress response in offspring emerging from the hyporesponsive period. Overall, these findings indicate that the impacts of perinatal HFD manifest early in postnatal development, potentially setting the stage for life-long impacts on stress-related phenotype. Understanding these impacts during this labile period of development will aid in developing methods to prevent or overcome its negative impacts.

Chapter 4

Perinatal HFD alters ultrasonic vocalization characteristics in rat pups

4 Perinatal HFD alters ultrasonic vocalization characteristics in rat pups

4.1 Introduction:

Mother-infant interaction is one of the first social experiences that animals encounter. Rats give birth to altricial offspring that depend greatly on their mothers for survival. Upon separation form their mothers and litter mates, pups start emitting distress calls at mean frequency of 40kHz (Hofer et al., 2002). A number of hypotheses were proposed to explain the functional significance of these USVs. One hypothesis suggested that USV production is purely physiological, where they are just a by-product of abdominal compression the pups engage in to increase blood flow when they are thermally challenged by low temperature or rough handling (Hofer, 1996). Others have argued that USVs are ethologically relevant and mediate the survival of the helpless neonates through the elicitation of maternal search, pup retrieval, and nest building behaviours in the dams (Brouette-Lahlou et al., 1992; Brunelli et al., 1994; D'Amato et al., 2005). It is believed that these elicited maternal behaviours could be associated with the induction of prolactin in dams in response to pup USVs (Hashimoto et al., 2001). Additionally, brief maternal reunion after social isolation was shown to potentiate USVs in pups (Hofer, 1996).

There is evidence suggesting that these distress calls are a reflection of a negative affective state of the pups. For example, it has been shown that anxiolytic and anxiogenic drugs attenuate and enhance USV counts, respectively (Insel and Harbaugh, 1989). Further, rats bred for high USVs during infancy (PND10) show increased anxiety-like behaviours in adulthood compared to ones bred for low USVs (Zimmerberg et al., 2005). Interestingly, two independent studies have explored the relationship between natural variation in maternal behaviour and USVs as an index of anxiety-like behaviour in infant rats (Brunelli et al., 2015; Wohr and Schwarting, 2008). One study reported that pups that had high licking and grooming dams emitted fewer USVs, while pups from low licking and grooming dams emitted more USVs (Wohr and Schwarting, 2008). Similarly, when examining maternal behaviour in animals bred for high- and low-USVs lines, dams from the low-USV line engaged in more high-arch nursing and licking and grooming than the high-USV line (Brunelli et al., 2015). These findings indicate that increased maternal care

behaviour, manifested in high licking and grooming and arched back nursing, is associated with lower anxiety-like behaviour in neonates, exhibited by lower numbers of USVs emitted by pups.

Other evidence of the ethological relevance of USVs is their suppression upon rat pup encounters with a predator, such as an unrelated adult male rat or its cues (Takahashi, 1992). It was proposed that the cessation of USVs is to reduce the rate of detection by the predator and increase survival (Takahashi, 1992). This modulation of USVs by predator presence is suggested to be mediated in part by the HPA axis (Takahashi, 1994; Takahashi and Kim, 1995). For example, adrenalectomized pups do not exhibit suppression of USVs when encountering an adult male rat, suggesting that Cort secreted from the adrenals might be a factor mediating USV suppression (Takahashi and Kim, 1995). In fact, adrenalectomized pups do not show USV suppression when confronted with anesthetized adult male rat, however, when these pups are injected with Cort, USV suppression is reconstituted in the presence of the predator (Takahashi, 1994). Moreover, USV numbers are modulated by CRH, the central mediator of the HPA axis, in an inverted U shape response, where intermediate levels of CRH induce USVs and high levels suppress them (Insel and Harbaugh, 1989).

Different aspects of USVs has been shown to exhibit a developmental profile. In rats, USV numbers are low right after birth and reach a peak by PND7 then they start decreasing again to disappear by weaning (Brudzynski et al., 1999). Other standard acoustic parameters such as call durations, peak frequencies, and peak amplitude are also examined in the literature and were shown to generally increase with age (Tonkiss et al., 2003). More recently, qualitative dimensions of USVs have been documented to also follow a developmental pattern with a proposed neurological function. For example, earlier work has demonstrated that calls with two frequency sweeps, which is defined as a rapid change of sound frequency in a single call, increase with age in pups (Brudzynski et al., 1999). These call types described as sharing features of a siren, as they are characterized by quicker and more evident frequency changes. They have been postulated to serve a significant function in capturing the attention of the dam and eliciting maternal care, however this hypothesis has not been tested (Brudzynski, 2005). Moreover, call types featuring abrupt discontinuous frequency changes, frequency shifts, are a feature of in human infants and rat pups prenatally exposed to cocaine (Zeskind et al., 2011).

Several studies have utilized infant rat USVs as a phenotypic outcome of neurodevelopment in response to different prenatal insults. For example, pups exposed to cocaine prenatally showed a decrease in USV call numbers on PND1 and PND5, while exhibiting a higher percentage of USVs containing frequency shifts (Zeskind et al., 2014). Lower USV counts across postnatal development were also observed in pups from mothers restricted from rapid eye movement sleep during gestation along with significant variation in the distribution of call type compared to the control animals, which could be explained by a shift in the developmental pattern of the different call types between the treatment groups (Gulia et al., 2014). Maternal hypothyroidism during gestation was associated with an increase in the number of USVs during the second postnatal week (PND10 and 15), with a decrease in the percentage of calls with frequency modulation during the first postnatal week (PND5) (Wada, 2017). Although maternal malnutrition during gestation did not affect the numbers of USVs, it reduced the range of call type emitted by the pups during the early postnatal days (Tonkiss et al., 2003). Further, maternal malnutrition increased the peak frequency at PND7 and altered the percentage of different call types across the second postnatal week (Tonkiss et al., 2003). These studies have all proposed that alteration in quantitative and qualitative measures of pup USVs could be an indication of disrupted neurodevelopmental trajectory of the central nervous system.

As previously demonstrated in earlier chapters, perinatal HFD programs anxiety-like behaviour in adults (Chapter 2) and neonates (Chapter 3) (Abuaish et al., 2018; Sasaki et al., 2013). In addition, as shown in chapter 3 HFD was associated with early activation of the HPA axis in neonatal pups that are typically known to have a hyporesponsive HPA axis to stress, which might suggest alteration in the neurodevelopmental trajectory in offspring (Abuaish et al., 2018). I also reported alteration in maternal care behaviour in HFD dams in the above chapters. Given the suggested function of USV as an index of the affective state in neonates, reported effects of early life adversity on developmental profiles of USVs, and their responsiveness to alterations in maternal behaviour, the aim of this study was to characterize quantitative and qualitative features of USVs and their developmental patterns in response to perinatal HFD in both sexes and their relationship to maternal care. I hypothesize that perinatal HFD would alter the developmental pattern of USV quantitative acoustic measures and qualitative call types, which would be associated with impaired maternal pup retrieval.

4.2 Material and methods

4.2.1 Animals and diets

Adult female Long Evans rats (7 week) were purchased from Charles River and housed in same sex pairs until mating and maintained on a 12:12-h light– dark cycle (lights on 7:00 am–7:00 pm) with *ad libitum* access to food and water. Females were either maintained on chow diet (CHD; Purina Lab Diets (St. Louis, MO: cat. no. 5001) consisting of 28.5% protein, 13.5% fat, and 58% carbohydrate, or high fat diet (HFD; Research Diets, Inc. (New Brunswick, NJ: cat. no. D12492), consisting of (by kcal): 20% protein, 60% fat, 20% carbohydrate.) for 3 weeks prior to mating. Two different cohorts were used in this study; cohort one (CHD= 11 litters, HFD= 9 litters) generated offspring tested on PND7 and PND 13. Cohort two (CHD= 7 litters, HFD=7 litters) was generated to measure maternal retrieval.

Adult females were housed with males for a week and sperm plugs were checked twice a day (9am and 5pm) to determine the onset of pregnancy. All females were housed for a week with males regardless of when the plug was observed to increase the chances of pregnancy, as the presence of vaginal plugs does not always guarantee pregnancy. The presence of vaginal plugs was noted and this information was combined with the monitoring of maternal body weight to confirm pregnancy. After parturition, the dams were moved into clean cages with paper towel strips used as nesting material. Pups were weighed and culled to 12 pups/ litter (6 males and 6 females) when possible. Cages were moved into a separate room and kept there until the end of the experiment on PND13. Dams and pups were weighed on PND0, PND7 and PND13. Experimental protocols were approved by the Local Animal Care Committee at the University of Toronto, Scarborough, and were in accordance with the guidelines of the Canadian Council on Animal Care.

4.2.2 Maternal retrieval

On PND7, dams and their pups (8 pups/litter, 2 pup/sex were euthanized for measures in chapter 3), were transported into the testing room and were habituated to the room for 10 min, after which pups were removed into a new cage and were placed on a heating pad maintained at 34°C for 10 min. Pups were later returned to their home cage by placing 2 pups in each corner of the cage independent of the location of the nest, after which the dams were introduced to the cage

and their behaviour was recorded for 15 min. The following measures were coded using Observer XT 10.0 software: latency to start retrieval, latency to finish retrieval, and percentage of pups retrieved at the end of the test.

4.2.3 Pup testing procedure

Modified operant conditioning chambers inside sound attenuating boxes were set up with clean bedding in the collecting tray of the chambers. The electrical grid was lined with vinyl shelf liners to prevent the pups from sliding through the electrical grid. Condenser microphones Avisoft Bioacoustics CM16/CMPA were suspended 15cm above the center of the chambers. Cameras were mounted on the inside of the boxes to record pup behaviour during the test.
Pup testing was carried out on PND 7 and PND 13, similar to (Hofer et al., 2002). On both days, one pup/sex of similar weights/litter were transferred into a mouse cage with bedding from their home cage and then transferred to an adjacent testing room where they were placed onto a heating pad set at 34°C. The pups were allowed to habituate for 10 min prior to placing them in the isolation chambers. After habituation, pups were placed in the center of the isolation chambers at room temperature for 10 min, where their USVs and behaviour were recorded. The chambers were wiped clean with Virox between trials and dried with paper towels.

4.2.4 Pup ultrasonic vocalization recording and analysis

The condenser microphones Avisoft Bioacoustics CM16/CMPA are sensitive to frequencies between 10 kHz-200 kHz and were connected to Avisoft UltraSoundGate 416 USB Audio device, which was connected to a personal computer. Settings included sampling rate at 166.66 kHz, format 16 bit, and high-pass filter set at 20 kHz, detecting a range of 20-80 kHz. Acoustic analysis was performed using Avisoft SASLab Pro where a fast Fourier transform was done (512 FFT length, 100% frame, Hamming window and 50% time window overlap) with 20 ms hold time. Calls were also inspected manually to ensure that all USVs detected using the software's automatic processing algorithms were valid calls. All false signals were removed before statistical analysis was performed. Call numbers, mean call duration, and peak frequency were determined.

Earlier work classified the sonographic structures based on the number of frequency modulations the call has and termed them frequency sweeps, which is defined as a rapid change of sound

frequency in a single call and ranges in numbers within each call (Brudzynski et al., 1999). Another study identified calls termed frequency steps that had a sudden shift in frequency with no interruption in time (Zeskind et al., 2011). This type of calls has been proposed to be modeling human infants' hyperphonotation cries, which are believed to be evidence of neurobehavioural dysregulation in humans (Zeskind et al., 2014).

The first 100 calls during the 10-minute test were classified into categories (call type 1-12), similar to (Brudzynski et al., 1999; Zeskind et al., 2011) and based on visual inspection of their sonographic structures. In addition, we identified 7 new call types (call type 13-20) that did not fit the categories in the earlier studies. The total of 20 call types were grouped into 6 different call categories, based on the number of frequency sweeps, the presence of frequency shifts, and combination of sonographic structures (Figure 4.1). If pups failed to emit 100 calls within the recorded period, they were excluded from the sonographic structures analyses. A total of 11629 calls were classified, with a total of 11100 calls after exclusions. The sample sizes of animals tested as follows: PND7: CHD-males n=9, CHD-females n=8, HFD-males n=7, HFD-females n=8; PND13: CHD-males n=9, CHD-females n=8, HFD-males n=8, HFD-females n=7.

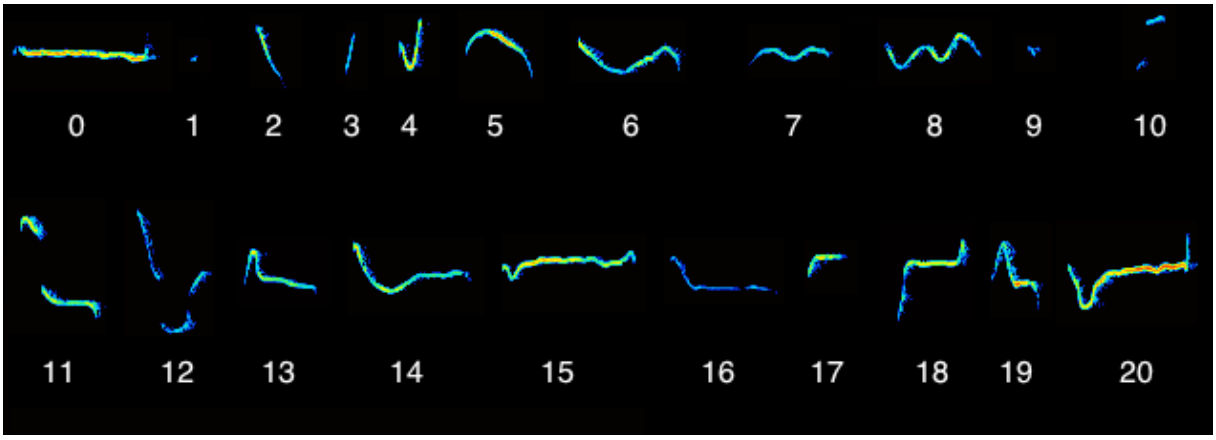

Figure 4.1 Representative sonograms of the different call types analyzed. Call types were grouped into 6 different call categories. Simple calls (0-1), 1-sweep calls (2-3), 2-sweep calls (4-5), ≥ 3-sweep calls (6-8), shift calls (10-12), and complex calls (9, 13-20).

4.2.5 Statistical analysis

Statistical analysis was carried out using SPSS (IBM). Shapiro-Wilk test was used to test for normality for data sets with $n < 30$. When data sets were not normality distributed, extreme outliers were examined using box plots and removed if they were 3X outside the interquartile range of the data (Ghasemi and Zahediasl, 2012). If the data set did not have outliers and was not normal, square root values were calculated to normalize the data. For data sets with $n > 30$ normality was assumed and subsequent analysis was carried out (Ghasemi and Zahediasl, 2012). Two tailed t-tests were used to analyze the diet effect on maternal retrieval measures and PND7 pup body weight and temperature. A Mann-Whitney non-parametric test was used to analyze the effects of diet on percent of pups retrieved. Chi-square analysis was used to measure the proportion of dams that retrieved all of its pups by the end of the test between the two diet groups. Factorial linear mixed models (LMM) (PND x diet x sex) were used to analyze data sets to control for dam ID as a random factor. Dam ID did not contribute to the variation in pup behaviour USVs acoustic measures during initial analysis using LMM, therefore a factorial (PND x diet x sex) general linear models (GLM) was used. Tukey's post hoc testing was used for pairwise comparisons between diet groups across diet groups and PNDs. Factorial multivariate analysis of variance (MANOVA) (PND x diet x sex) was used to analyze the percentage of USV call types followed by a factorial (PND x diet x sex) ANOVA to analyze these effects on each call type.

4.3 Results:

4.3.1 Maternal pup retrieval

HFD dams did not differ in the latency to start retrieving their pups from CHD dams ($t_{(12,1)}= 1.70$, $p= 0.11$; Figure 4.2A). However, HFD dams retrieved fewer pups overall during the test compared to CHD dams (Mann-Whitney $U= 9$, $p= 0.04$; Figure 4.2B). In addition, HFD (3/7 retrieved all pups) dams tended to fail to complete the retrieval during the test compared to CHD (6/7 retrieved all pups) ($X^2_{(1,14)}= 2.8$, $p=0.09$; Figure 4.2C).

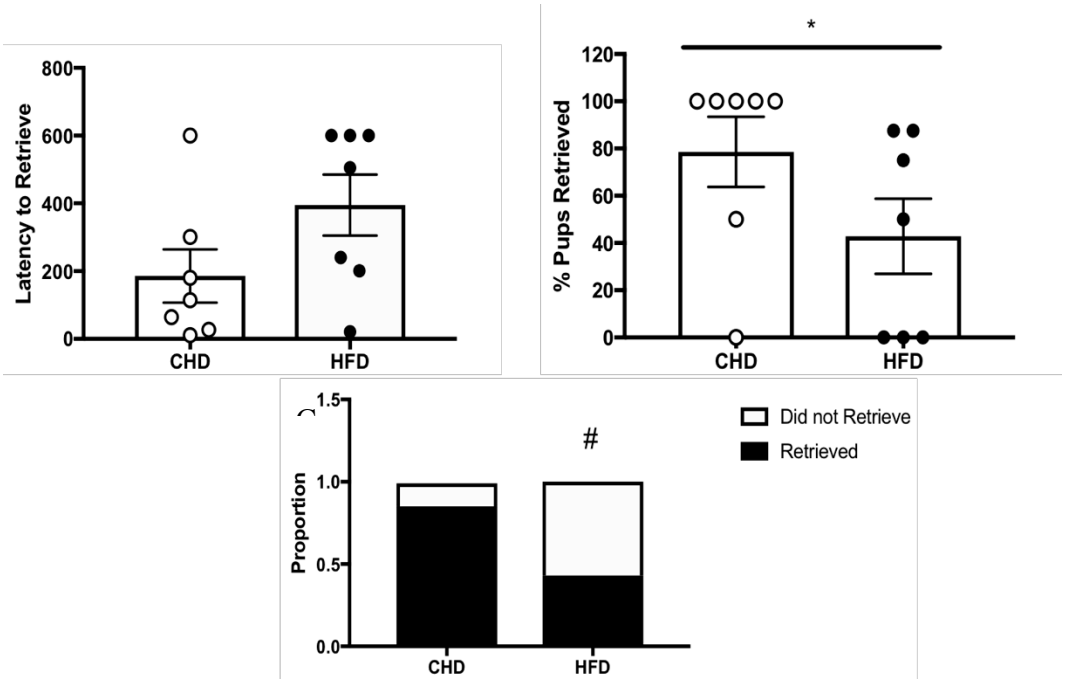

Figure 4.2 Maternal pup retrieval at PND7. (A) HFD dams did not significantly differ in their latency to retrieve their pups. (B) HFD dams retrieved less pups than CHD dams. (C) HFD group tended to have a higher proportion of dams that failed to retrieve all pups. Data presented are means ± standard error. *p≤0.05, #p=0.09.

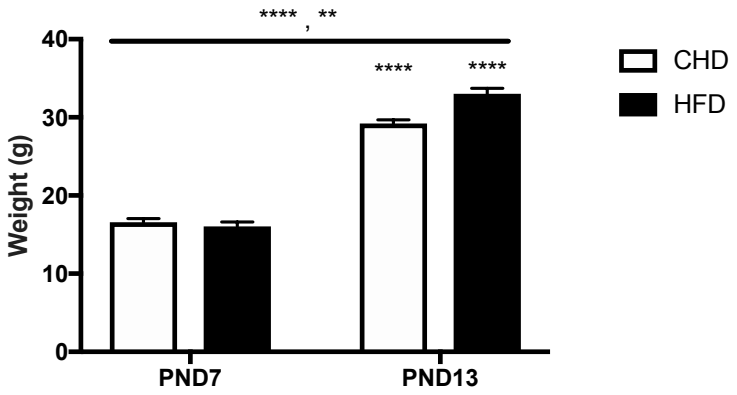

Figure 4.3 Offspring weights. PND13 offspring were heavier than PND7 offspring. HFD pups were heavier than CHD pups on PND13 and not PND7. Data presented are means ± standard error. PND effect: ****p≤0.001, diet effect: **p≤ 0.01. Tukey post hoc: ****p≤0.0001 HFD-13 vs. all, CHD-13 vs. all.

Table 4.1 Pups USV acoustic parameters at PND 7 and 13

Call Parameters	**CHD**		**HFD**	
PND	7	13	7	13
Call counts**	382.17 ± 54.28**abc**	139.45± 26.16**a**	185.47 ± 42.80**b**	215.83 ± 41.97**c**
Total Duration (s)	46.72 ± 8.19**a**	24.52 ± 5.05	17.50 ± 4.49**a**	36.59 ± 6.93
Mean Duration (s)****	0.121 ± 0.009	0.159 ± 0.019**d**	0.070 ± 0.008**de**	0.163 ± 0.013**e**
Peak Frequency (kHz)	40.10 ± 0.70	44.35 ± 2.23	44.67 ± 2.34	39.34 ± 1.55

PND effect: ****p≤0.0001, **p≤0.01. Values sharing the same letters are significantly different by Tukey's post hoc

4.3.2 Call quantitative acoustic parameters

As expected, pups weights changed over development with PND13 pups weighing more than PND7 pups (PND effect: $F_{(1, 64)} = 644.1$, $p \leq 0.0001$; Figure 4.3). There was an overall effect of diet on body weight with HFD pups being heavier than CHD pups (Diet effect: $F_{(1, 64)} = 7.95$, $p=0.006$). More specifically, HFD pups were only heavier than CHD pups at PND13 and not PND7 (Diet x PND interaction: $F_{(1, 64)} = 13.70$, $p=0.0004$; Tukey's post hoc $p \leq 0.002$).

Table 4.1 presents the acoustic measures of pup USVs measured at PND7 and 13. Overall, as pups aged they produced fewer USV calls (PND effect: $F_{(1,64)}= 6.22$, $p=0.01$). However, this was only evident in CHD pups (Diet x PND interaction: $F_{(1,64)}= 10.42$, $p=0.002$), where PND7 CHD pups vocalized more than PND13 CHD pups (Tukey's post hoc $p=0.002$), while HFD pups showed no difference in the number of USVs between the two ages. In addition, at PND7 HFD pups vocalized less than CHD pups (Tukey's post hoc $p=0.01$). There were no effects of PND or diet on the total call duration. However, the total duration that HFD pups vocalized on PND7 was shorter compared to CHD offspring at the same age (Diet x PND interaction: $F_{(1,64)}= 10.30$, $p=0.002$; Tukey's post hoc $p=0.01$). The mean call duration was significantly shorter in PND7 pups compared to PND13 pups (PND effect: $F_{(1,64)}= 20.46$, $p \leq 0.0001$). This effect was mainly driven by HFD pups on PND7, when they had significantly shorter calls compared to PND13 HFD and CHD pups (Tukey's post hoc $p \leq 0.0001$), which was not observed between CHD pups at PND7 and PND13. Only a main diet by PND interaction effect on the average peak frequency was observed (Diet x PND interaction: $F_{(1,64)}= 6.32$, $p=0.01$), indicating that diet affected the average peak frequency of pups in a PND dependent manner. However, differences did not reach significance using the post hoc multiple comparison correction.

There were no correlations observed between body weight and any of the acoustic measures examined in either testing days. At PND7, call counts were positively correlated with total call duration ($r=0.89$, $p \leq 0.0001$) and mean call duration ($r=0.72$, $p \leq 0.0001$), while peak frequency was negatively correlated with mean call duration ($r= -0.35$, $p=0.04$). At PND13, call counts were correlated positively with total call duration ($r=0.93$, $p \leq 0.0001$), while peak frequency

correlated negatively with call counts (r= -0.43, p=.007), total call duration (r=-0.59, p≤0.0001), and mean call duration (r=-0.75, p≤0.0001).

Given the possible contribution of body temperature to USV production, in a separate control cohort, we measured body temperature of pups at PND7 and found that HFD pups had significantly higher body temperature ($t_{(2, 26)}$= 2.75, p=0.01; HFD= 34.52±0.33ºC, CHD= 33.21±0.34 ºC) than CHD pups. However, in this cohort, HFD pups were also significantly heavier than CHD pups ($t_{(2, 28)}$ = 3.20, p=0.003; HFD= 17.6±0.45g, CHD= 16±0.18g). Body weight positively correlated with body temperature (r=0.50, p=0.007).

4.3.3 Call qualitative characteristics of sonographic structures

Pups from both ages and diet groups emitted a variety of call types during the isolation test. Similar to previous reports (Brudzynski et al., 1999; Zeskind et al., 2011) we were able to identify 12 different call types (Figure 4.1). In addition, we also identified categories of calls that did not fit the described call types (C13-C120). We further grouped the different calls into 6 different categories based on the presence and the number of frequency sweeps (simple calls, 1-sweeps, 2-sweep, ≥ 3-sweep), the presence of frequency shifts (call shifts), or have different combinations of the other categories (complex calls) similar to earlier report (Brudzynski et al., 1999; Zeskind et al., 2011).

A multivariate ANOVA of the 6 different categories revealed that age affected the percentages of call categories of the calls pups emitted (PND effect: $F_{(5,52)}$= 5.67, p≤0.0001; Figure 4.4A). More specifically, PND7 pups emitted more 1-sweep calls (PND effect: $F_{(1,56)}$= 10.62, p=0.002) and fewer 2-sweep calls (PND effect: $F_{(1,56)}$= 6.28, p=0.01) compared to PND13 pups. In addition, there was a non-significant trend of lower simple calls emitted by PND7 pups compared to PND13 (PND effect: $F_{(1,56)}$= 3.26, p=0.07). Pups from both diet groups did not differ in their call patterns (Diet effect: $F_{(5,52)}$= 0.32, p= 0.9; Diet x PND interaction: $F_{(5,52)}$= 0.87; Figure 4.4B). However, maternal diet affected the call patterns in a sex specific manner (Diet x Sex interaction: $F_{(5,52)}$= 3.14, p=0.01; Figure 4.4C). This interaction was specific to simple calls (Diet x Sex interaction: $F_{(5,56)}$= 4.67, p= 0.03; Figure 4.5A), 1-sweep calls (Diet x Sex interaction: $F_{(5,56)}$= 8.76, p=0.005; Figure 4.5B), and 2-sweep calls (Diet x Sex interaction: $F_{(5,56)}$= 9.96, p=0.003; Figure 4.5C). HFD female offspring tended to emit more 1-sweep calls compared to CHD female

offspring (Tukey's post hoc p= 0.058). HFD male offspring on the other hand, emitted more 2-sweep calls compared to CHD male offspring (Tukey's post hoc p= 0.04).

4.3.4 Acoustic parameters of simple, 1-sweep, and 2-sweep call categories

Call types across age and diets differed in their peak frequency ($F_{(5, 374)}$ = 10.26, p≤0.0001), mean duration ($F_{(5, 374)}$= 29.74, p≤0.0001), and peak amplitude ($F_{(5, 374)}$= 18.96, p≤0.0001). Since we found that simple, 1-sweep, and 2-sweep calls were the only call types affected by age, diet, and sex (see section 4.3.3 above), therefore we focused further analyses on these call types. A multivariate ANOVA of the three call types revealed that age has affected the peak frequency of the three call types (PND effect: $F_{(3,53)}$= 6.48, p=0.001), while the diet affected the frequency in an age dependent manner (Diet x PND interaction: $F_{(3,53)}$= 3.86, p=0.01). CHD and HFD offspring emitted simple and 1-sweep calls with frequencies that changed across age (simple: Diet x PND interaction: $F_{(1,55)}$= 5.06, p=0.03; 1-sweep: Diet x PND interaction: $F_{(1,55)}$= 10.16, p=0.002; Figure 4.6A,C). 2-sweep calls had higher frequency on PND7 compared to PND13 (PND effect: $F_{(1,55)}$= 4.97, p=0.03; Figure 4.6E). Among CHD offspring, 1-sweep calls increased in frequency on PND13 compared to PND7 calls (Tukey's post hoc p=0.03; Figure 4.6C). In addition, on PND13, 1-sweep calls among CHD offspring were of higher frequency than those of HFD offspring (Tukey's post hoc p=0.02; Figure 4.6C).

The mean duration of the calls changed across age and between diets (MANOVA: PND effect: $F_{(3,53)}$= 17.38, p≤0.0001; Diet effect: $F_{(3,53)}$= 3.96, p=0.01). Mean call duration of all three call types increased with age in both groups (PND effect: simple: $F_{(1,55)}$= 15.16, p≤0.0001, Tukey's post hoc: CHD-7 vs. CHD-13 (p=0.059), HFD-7 vs. HFD-13 (p=0.01); 1-sweep: $F_{(1,55)}$= 23.95, p≤0.0001, Tukey's post hoc: CHD-7 vs. CHD-13 (p=0.01), HFD-7 vs. HFD-13 (p=0.002); 2-sweep call: $F_{(1,55)}$= 51.41, p≤0.0001, Tukey's post hoc: CHD-7 vs. CHD-13 (p≤0.0001), HFD-7 vs. HFD-13 (p≤0.0001)). Maternal HFD decreased call mean duration for the simple (Diet effect: $F_{(1,55)}$= 5.42, p=0.02; Figure 4.6B) and 2-sweep calls (Diet effect: $F_{(1,55)}$= 7.79, p=0.007; Figure 4.6F) compared to CHD offspring.

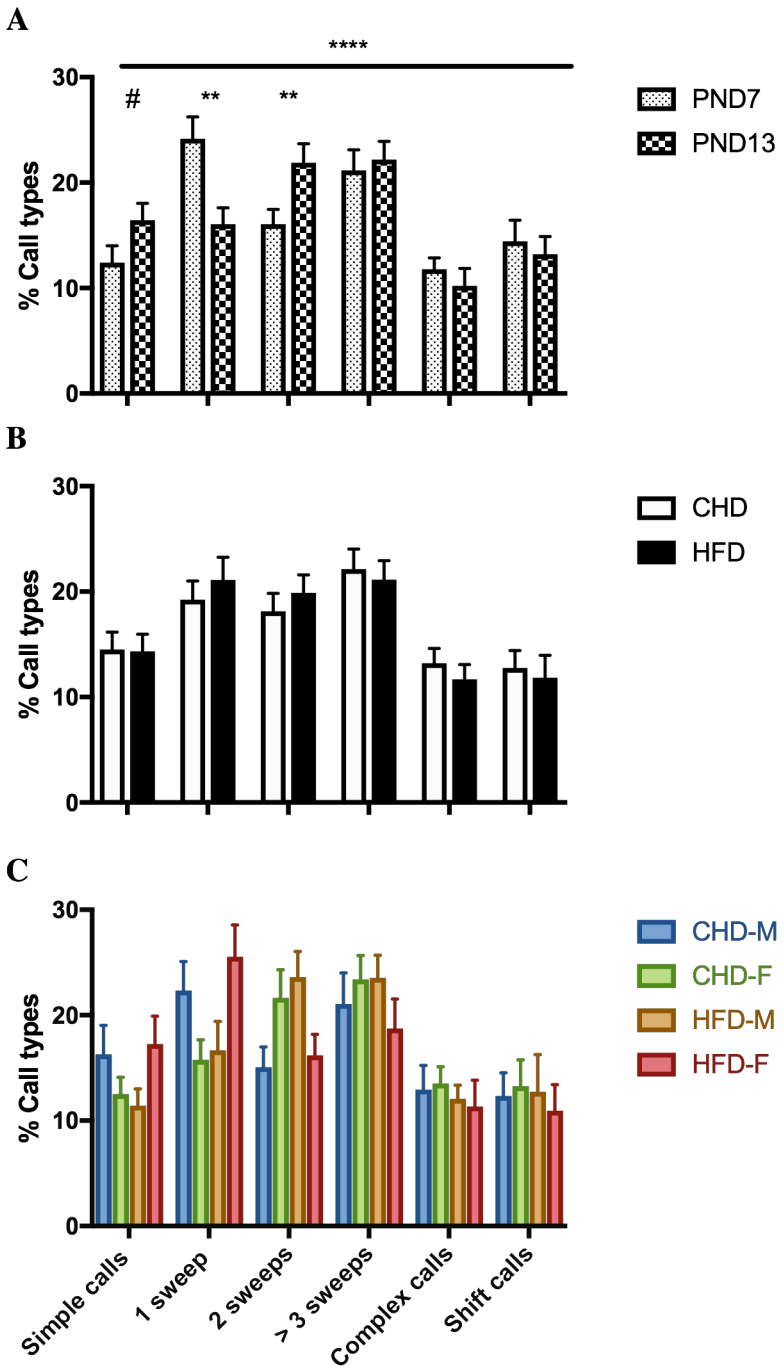

Figure 4.4 The percentages of call categories across age, diet, and sex. (A) PND affected the patterns of calls emitted by pups independent of their maternal diet. (B) There were no main maternal diet effects on the call patterns. (C) Diet affected the pattern of calls emitted in a sex dependent manner. Data presented are means ± standard error. PND effect: ****p≤0.001, **p≤ 0.01, #p=0.

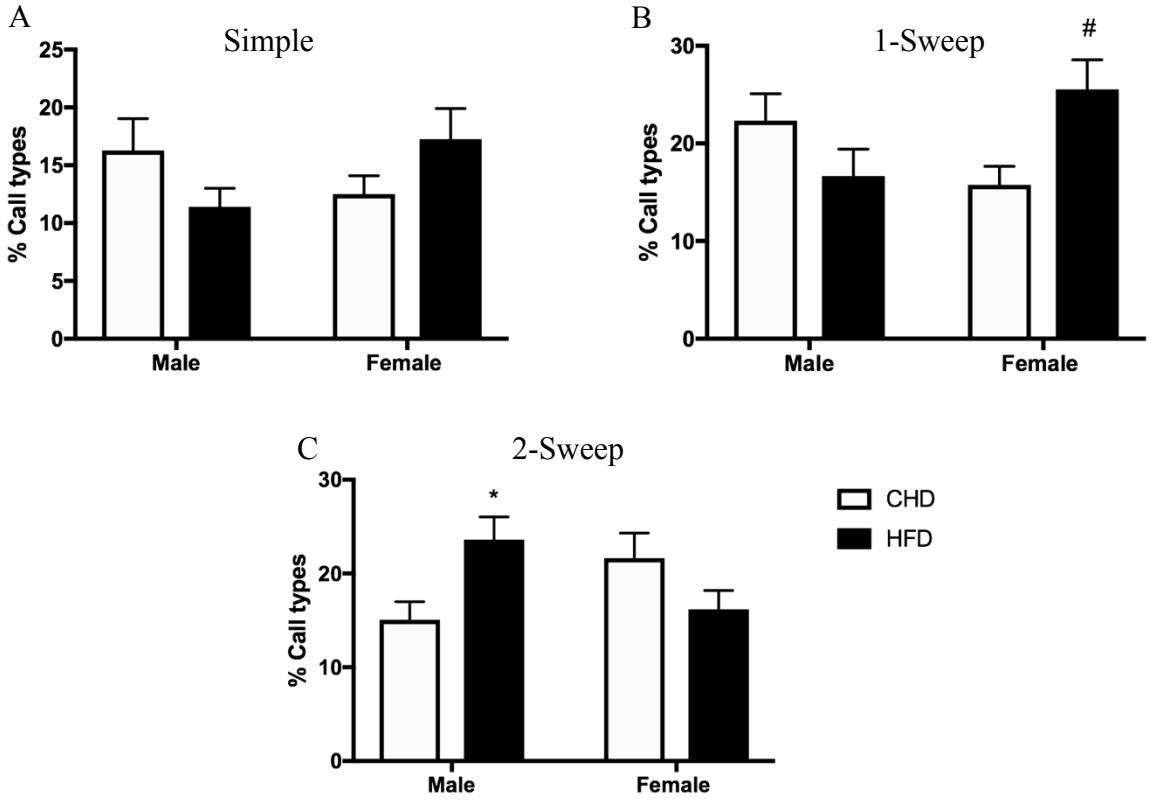

Figure 4.5 USV calls affected by HFD and sex. (A) maternal HFD affected the patterns of simple calls emitted by offspring in a sex specific manner. (B) HFD female offspring tended to emit more 1-sweep calls compared to CHD female offspring. (C) HFD males emitted more 2-sweep calls than CHD males. Data presented are means ± standard error. Tukey post hoc: *p≤0.05 2-sweep: CHD-male vs. HFD-male, #p=0.05 1-sweep: CHD-female vs. HFD-female.

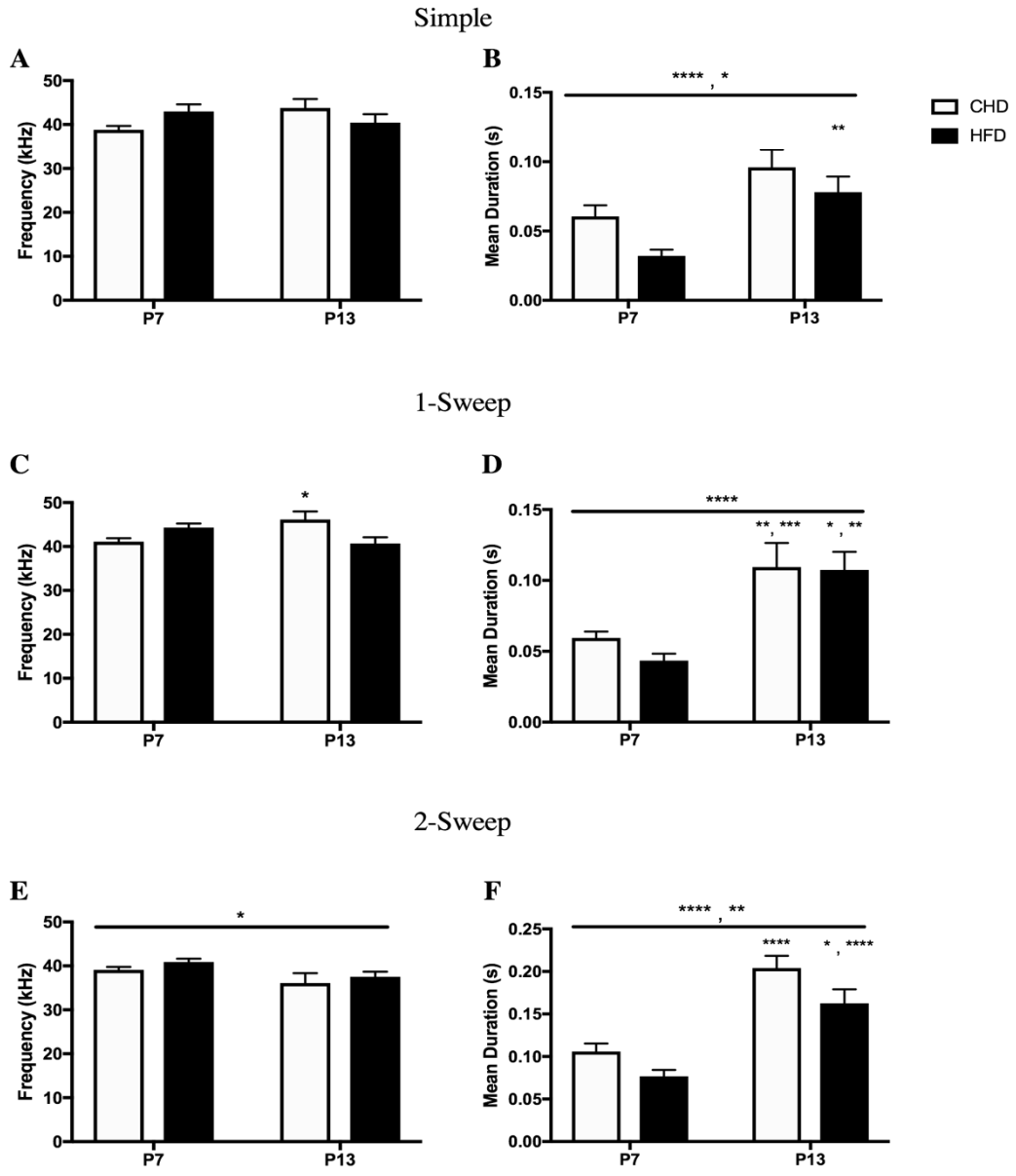

Figure 4.6 Acoustic characteristics of simple, 1-sweep, and 2-sweep call types. (A) diet and PND interaction effects were observed on simple calls frequency. (B) PND13 simple calls were longer than PND7, while HFD offspring simple calls were shorter than CHD offspring. (C) CHD offspring at PND13 had higher 1-sweep call frequency than PND7 CHD offspring and PND13 HFD offspring. (D) PND13 pups had longer 1-sweep calls than PND7 pups. E) PND13 pups had lower 2-sweep frequency than PND7 pups. (E) PND13 simple calls were longer than PND7, while HFD offspring simple calls were shorter than CHD offspring. Data presented are means ± standard error. PND effect ****p≤0.0001, *p≤0.05; diet effect **p≤0.01, *p≤0.05.

4.4 Discussion

In this chapter I examined for the first time the effects of maternal HFD on offspring USV characteristics across development in both sexes. I found that maternal HFD impaired maternal retrieval in dams. In addition, maternal HFD affected the acoustic parameters of the USVs emitted during development. The repertoire of call types was affected by age and maternal HFD. Maternal HFD also affected the call types emitted by offspring in a sex-specific manner.

4.4.1 Maternal Retrieval

An established function of pup USVs is their induction of maternal pup retrieval (Brunelli et al., 1994), therefore one of the aims of this study was to examine maternal pup retrieval in HFD dams. I found that although there were no differences in the latency to retrieve pups between CHD and HFD dams, HFD dams retrieved less pups by the end of the retrieval test. In addition, a higher proportion of HFD dams failed to retrieve all of their pups by the end of the retrieval test. An earlier report showed that HFD consumption pregestationally alone in mice impairs pup retrieval in dams, as HFD dams show higher latency to approach and spend more time sniffing their pups at PND1, which was suggested to possibly be related to reduced olfactory discrimination in obese animals (Bellisario et al., 2015). In this chapter I also report that HFD pups emitted fewer USVs compared to CHD offspring at PND7. The reduced USV numbers in HFD pups could be insufficient in recruiting the dam explaining the impaired maternal retrieval we observed.

4.4.2 Call quantitative acoustic parameters

CHD pups call counts were affected by PND, where CHD pups showed a significant 36% reduction in their USV counts as they developed. This typical call reduction with age has been previously documented by many studies (Barron and Gilbertson, 2005; Brudzynski et al., 1999; Tonkiss et al., 2003; Williams et al., 1998). However, HFD pups failed to show this developmental reduction of USV call counts, as PND7 call counts were not significantly different than the number of calls at PND13. HFD pups show already lower calls at PND7 compared to CHD pups and comparable to PND13 pups. In addition, HFD pups at PND7 had

shorter call durations overall and a shorter mean duration of calls compared to CHD pups of the same age.

As discussed in chapter 3 (Section 3.4.2), the lower call counts observed in HFD pups at PND7 could be associated with early activation of the HPA axis and the higher CRH transcript levels observed in these animals (Abuaish et al., 2018). Another factor that might be linked to this decrease in call counts at this age is body temperature differences between HFD and CHD pups. It has been shown that the USVs of neonates younger than PND10 are more influenced by temperature differences (Allin and Banks, 1971; Brunelli and Hofer, 1996). In a separate control cohort, I found that HFD pups had significantly higher body temperature than CHD pups. However, in this control cohort, HFD pups were heavier than CHD at PND7, while the main cohort of this study did not show body weight differences. The body temperature differences were correlated with the body weight, and therefore might not be the direct factor interpreting these results. In depth examination of the effects of maternal HFD on pup thermogenesis, including programming of BAT and their contribution to body temperature differences, during the first postnatal week, when USV numbers are more influenced by temperature differences, is needed. A previous study examining a rat model of maternal malnutrition has reported no relationship between body temperature and mean call duration or call peak frequency in PND7 pups in control animals (Tonkiss et al., 2003). In the present investigation, mean call duration and peak frequency were another acoustic parameter that were affected by diet. This might indicate that maternal diet effects on pup USV acoustic measures are not merely due to differences in thermoregulatory capacity of the pups, which could only confound the USV number findings and not call frequency.

In offspring of rats fed a control chow diet, mean call duration and peak call frequency were reported to not change across development in younger pups (PND7-PND11) (Tonkiss et al., 2003), whereas they increase with age when examining older pups (PND10-PND17) (Brudzynski et al., 1999). Here, I saw a strong PND effect on mean call duration, however this was only significant in HFD pups, as they emitted a reduced mean duration of calls at PND7 compared to CHD pups. The shorter call duration was not due to differences in the types of calls HFD pups emitted at PND7 relative to CHD pups. In fact, when examining the first three call types' (simple, 1-sweep, and 2-sweep) mean duration, HFD pups were consistently showing shorter calls across the different call types. CHD pups did not show a significant difference in

their call mean duration across development, which is in line with a previous study examining similar developmental time frame as our study (PND7, PND9 and PND11) (Tonkiss et al., 2003). I observed a significant interaction of PND and diet on call peak frequency; CHD pups had higher peak frequency at PND13 compared to PND7, while HFD pups had lower peak frequency at PND7 relative to PND13. In addition, HFD pups had a non-significant trend of a higher peak frequency compared to CHD pups at PND7. On PND13, HFD pups had a non-significantly lower peak frequency compare to CHD pups. Similar PND by diet interaction effects were observed when examining peak frequencies of the first three call types (simple, 1-sweep, and 2-sweep). Only 1-sweep calls showed a significant increase in peak frequency with age in CHD pups but not in HFD pups. Peak frequency, the loudest frequency of the call, has been suggested to play an active role in communication as it is not influenced by physical maturation of the larynx (Brudzynski et al., 1999). These results indicate an alteration in the communication signature of HFD pups typically observed during development, as they show significantly lower call duration at PND7 and altered peak frequency pattern across the two PNDs, which could impact pup-dam communication and maternal care received.

4.4.3 Call qualitative characteristics of sonographic structures

In this chapter, I also examined other aspects of USVs represented by sonographic structures of the calls. After the first characterization of the different call types rat pups emit by Brudzynski et al. (1999) other studies have utilized this schema and expanded it to examine perinatal insults on developmental patterns of these call types (Barron and Gilbertson, 2005; Tonkiss et al., 2003; Zeskind et al., 2014). I was able to identify the 9 different calls types first identified by Brudzynski et al. (1999) and 3 additional call types identified by Zeskind et al. (2014). I have identified 8 additional call types, which featured a combination of the frequency sweeps and simple calls that were not previously described. All call types were later classified into 6 different categories based on the number of their frequency sweeps or shifts similar to (Brudzynski, 2005; Zeskind et al., 2014). This classification aid in relating call types to a possible functional significance based on the proposed function of these frequency modulations. Frequency sweeps are defined as a rapid change of sound frequency in a single call and ranges in numbers within each call, and are suggested to be more effective acoustic features for sound localization (Brudzynski, 2005; Brudzynski et al., 1999). Two frequency sweeps (U and inverted U sonographs), which contains two alternating frequency sweeps, resemble siren sounds making

them more effective in localizing the pup (Brudzynski, 2005). Pups have been reported to be more proficient in producing these calls by PND17, suggested by the increase of the numbers of these calls by age. Other calls that were emitted more frequently in younger animals (PND10 and PND15) were regarded as either incomplete attempts to produce the ideal two sweep frequency calls or "practicing" calls before being able to emit the two-sweep calls (Brudzynski et al., 1999). Although I examined an earlier time frame of development compared to Brudzynski et al. (1999), I found similar results, indicating an increase of 2-sweep calls with age. In addition, PND13 pups decreased the number of 1-sweep calls and showed and trend of increase of simple calls (with no frequency modulations). The decrease in 1-sweep calls is in line with the general developmental pattern proposed by Brudzynski et al., (1999), however another study examining a similar developmental time frame as ours PND (7-11) did not report effects on the number of 1-sweep calls (Tonkiss et al., 2003). Other call categories such as ≥3-sweeps did not differ across age in our study, and were previously reported to significantly decrease by PND17 (Brudzynski, 2005), a time point outside our testing time frame. The lack of consistency across studies could be due to the different ages examined and whether calls are grouped into categories or not. Despite these differences, the increase of 2-sweep calls with age appear to be a consistent feature of development (our study: PND7-13 and Brudzynski et al. (1999): PND10-17).

Maternal HFD did not affect the overall patterns of call types emitted by pups nor did it effect the developmental patterns of these calls. Interestingly, however, maternal HFD differentially affected the patterns of calls emitted by males and females. HFD females tended to emit more 1-sweep calls compared to CHD females, while HFD males emitted significantly more 2-sweep calls compared to CHD males. There were no significant differences between the sexes within either diet group, however. In addition, we did not find main effects of sex in the acoustic parameters examined. Earlier studies on USV call types have examined either males only (Brudzynski et al., 1999; Tonkiss et al., 2003) or reported no sex differences (Barron and Gilbertson, 2005; Zeskind et al., 2014). To my knowledge only one study has reported sex differences in acoustic parameters in rats, where PND4 males emitted more USVs at lower frequencies with softer amplitudes compared to females (Bowers et al., 2013). These sex differences were associated with gene expression differences of Foxp2, a gene linked to speech and language impairment in humans when mutated, in several brain regions, including amygdala, cortex, cerebellum, and thalamus, between the sexes (Bowers et al., 2013). However, this

previous study did not characterize the different call types emitted by the pups. These findings indicate while HFD males are emitting more 2-sweep calls, which are hypothesized to be more efficient in localizing the pups by the dam (Brudzynski, 2005), HFD females are shifting their calls to a less efficient 1-sweep call. In addition to localizing pups, USVs have been shown to induce anogenital licking and nest building by the rat dams (Brouette-Lahlou et al., 1992; Noirot, 1972). It still not clear whether USVs simply just promote maternal arousal and general caregiving behaviour or they also communicate specific pup needs (i.e. retrieval vs. tactile stimulation) through their features that have been characterized thus far in previous research. Our results might indicate that HFD males are engaging in a more effective form of communication than HFD females, which could be associated with better recruitment of the dam and maternal behaviour. Within-litter maternal behaviour variation has been previously documented, and been associated with alterations in adult offspring behaviour and neural gene expression (Pan et al., 2014). Future studies should examine individual maternal behaviour received by males and females within HFD litters in order to discern whether the differences in call types between males and females are associated with differences in maternal behaviour received. Previous results from adult offspring indicate that females are more impacted by maternal HFD, as they show increased anxiety-like behaviours and stress reactivity, and differential gene expression of HPA axis related genes in the hippocampus and amygdala (Sasaki et al., 2013). One hypothesis could be that these impacts might be mediated in part by impaired pup-dam communication in females, leading to less than optimum maternal behaviour received and consequently programming of HPA axis related behaviours and genes in females.

4.4.4 Conclusion

Pup-dam interaction is important for the development, survival, and later programming of offspring phenotype. USVs are a mean of communication between the pups and their mothers. In this chapter, I explored the effects of maternal HFD and development on several aspects of USVs including acoustic parameters and call types. The results show that perinatal HFD exposure altered different features of USVs in a developmental stage and sex specific manner. These findings indicate alteration in the mode of communication between the offspring and their mother in HFD litters. This sets the stage for future examination of the relationship of these alterations in relation to maternal behaviour and downstream programming in adult offspring.

Chapter 5

Maternal High Fat Diet Effects on Offspring Epigenetic Signature

5 Maternal High Fat Diet Effects on Offspring Epigenetic Signature

5.1 Introduction:

The epigenome governs phenotypic variation among individuals through its interaction with the environment during development. A signature of differential DNA methylation has been observed in the brains of adult individuals exposed to different environmental insults during development, such as prenatal and postnatal stress in humans and animal models and it is linked to individual phenotypic outcomes (Mychasiuk et al., 2011b; Suderman et al., 2012). Although DNA methylation has been studied in the context of differential methylation as a consequence of early exposure to environmental factors in adult brains, it also plays an essential role in animal development starting at the embryonic stage and continues onto postnatal life regulating cell differentiation (Geiman and Muegge, 2009). Several studies have reported a gradual increase and a dynamic regulation of DNA methylation in the brain during early postnatal development, which coincides with the process of synaptogenesis and circuit maturation (Lister et al., 2013a; Numata et al., 2012; Siegmund et al., 2007; Szulwach et al., 2011). Moreover, DNA methylation has a role in mediating activity-dependent plasticity of the brain, an important process in shaping circuits development in neonatal and adult brains (Tognini et al., 2015).

The impact of maternal diet on the epigenome was first illustrated by early work on agouti obese mice demonstrating the responsiveness of offspring DNA methylation status to maternal diet and consequently their phenotype (Waterland et al., 2008). Global DNA hypomethylation has been reported in the PFC and hypothalamus of adult mouse offspring from HFD dams, which was associated with reduced methylation in the μ–opioid receptor, a regulator of reward seeking behaviours (Grissom et al., 2013; Vucetic et al., 2010). In addition, offspring from HFD dams have increased methylation in the POMC gene promoter, a precursor to ACTH, and α-melanocyte-stimulating hormone (α-MSH), an anorexigenic neuropeptide, in the arcuate nucleus of the hypothalamus of HFD offspring (Marco et al., 2014). To date however, there are no reports on the effects of perinatal HFD on genome-wide differential DNA methylation in offspring.

There are multiple high-throughput methods to assess DNA methylation and determine differential DNA methylation between experimental treatments. Methylated cytosine across the genome could be experimentally screened for either by using methylated DNA immunoprecipitation (MeDIP) or sodium bisulfate treatment followed by sequencing. The latter is considered the gold standard for assessing DNA methylation (Frommer et al., 1992). Bisulfate treatment leads to the deamination of unmethylated cytosine that is converted later to uracil, while methylated cytosine remains unaffected by the treatment, allowing the detection of DNA methylation pattern (Frommer et al., 1992). Bisulfate conversion is incorporated into two sequencing techniques: reduced representation (RRBS) or whole genome bisulfate sequencing (WGBS). RRBS utilizes the methylation insensitive resection enzyme, MspI, to digest the DNA followed by bisulfate conversion and sequencing. MspI digestion enrich for CpG rich regions of the genome providing a "reduced representation" of 1% of the genome (Meissner et al., 2005). Bisulfate sequencing methods provide better resolution compared to MeDIP methods and devoid from background noise created by non-specific binding of antibodies used for immunoprecipitation (Lentini et al., 2017). Although RRBS has a rather low coverage of the genome compared to WGBS, it is more cost effective and requires less intensive computation, which is demanded by WGBS due to its lower methylation mapping efficiency that is caused by the presence of repeated non-coding genomic regions in the final reads (de Vega et al., 2017).

We have demonstrated that perinatal HFD affects adult and neonatal offspring stress reactivity phenotype by increasing Cort response to stress, anxiety-like behaviour, and dysregulating expression of HPA axis related genes in the amygdala, hippocampus and PVN (Abuaish et al., 2018; A Sasaki et al., 2013). Although I did not observe sex specific effects of perinatal HFD on neonatal HPA axis phenotype, others have reported that neonatal female offspring had more differentially expressed genes in response to perinatal HFD relative to male offspring in the hypothalamus (Barrand et al., 2017). On the other hand, earlier work from our laboratory has reported that adult female offspring showed stronger behavioural and physiological phenotypic alterations in response to perinatal HFD in comparison to their male counterparts (Sasaki et al., 2013). In addition, adult females showed more pronounced differences in gene expression in the amygdala compared to the hippocampus (Sasaki et al., 2013). The effects of perinatal HFD on females are observed as early as embryonic stages, where female placentas show epigenetic and gene expression differences that are not observed in males (Gabory et al., 2012; Gallou-Kabani

et al., 2010). These reports indicate that females are more sensitive to perinatal HFD effects in general. In addition, HPA axis dysregulation observed in females offspring could be mediated in part via differential gene expression in the amygdala, a key brain region in modulating emotionality and activating the HPA response (Joels et al., 2008).

Therefore, in this chapter I examined the underlying epigenetic signature of perinatal HFD in the form of DNA methylation in the amygdala of neonatal and adult female rat offspring from mothers exposed to HFD prior and throughout gestation and lactation. Pups exposed to perinatal HFD manifest a metabolic phenotype that is absent from adult offspring that are weaned onto control CHD, including higher weights, leptin and insulin levels (Tamashiro et al., 2009). This metabolic milieu might act on epigenome dynamics during this critical period to influence the trajectory of development and later epigenetic signatures in adult animals and consequently affect their phenotype.

5.2 Material and Methods:

5.2.1 Animals and diets

Adult female Long Evans rats (7 week) were purchased from Charles River and housed in same sex pairs until mating and maintained on a 12:12-h light– dark cycle (lights on 7:00 am–7:00 pm) with *ad libitum* access to food and water. Females were either maintained on chow diet (CHD; Purina Lab Diets (St. Louis, MO: cat. no. 5001) consisting of 28.5% protein, 13.5% fat, and 58% carbohydrate, or high fat diet (HFD; Research Diets, Inc. (New Brunswick, NJ: cat. no. D12492), consisting of (by kcal): 20% protein, 60% fat, 20% carbohydrate.) for 3-4 weeks prior to mating. Two different cohorts were used in this study; cohort one (CHD= 4 litters, HFD= 4 litters) generated postnatal day (PND)7 offspring. Cohort two (CHD= 4 litters, HFD=4 litters) generated PND90 offspring.

Females were housed with males for a week and sperm plugs were checked twice a day to determine the onset of pregnancy. Females were then separated from males and singly housed throughout their pregnancy. After parturition, the dams were moved into clean cages with paper towel strips used as nesting material. Pups were weighed and culled to 12 pups/ litter (6 males and 6 females) when possible.

Experimental protocols were approved by the Local Animal Care Committee at the University of Toronto, Scarborough, and were in accordance with the guidelines of the Canadian Council on Animal Care.

5.2.2 Tissue preparation and nucleic acid extraction

PND7 animals were removed from their litters and rapidly decapitated and brains were collected. PND90 were scarified by CO2 inhalation and decapitated to collect brains. Brains obtained from all animals were flash frozen in isobutene and stored in -80°C. Brain were later cryosectioned into 50 μM sections using Research Cryostat Leica CM3050 S (Leica Biosystems) and the amygdala was microdisected based on stereotaxic coordinates (PND7: bregma: -0.20mm to -1.60 mm (Paxinos et al., 1991); PND90 bregma: −1.72mmto −3.00mm (Paxinos and Watson, 1998)). DNA and RNA were extracted using ZR-Duet™ DNA/RNA MiniPrep (Zymo Research) following the manufacturing instructions. The concentration of genomic DNA was quantified using the PicoGreen dsDNA Quantitation kit (Thermo Scientific). Quantification and purity of the RNA were assessed with a spectrophotometer (Nanodrop ND-2000C, Thermo Scientific).

5.2.3 Reduced representation bisulfate sequencing (RRBS)

Genomic DNA (100ng) from amygdala was used to construct libraries for RRBS using the Ovation RRBS Methyl-Seq System (NuGEN) as per the manufacture's guidelines. EpiTect Fast DNA Bisulfite Kit (Qiagen) was used for Bisulfte conversion. Libraries were sequenced on NextSeq 500 (Illumina) at the Princess Margaret Genomics Centre, part of the University Health Network, Toronto. Sequencing was done using a single end with a 70-base-read length and multiplexed at 8–10 samples per flowcell.

5.2.4 Differentially methylated region analysis (DMR)

RRBS fastq files were processed similar to our previous study (Ashbrook et al., 2018). Briefly, files were trimmed to remove low quality reads ($q<30$) and adaptors and later aligned to RGD Rnor_6.0. The average reads per sample were $20.8X10^{6}$ with a mapping efficiency of 50-60%. DMRs coincides with the size of gene regulatory regions and their analysis help detect weak differences in DNA methylation, where methylation levels of neighbouring CpGs correlate with each other giving rise to stronger significant differences that could be missed by examining single sites (Bock, 2012). DMRs were identified using a dynamic sliding window approach

annotated using the methylPipe and compEpiTools R packages (Kishore et al., 2015). The bisulfite conversion rate was >99% for all samples. Regions were declared to be differentially methylated if the average methylation of at least 10 consecutive CpG sites within 1kp region was ≥ 5% difference between HFD and CHD and passed an FDR < 0.05 statistical cutoff.

5.2.5 Enrichment analysis

Genes sets from the DMR analysis from both ages were used in Gene Ontology Biological Process (GO BP) and KEGG pathway enrichment analyses. GO terms are hierarchal vocabularies composed of annotated genes that aim to describe different biological aspects of genes, such as biological process, molecular function, and cellular component (Ashburner et al., 2000). Enrichment analysis determines the statistical associate between the genes identified from the RRBS analysis and GO terms and the enrichment of GO terms relative to a background gene set, which is in this case was the whole rat genome. KEEG Pathway database is a set of genes organized in manually drawn maps representing current knowledge of the interactions of these genes (https://www.genome.jp/kegg/pathway.html). Both analyses provide biological interpretation of the genes identified by associating them to biological function and molecular pathways. The enrichment analysis was performed using gProfiler with an FDR ≤ 0.05 cutoff.

Networks of GO terms were constructed and visualized using Enrichment Map on Cytoescape 3.6.1 (Merico et al., 2010). Using this network analysis reduces the redundancy inherent in GO analysis and help to depict the themes associated with the GO results, aiding in the biological interpretation of the significant GO terms associated with the differentially methylated gene list. Enrichment Map represents GO terms as nodes, where node size is representing the number of genes within each GO term, and edges between nodes represent shred genes between nodes, where edge thickness is proportional to the number of common genes. Clusters of GO terms were organized into themes associated with the GO terms within the clusters. Using yFiles algorithm and WordCloud plugin on Cytoescape 3.6.1, clusters were arranged and labeled. GO analysis was also performed using DAVID algorithm to compare the results generated by gProfiler.

5.3 Results:

5.3.1 Diet differentially methylated regions

Dams were weighed every week throughout the study; HFD dams were heavier than CHD dams after being on the HFD for three weeks and throughout gestation ($F_{(6, 14)}$= 1.85, p=0.032; Figure 5.1A). Moreover, while all pups increased in weight during the first postnatal week (postnatal day effect: $F_{(1,13)}$= 953.7, p≤0.0001), HFD were heavier at PND7 relative to CHD offspring (Bonferroni post hoc p= 0.006; Figure 5.1B).

RRBS and DMR analysis revealed 777 DMRs at PND7 and 1050 DMRs at PND90. At PND7, 69% of DMRs were hypomethylated and 31% were hypermethylated, while at PND90 61% of DMRs were hypomethylated and 39% were hypermethylated (Figure 5.2). About 50% of significant DMRs at PND7 were found in intergenic regions, while 21% and 29% were found in promoters and gene bodies, respectively. At PND90, 56% of significant DMRs were found in intergenic regions while only 6% in promoters and 38% in gene bodies.

Fifty-seven DMRs were shared between the two age groups (Figure 5.3), corresponding to 26 genes listed in table 5.1. About 33% of the overlapping DMRs had opposite methylation profiles between the two age groups. Specifically, we found that 7 hypomethylated and 12 hypermethylated DMRs at PND7 showed the opposite methylation pattern at PND90. Consequently, 6 genes showed opposite methylation profiles between early life and adulthood, including Polr3g, Prpf38b, and Htatsf1, that were hypomethylated at PND7 and hypermethylated at PND90 and Ank1, Dupd1, and Tmprss9, that were hypermethylated at PND7 and hypomethylated at PND90.

5.3.2 Gene Ontology enrichment analysis

A total of 379 and 444 DMRs were localized to genic regions at PND7 and PND90, respectively. In order to determine the biological relevance of these genes, we carried out GO analysis using gProfiler to identify enriched biological processes.

GO analysis identified 163 significantly enriched biological processes at PND7 that clustered into 10 groups (Figure 5.4). Of the 10 groups, biological processes involving development that included organism (7 terms), cellular (21 terms), and neuronal (28 terms) GO terms comprised

three groups. Protein modification, consisting of protein phosphorylation (12 terms) and catabolic process (21 terms) such as proteolysis clustered into two groups. GO terms of cellular response to stimuli (13 terms), such as response to growth factors, stress, and organic compounds. The other clusters included GO terms involving cell secretion and signal transduction (21 terms), metabolic regulation (18 terms), apoptosis (7 terms), and gene expression regulation (15 terms).

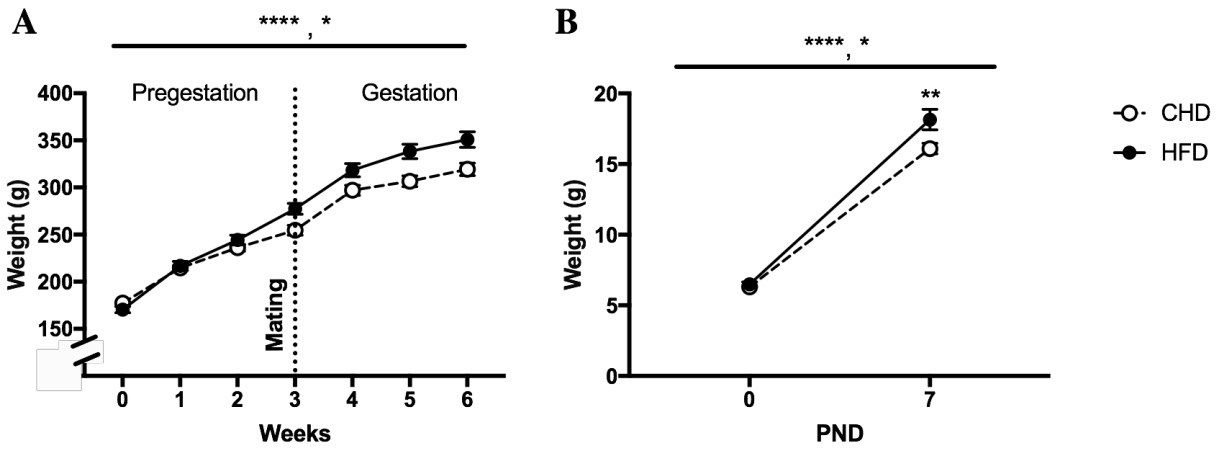

Figure 5.1 Maternal and offspring body weights. (A) HFD dams weighed more than CHD dams at conception. (B) HFD offspring were heavier than CHD offspring at PND7. Data presented are means ± standard error. Diet effect: *p≤0.05; postnatal day effect: ****p≤0.0001. Bonferroni post hoc: CHD-PND7 vs. HFD-PND7 **p≤0.01

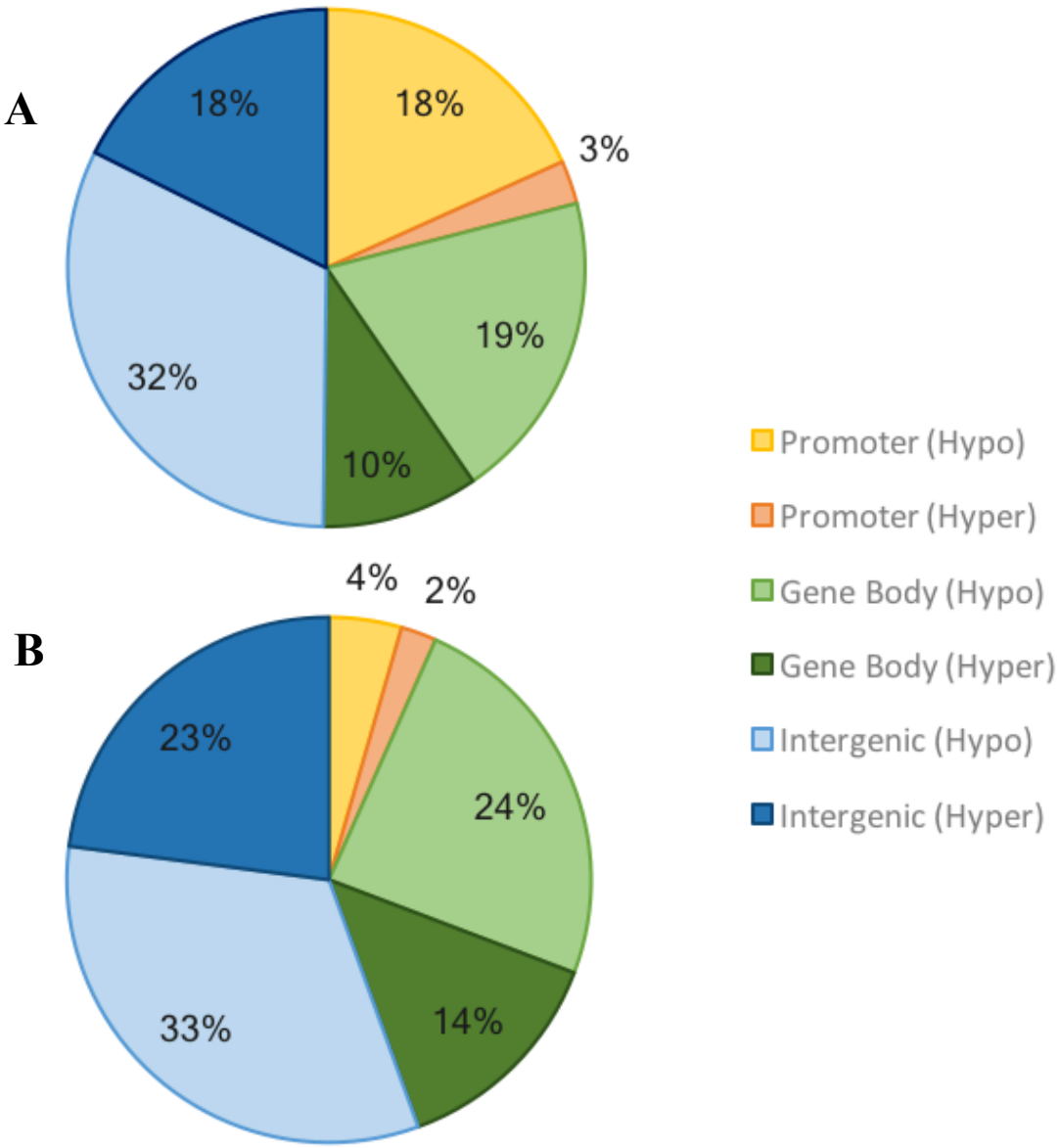

Figure 5.2 Distribution of significant differentially methylated regions (DMR) in HFD compared to CHD offspring at (A) PND 7 and (B) PND90. Distribution of hyper- and hypo-methylated regions are shown according to genic location including promoter regions, gene bodies, and intergenic regions.

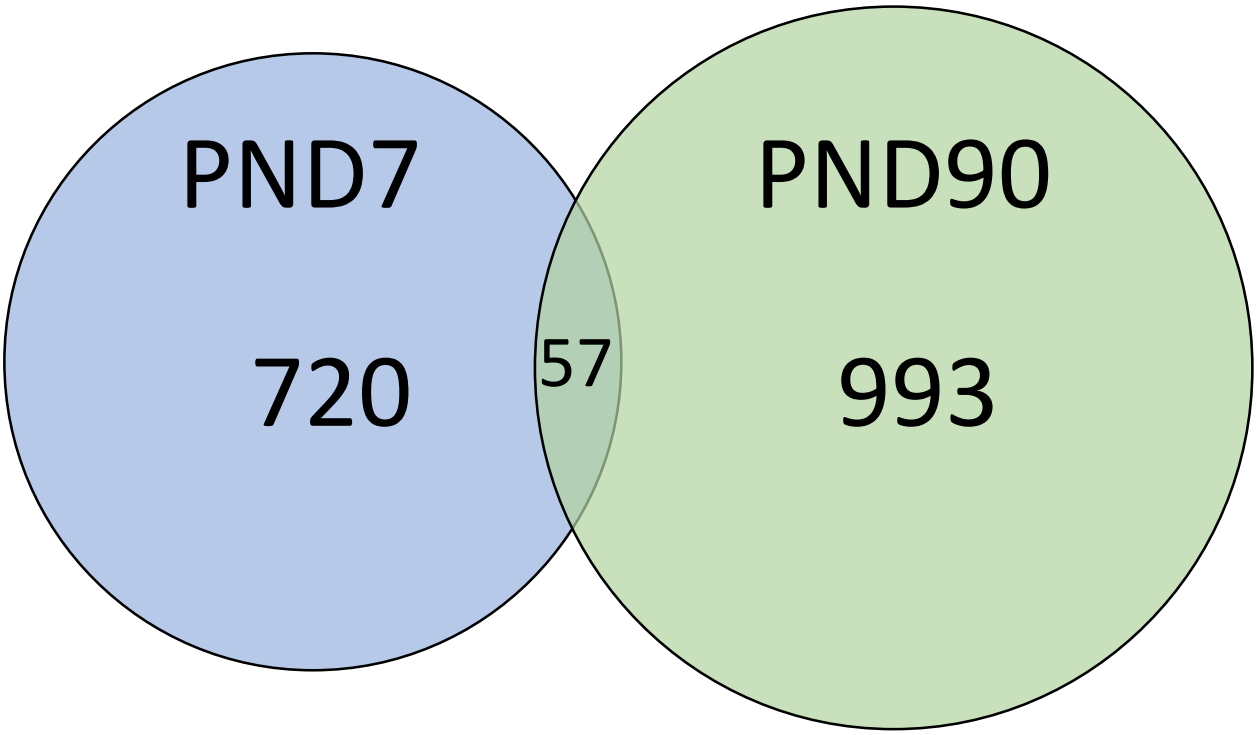

Figure 5.3 Venn diagram illustrating the number of DMRs in HFD compared to CHD offspring at PND7 (blue) and PND90 (green) and the number of overlapping DMRs across the two ages. At PND7, HFD offspring had a total of 777 DMRs, while at PND90 a total of 1050 DMRs were found in HFD offspring compared to CHD. There were 75 DMRs shared between the two ages**.**

Table 5.1 Genes containing significant DMRs in both PND7 and PND90 offspring, along with the percentages of differential methylation between HFD and CHD at each age and the location of the DMR

Chromosome	Gene Symbol	Gene Name	PND7 Methylation differences (%)	PND90 Methylation differences (%)	Gene Location
chr1	LOC310926	Hypothetical protein LOC310926	-21.01	-10.295	Gene body
chr1	Tbc1d10b	TBC1 domain family, member 10b	-11.427	-30.145	Promoter
chr2	Paqr6	Progestin and adipoQ receptor family member 6	-40.93	-11.022	Gene body
chr2	Polr3g	RNA polymerase III subunit G	-19.942	5.273	Gene body
chr2	Prpf38b	Pre-mRNA processing factor 38B	-8.727	5.153	Gene body
chr3	LOC499742	LRRG00137	27	34.943	Gene body
chr5	Rgs3	Regulator of G-protein signaling 3	-8.432	-7.377	Gene body
chr6	Unc79	Unc-79 homolog	-17.035	-13.765	Gene body
chr7	Tmprss9	Transmembrane protease, serine 9	12.28	-10.145	Gene body
chr8	Icam4	Intercellular adhesion molecule 4, Landsteiner-Wiener blood group	-12.475	-15.715	Promoter
chr10	Anxa6	Annexin A6	20.15	34.822	Gene body
chr11	Gtf2e1	General transcription factor IIE subunit 1	-10.285	-6.048	Gene body
chr11	Hic2	HIC ZBTB transcriptional repressor 2	-7.472	-5.325	Gene body
chr12	Tpst2	Tyrosylprotein sulfotransferase 2	-8.54	-15.268	Gene body
chr14	Rab5a	Ras-related protein Rab-5A	-7.45	-7.975	Promoter
chr15	Dupd1	Dual specificity phosphatase and pro isomerase domain containing 1	11.21	-8.108	Gene body
chr16	Ank1	Ankyrin 1	5.695	-30.753	Gene body
chr16	Plvap	Plasmalemma vesicle associated protein	7.403	5.117	Promoter
chr17	Rsu1	Ras suppressor protein 1	5.56	7.537	Gene body
chr18	Nfatc1	Nuclear factor of activated T-cells 1	-9	-17.6	Gene body

chr19	Zfp423	Zinc finger protein 423	9.005	8.777	Gene body
chrX	Nhs	NHS actin remodeling regulator	-5.795	-18.252	Gene body
chrX	Lancl3	LanC like 3	-14.728	-11.385	Promoter
chrX	Mecp2	Methyl CpG binding protein 2	-5.84	-5.72	Promoter
chrX	Htatsf1	HIV-1 Tat specific factor 1	-5.308	14.31	Promoter
chrX	Ndufb1	NADH:ubiquinone oxidoreductase subunit B11	-5.608	-17.19	Promoter

Enrichment analysis on genes identified at PND90 identified 105 GO terms clustered into 7 different groups (Figure 5.5). Similar to PND7, biological processes involving development including organism (12 terms), cellular (17 terms), and neuronal (16 terms) were enriched and clustered into three different groups. Twenty-two GO terms were clustered into protein phosphorylation regulation and signaling, which included GTPase activity regulation and intracellular signal transduction. Protein localization and secretion clustered 21 GO terms and ion transport clustered 10 GO terms. Cellular stimulus response (5 terms) included response to lipopolysaccharide and lipids.

Using the 26 genes identified from the common DMRs among the two ages in the enrichment analysis did not yield any significant GO terms, therefore we examined the common GO terms identified at each of the two ages and clustered them into groups. A total of 35 enriched GO terms were overlapping across the two age groups and were clustered into 5 different groups (Figure 5.6). PND7 and PND90 shared GO terms associated with cellular morphogenesis and organism development (10 GO terms) and neuronal projection and nervous system development (11 terms). In addition, GO terms associated with protein phosphorylation (6 terms) and protein transportation and secretion (7 terms) were shared between PND7 and PND90. The lipid response GO term was common among the two age groups and did not cluster with any other terms. Similar GO terms were obtained using DAVID (Figure 5.7-5.9).

5.3.3 KEGG pathway enrichment analysis

We examined the enrichment of the identified genes in biological pathways using the KEGG pathway database via gProfiler. The KEGG database contains pathways representing our current experimental knowledge of molecular interactions organized in networks that include cellular processes, organismal systems, environmental information processing, and metabolism. Analysis at PND7 and PND90 revealed 8 shared KEGG pathways despite the presence of different sets of genes used for the enrichment analysis (Table 5.2). A set of 4 interrelated KEGG pathways were enriched with the differentially methylated genes, which included MAPK, cGMP-PKG, cAMP, and calcium signaling pathways. In addition, the aforementioned signaling pathways are involved in the axon guidance pathway, which was also enriched with a number of differentially methylated genes.

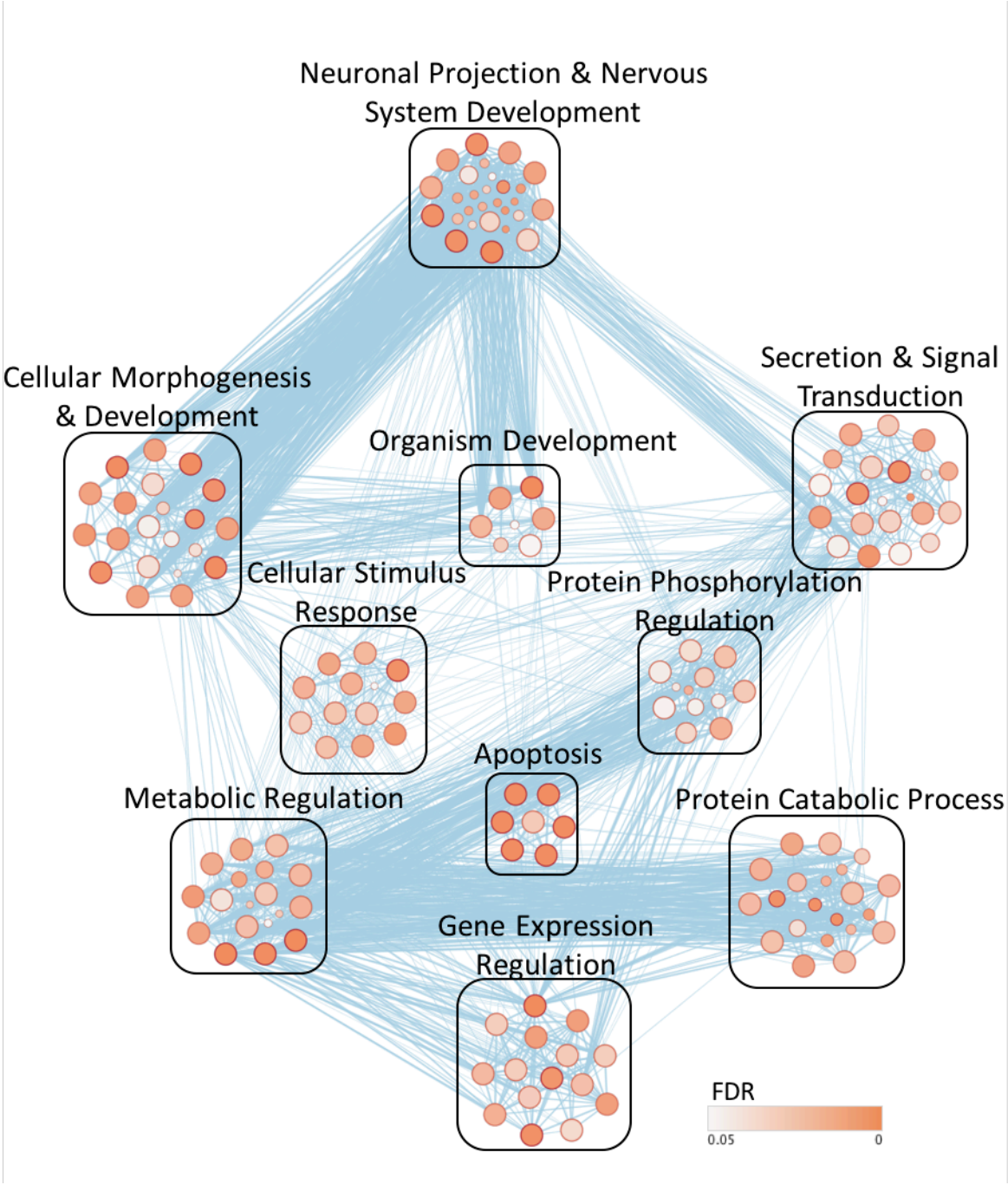

Figure 5.4 gProfiler clusters of Gene Ontology (GO) Biological Processes terms significantly enriched at PND7. The size of the nodes represents the number of genes, while the color indicates the FDR p-value. The edges between the nodes indicate shared genes, with edge thickness representing the number of genes in common.

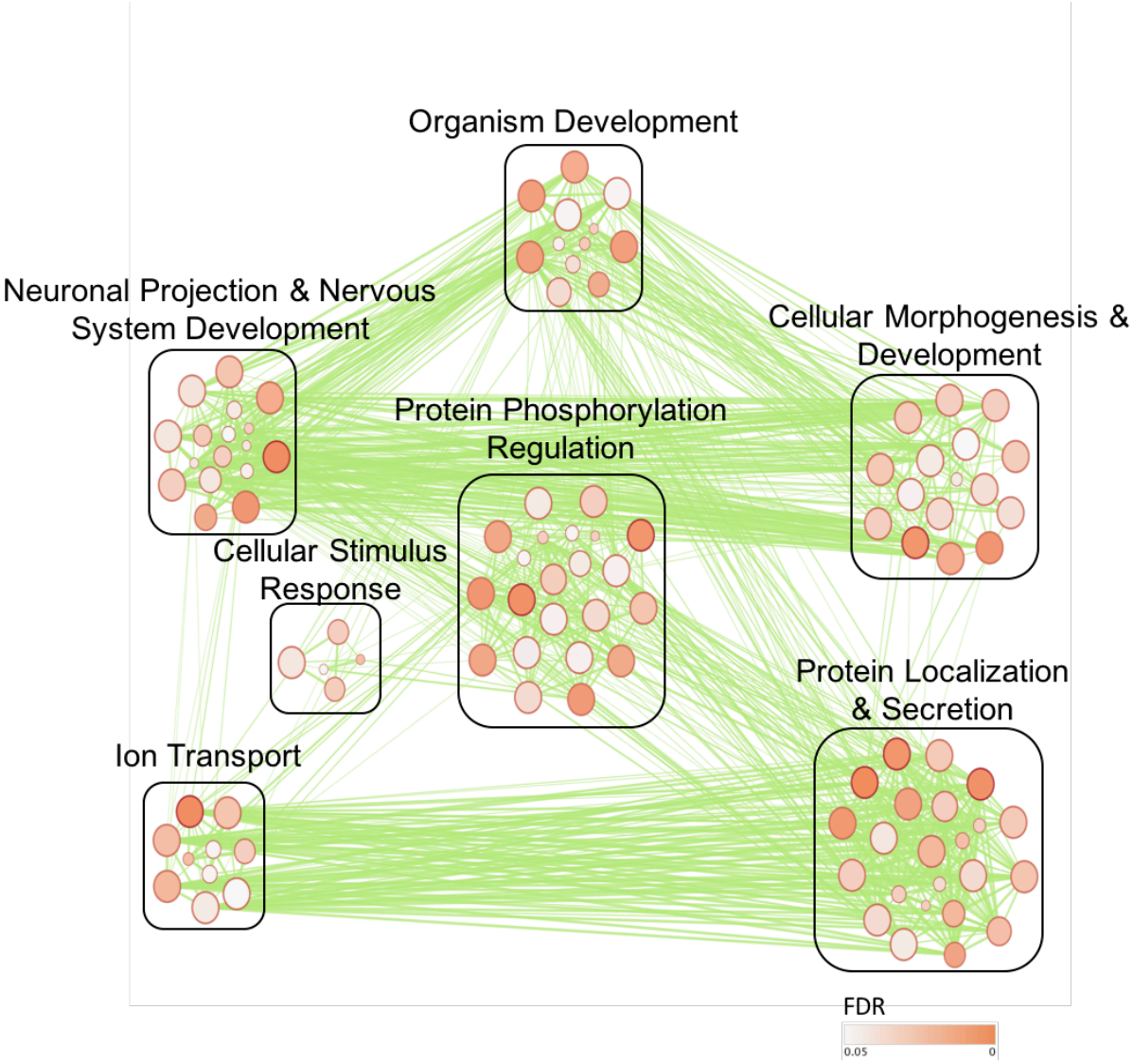

Figure 5.5 gProfiler clusters of Gene Ontology (GO) Biological Processes terms significantly enriched at PND90. The size of the nodes represents the number of genes, while the color indicates the FDR p-value. The edges between the nodes indicate shared genes, with edge thickness representing the number of genes in common.

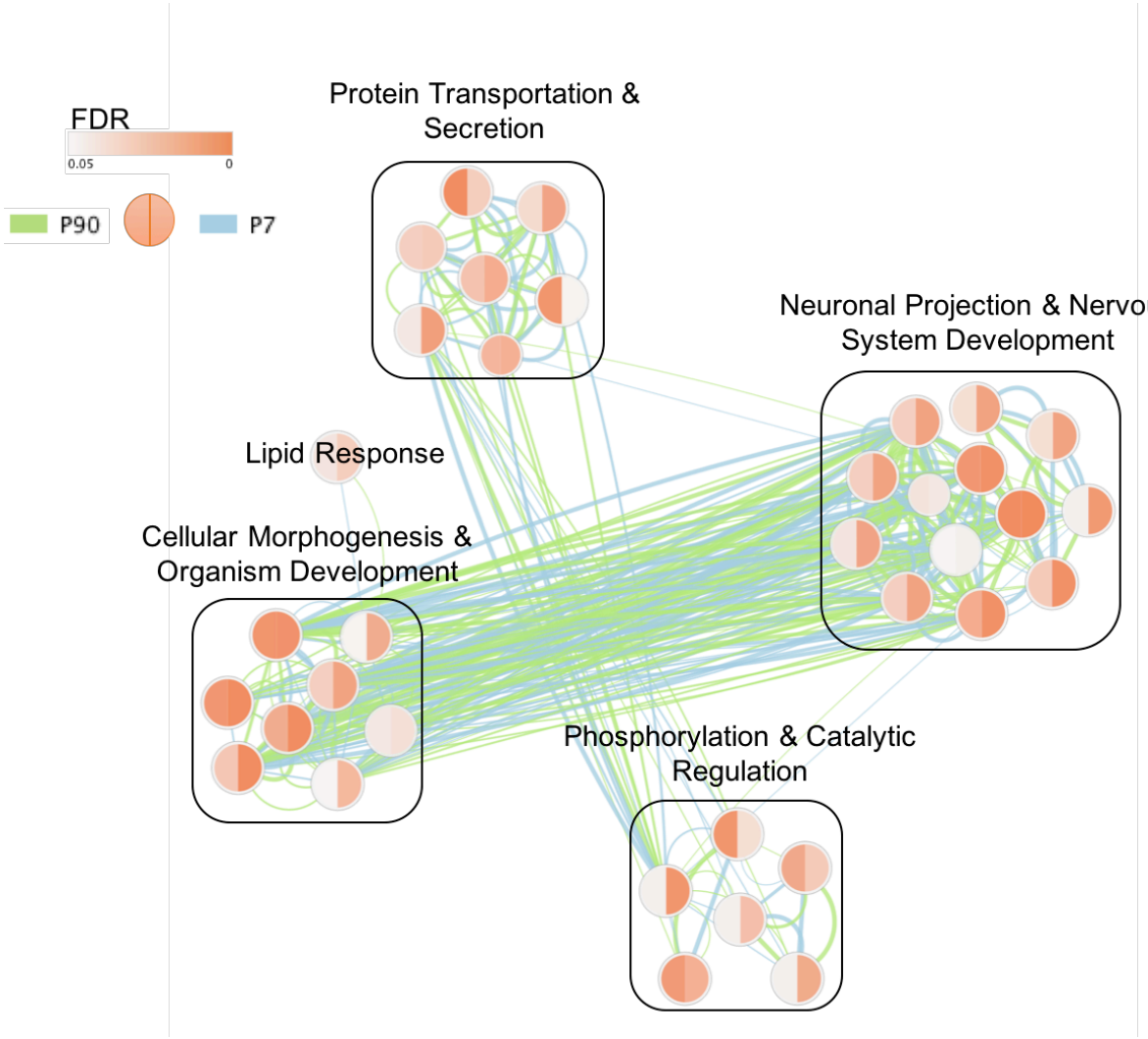

Figure 5.6 Overlapping gProfiler clusters of Gene Ontology (GO) Biological Processes enriched in PND7 (right half of nodes and blue edges) and PND90 (left half of nodes and green edge) differentially methylated gene set. The size of the nodes represents the number of genes, while the color indicates the FDR p-value. The edge between the nodes indicate shared genes, with edge thickness representing the number of genes shared.

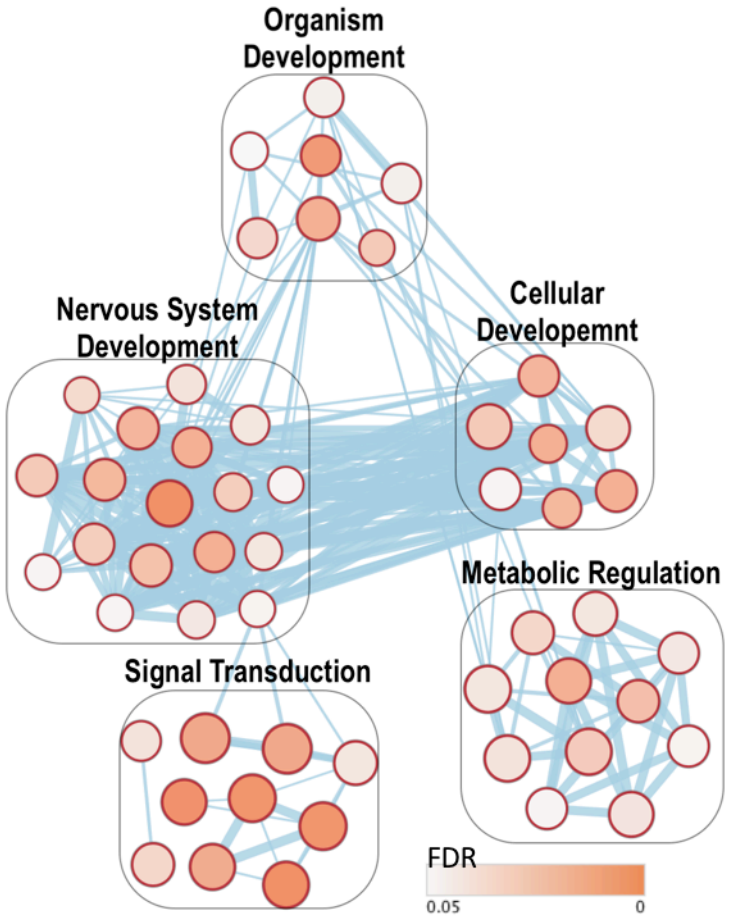

Figure 5.7 DAVID clusters of Gene Ontology (GO) Biological Processes terms significantly enriched at PND7. The size of the nodes represents the number of genes, while the color indicates the FDR p-value. The edges between the nodes indicate shared genes, with edge thickness representing the number of genes in common.

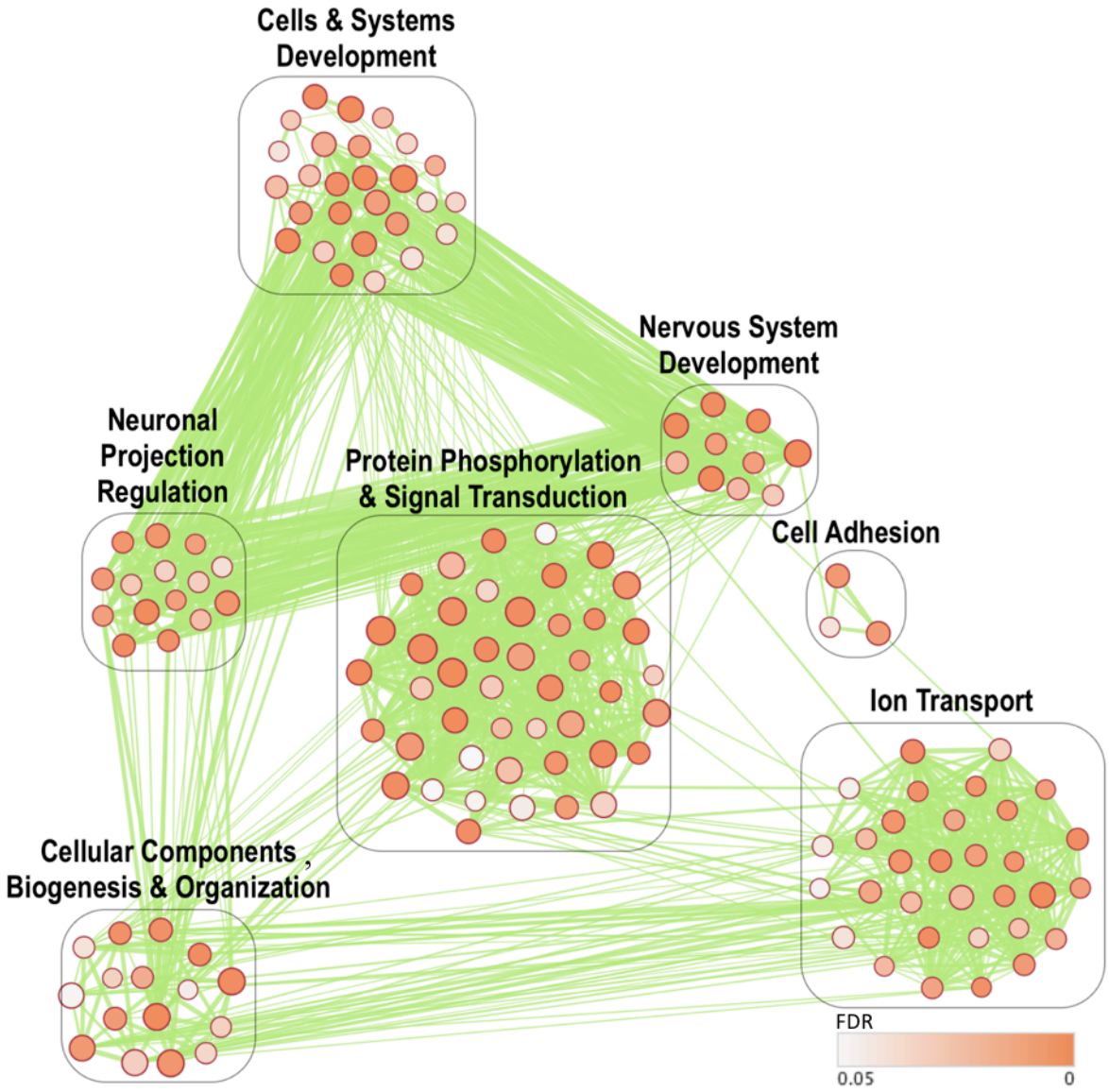

Figure 5.8 DAVID clusters of Gene Ontology (GO) Biological Processes terms significantly enriched at PND90. The size of the nodes represents the number of genes, while the color indicates the FDR p-value. The edges between the nodes indicate shared genes, with edge thickness representing the number of genes in common.

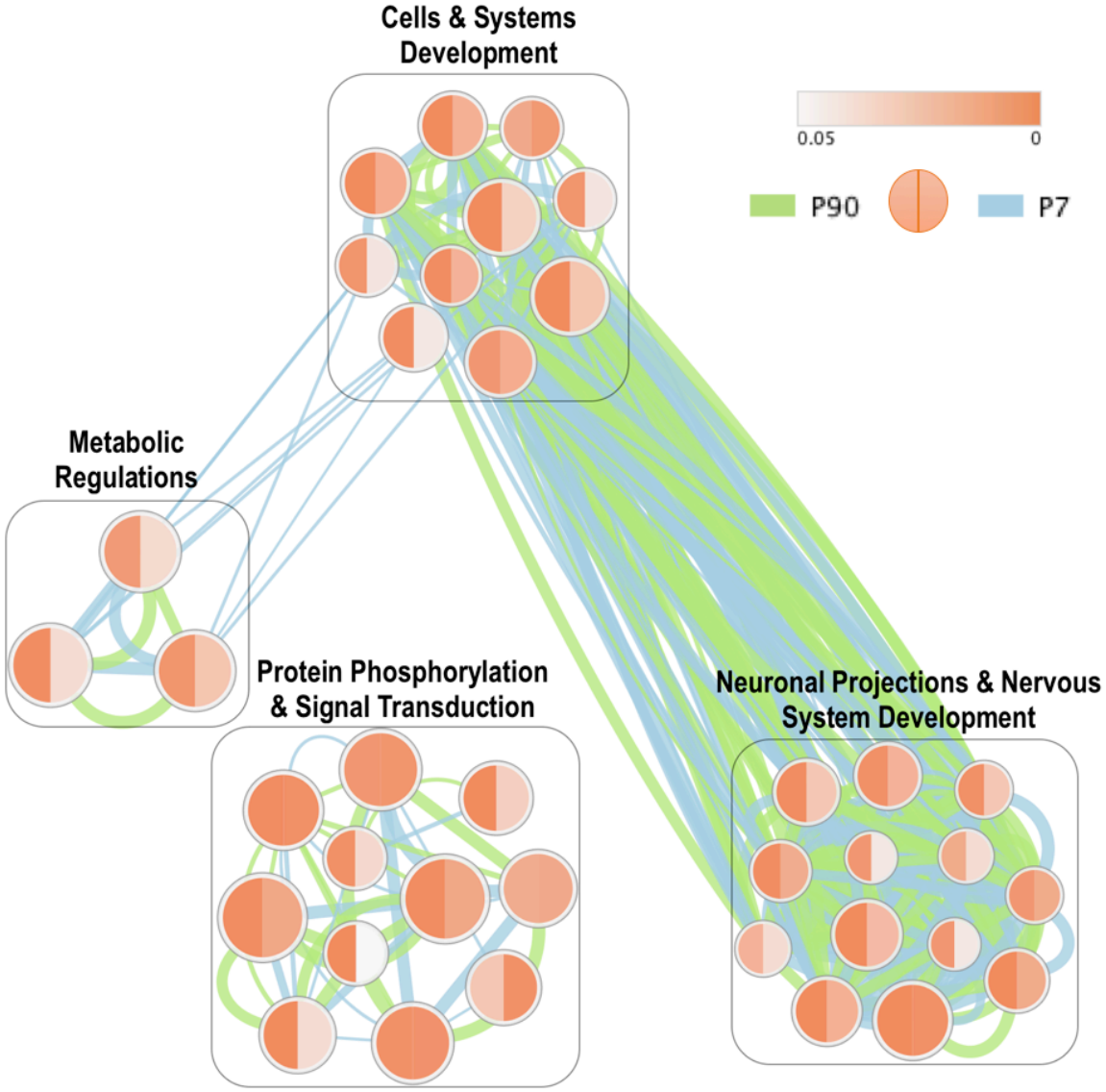

Figure 5.9 Overlapping clusters DAVID Gene Ontology Biological Processes enriched in PND7 (right half of nodes and blue edges) and PND90 (left half of nodes and green edge) differentially methylated gene set. The size of the nodes represents the number of genes, while the color indicates the FDR p-value. The edge between the nodes indicate shared genes, with edge thickness representing the number of genes shared.

Oxytocin signaling, which involves MAPK and calcium signaling, axonal guidance, tight junction and circadian entrainment KEGG pathways were also significantly enriched at both ages.

In addition to the shared pathways, KEGG enrichment analysis of differentially methylated genes at PND7 yielded 10 KEGG pathways that were specific to PND7 (Table 5.3). The wnt signaling pathway was enriched with genes differentially methylated at PND7. Neurotrophin signaling pathway, associated with MAPK signaling, and mediates axonal outgrowth and long-term potentiation, was another pathway enriched with the differentially methylated gene set. Moreover, two endocrine system pathways were identified, thyroid hormone and GnRH signaling pathways, both of which also involve MAPK, cAMP, and/or calcium signaling. Other pathways were associated with human diseases affecting the nervous system; Alzheimer's disease and amphetamine addiction, and nervous system related signaling pathways involved in retrograde endocannabinoid signaling.

Enrichment analysis of differentially methylated genes at PND90 revealed 20 age specific KEGG pathways in addition to the pathways in common with PND7 (Table 5.4). A set of three interrelated signaling pathways were enriched at PND90, including the Ras, Rap1, and PI3K-Akt signaling pathways. Three nervous system specific pathways were enriched including, GABAergic synapse, glutamatergic synapse and long-term depression pathways. The analysis revealed three endocrine-related pathways including, insulin secretion, relaxin singling and aldosterone synthesis and secretion pathways. Two pathways important for cellular integrity, extracellular matrix (ECM)-receptor interaction and focal adhesion were identified. Interestingly, a number of cardiovascular related pathways were enriched at PND90 including three cardiomyopathy pathways and vascular smooth muscle contraction. In addition, pathways involved in cancer and transcriptional dysregulation in cancer were two additional pathways associated with human diseases that were enriched.

Table 5.2 List of common KEGG Pathways enriched by the genes identified from DMRs at PND7 and PND90

ID	Description	PND7 FDR	PND90 FDR	PND7 Genes	PND90 Genes
KEGG:04010	MAPK signaling pathway	4.98E-03	4.47E-02	MAPK10,RPS6KA6,MAP3K14,RPS6KA3,CACNA1C,CACNA1F,RASGRF1,NFATC1,MAP3K4,DUSP9,FGF16	CACNA1C,MAP3K6,MECOM,CACNA1D,NFATC1,NTRK2,NFKB1,CACNA2D3,FGF13
KEGG:04020	Calcium signaling pathway	5.30E-03	2.93E-03	PLCD3,CACNA1C,SLC8A1,CACNA1F,SPHK1,HTR2C,PLCB4,CAMK2B,ATP2B3	NOS1,P2RX7,MYLK,ITPKB,PHKA1,ADCY8,CACNA1C,EDNRB,CACNA1D,SLC25A5
KEGG:04022	cGMP-PKG signaling pathway	8.64E-03	5.38E-03	CACNA1C,SLC8A1,CACNA1F,NFATC2,NFATC1,MRVI1,PLCB4,ATP2B3	ATF6B,MYLK,ADCY8,CACNA1C,EDNRB,CACNA1D,NFATC1,GUCY1A2,SLC25A5
KEGG:04024	cAMP signaling pathway	2.73E-03	1.21E-02	MAPK10,CACNA1C,GRIA3,ABCC4,CACNA1F,GRIN3B,NFATC1,TIAM1,CAMK2B,ATP2B3	ADCY8,CACNA1C,GABBR2,CACNA1D,NFATC1,VAV3,TIAM1,NFKB1,RAPGEF3
KEGG:04360	Axon guidance	6.80E-04	9.09E-03	FYN,NTN1,PAK3,EFNB1,NFATC2,RGS3,SLIT1,SEMA6B,CAMK2B,BMPND7,PLXNA3	FYN,EPHB1,DPYSL5,EPHB2,LIMK2,SEMA4A,RGS3,PARD3,BMPND7
KEGG:04530	Tight junction	2.69E-02	1.52E-03	RUNX1,MAPK10,RAP2C,DLG3,TIAM1,ARHGEF18,EPB41L4B	DLG3,MYH10,SLC9A3R1,CLDN11,CACNA1D,TJP3,TIAM1,MAGI1,ARHGEF18,PARD3,CGNL1
KEGG:04713	Circadian entrainment	3.82E-02	1.41E-02	CACNA1C,GRIA3,PER3,PLCB4,CAMK2B	NOS1,ADCY8,CACNA1C,CACNA1D,GNG7,GUCY1A2
KEGG:04921	Oxytocin signaling pathway	6.94E-03	2.84E-02	CDKN1A,CACNA1C,CACNA1F,NFATC2,EEF2K,NFATC1,PLCB4,CAMK2B	MYLK,ADCY8,CACNA1C,CACNA1D,NFATC1,GUCY1A2,CACNA2D3

Table 5.3 List of KEGG Pathways enriched by the genes identified from DMR at PND7

ID	Term	FDR	Genes
KEGG:00604	Glycosphingolipid biosynthesis - ganglio series	9.14E-03	ST8SIA1,ST8SIA5,ST6GALNAC3
KEGG:04010	MAPK signaling pathway	4.98E-03	MAPK10,RPS6KA6,MAP3K14,RPS6KA3,CACNA1C,CACNA1F,RASGRF1,NFATC1,MAP3K4,DUSP9,FGF16
KEGG:04020	Calcium signaling pathway	5.30E-03	PLCD3,CACNA1C,SLC8A1,CACNA1F,SPHK1,HTR2C,PLCB4,CAMK2B,ATP2B3
KEGG:04022	cGMP-PKG signaling pathway	8.64E-03	CACNA1C,SLC8A1,CACNA1F,NFATC2,NFATC1,MRVI1,PLCB4,ATP2B3
KEGG:04024	cAMP signaling pathway	2.73E-03	MAPK10,CACNA1C,GRIA3,ABCC4,CACNA1F,GRIN3B,NFATC1,TIAM1,CAMK2B,ATP2B3
KEGG:04261	Adrenergic signaling in cardiomyocytes	4.69E-02	CACNA1C,SLC8A1,CACNA1F,PLCB4,CAMK2B,ATP2B3
KEGG:04310	Wnt signaling pathway	1.55E-02	MAPK10,FZD6,NFATC2,CTNNBIP1,NFATC1,PLCB4,CAMK2B
KEGG:04360	Axon guidance	6.80E-04	FYN,NTN1,PAK3,EFNB1,NFATC2,RGS3,SLIT1,SEMA6B,CAMK2B,BMPND7,PLXNA3
KEGG:04530	Tight junction	2.69E-02	RUNX1,MAPK10,RAP2C,DLG3,TIAM1,ARHGEF18,EPB41L4B
KEGG:04713	Circadian entrainment	3.82E-02	CACNA1C,GRIA3,PER3,PLCB4,CAMK2B
KEGG:04720	Long-term potentiation	9.14E-03	RPS6KA6,RPS6KA3,CACNA1C,PLCB4,CAMK2B
KEGG:04722	Neurotrophin signaling pathway	2.69E-02	MAPK10,RPS6KA6,RPS6KA3,MAGED1,BEX3,CAMK2B
KEGG:04723	Retrograde endocannabinoid signaling	2.15E-02	MAPK10,CACNA1C,GRIA3,NDUFB11,CACNA1F,PLCB4,GABRE
KEGG:04912	GnRH signaling pathway	8.64E-03	MAPK10,CACNA1C,CACNA1F,MAP3K4,PLCB4,CAMK2B
KEGG:04919	Thyroid hormone signaling pathway	2.73E-03	NOTCH4,SLC16A2,PLCD3,MED14,MED12,BMP4,MED16,PLCB4
KEGG:04921	Oxytocin signaling pathway	6.94E-03	CDKN1A,CACNA1C,CACNA1F,NFATC2,EEF2K,NFATC1,PLCB4,CAMK2B
KEGG:05010	Alzheimer's disease	3.93E-02	APP,CACNA1C,NDUFB11,CACNA1F,TAF3,UQCRC1,PLCB4

Table 5.4 List of KEGG Pathway enriched by the genes identified from DMR at PND90

ID	Term	FDR	Genes
KEGG:00230	Purine metabolism	2.25E-02	PDE9A,ADCY8,GUCY2C,GUCY2E,POLR3G,PDE8A, POLD1,GUCY1A2
KEGG:04010	MAPK signaling pathway	4.47E-02	CACNA1C,MAP3K6,MECOM,CACNA1D,NFATC1,NTRK2 ,NFKB1,CACNA2D3,FGF13
KEGG:04014	Ras signaling pathway	2.25E-03	FLT1,KIT,RALB,FLT4,KSR1,IGF1R,RASA3,NTRK2,GNG7, TIAM1,NFKB1,FGF13
KEGG:04015	Rap1 signaling pathway	2.46E-03	FLT1,KIT,RALB,FLT4,ADCY8,IGF1R,TIAM1,MAGI1, PARD3,FGF13,RAPGEF3
KEGG:04020	Calcium signaling pathway	2.93E-03	NOS1,P2RX7,MYLK,ITPKB,PHKA1,ADCY8,CACNA1C, EDNRB,CACNA1D,SLC25A5
KEGG:04022	cGMP-PKG signaling pathway	5.38E-03	ATF6B,MYLK,ADCY8,CACNA1C,EDNRB,CACNA1D, NFATC1,GUCY1A2,SLC25A5
KEGG:04024	cAMP signaling pathway	1.21E-02	ADCY8,CACNA1C,GABBR2,CACNA1D,NFATC1,VAV3, TIAM1,NFKB1,RAPGEF3
KEGG:04151	PI3K-Akt signaling pathway	2.93E-03	ATF6B,FLT1,COL6A2,KIT,FLT4,CDK6,IGF1R,COL4A3, NTRK2,VWF,GNG7,NFKB1,PCK1,FGF13
KEGG:04270	Vascular smooth muscle contraction	2.84E-02	MYLK,PRKCH,ADCY8,CACNA1C,CACNA1D,GUCY1A2
KEGG:04360	Axon guidance	9.09E-03	FYN,EPHB1,DPYSL5,EPHB2,LIMK2,SEMA4A,RGS3, PARD3,BMPND7
KEGG:04510	Focal adhesion	1.41E-02	FYN,FLT1,COL6A2,MYLK,FLT4,IGF1R,COL4A3,VWF, VAV3
KEGG:04512	ECM-receptor interaction	2.84E-02	COL6A2,CD47,COL4A3,VWF,CD36
KEGG:04530	Tight junction	1.52E-03	DLG3,MYH10,SLC9A3R1,CLDN11,CACNA1D,TJP3, TIAM1,MAGI1,ARHGEF18,PARD3,CGNL1
KEGG:04713	Circadian entrainment	1.41E-02	NOS1,ADCY8,CACNA1C,CACNA1D,GNG7,GUCY1A2
KEGG:04724	Glutamatergic synapse	9.09E-03	ADCY8,SLC38A1,CACNA1C,CACNA1D,SLC1A3,GNG7, GLUL
KEGG:04727	GABAergic synapse	1.53E-03	ADCY8,SLC38A1,CACNA1C,GABBR2,SLC6A13, CACNA1D,GNG7,GLUL
KEGG:04730	Long-term depression	4.67E-02	NOS1,GNAZ,IGF1R,GUCY1A2
KEGG:04911	Insulin secretion	1.19E-02	ATF6B,ADCY8,CACNA1C,CACNA1D,KCNN3,ABCC8

KEGG:04921	Oxytocin signaling pathway	2.84E-02	MYLK,ADCY8,CACNA1C,CACNA1D,NFATC1,GUCY1A2, CACNA2D3
KEGG:04925	Aldosterone synthesis and secretion	2.84E-02	ATF6B,ADCY8,CACNA1C,CACNA1D,DAGLA
KEGG:04926	Relaxin signaling pathway	2.01E-02	ATF6B,NOS1,ADCY8,EDNRB,COL4A3,GNG7,NFKB1
KEGG:04964	Proximal tubule bicarbonate reclamation	2.79E-02	SLC4A4,SLC9A3,PCK1
KEGG:05165	Human papillomavirus infection	2.84E-02	COL6A2,DLG3,SLC9A3R1,TRAF3,CDK6,COL4A3,VWF, MAGI1,NFKB1,PARD3,AXIN2
KEGG:05200	Pathways in cancer	2.25E-03	KIT,RALB,ADCY8,TRAF3,CDK6,EDNRB,MECOM,IGF1R, COL4A3,GNG7,PLEKHG5,NFKB1,ZBTB16,FGF13,TRAF1, AXIN2
KEGG:05202	Transcriptional misregulation in cancer	2.74E-02	FLT1,AFF1,IGF1R,RUNX2,NFKB1,ZBTB16,TRAF1,NCOR1
KEGG:05410	Hypertrophic cardiomyopathy (HCM)	2.95E-02	CACNA1C,CACNA1D,SGCG,CACNA2D3,EMD
KEGG:05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	1.53E-03	CACNA1C,CACNA1D,SGCG,CDH2,ACTN2,CACNA2D3, EMD
KEGG:05414	Dilated cardiomyopathy (DCM)	1.19E-02	ADCY8,CACNA1C,CACNA1D,SGCG,CACNA2D3,EMD

5.4 Discussion:

In this chapter I aimed to characterize the effects of perinatal HFD consumption on the DNA methylome in the amygdala of female offspring at neonatal life and in adulthood. I report that perinatal HFD was associated with 777 DMRs at PND7 and 1050 DMRs at PND90 with a total of 379 and 444 genes were identified from the DMRs found at PND7 and PND90, respectively. In addition, there was an overlap of 57 DMRs between the two age groups, with 26 genes annotated to these regions. I preformed GO and KEGG pathway enrichment analyses to biologically contextualize the differentially methylated genes associated with perinatal HFD exposure. Protein transport and phosphorylation were two conserved GO clusters enriched with differentially methylated genes across the two developmental stages examined. In addition, KEGG pathway analysis revealed 4 core signaling pathways that were enriched with differentially methylated genes from both time points, which were MAPK, cGMP-PKG, cAMP, and calcium signaling pathways. Finally, PND specific GO terms and pathways were also determined and are discussed below.

5.4.1 Differentially methylated genes and enriched pathways shared across development in HFD offspring

Our DMR analysis revealed 26 genes associated with the 57 DMRs in common between PND7 and PND90. One of these shared genes was zinc finger protein 423 (Zfp423), a transcription factor involved in initiating adipogenesis. Interestingly, two independent studies have reported hypomethylation of Zfp423 promoter and increased transcript levels in embryonic tissues and adipocytes of weanling offspring exposed to maternal obesity (Liang et al., 2016; Yang et al., 2013). Our study revealed hypermethylation in the region associated with the gene body of Zfp423 in offspring exposed to perinatal HFD at both PND7 and PND90. While promoter methylation is linked to suppression of gene expression, methylation of gene bodies is more closely associated with gene expression activation (Jjingo et al., 2012). Zfp423 is also associated with neuronal differentiation and cerebellar development (Warming et al., 2006). Taken together, these findings provide evidence of the epigenetic programming of Zfp423 across tissues and developmental stages in response to maternal obesity.

The GO enrichment analysis has revealed a number of terms that are related to protein localization, transport, and secretion that were common across the two developmental stages.

Protein transport and secretion could take the form of endocytosis through the endosomal machinery or exocytosis to form exosomes. A number of genes differentially methylated in both PND7 and PND90 are known for their involvement in different aspects of protein transport and secretion. One of these genes was annexin A6 (Anxa6). Anxa6 is a lipid binding protein and is involved in the endocytic machinery via its interaction with membranes and the actin cytoskeleton (Grewal et al., 2010). A report has indicated that patients who are obese and type 2 diabetic have higher protein levels of Anxa6 in monocytes, with increasing levels positively correlated with body mass index (Stögbauer et al., 2009). In the brain, Anxa6 is associated with lipid rafts (membrane microdomains enriched with cholesterol) where Anxa6 plays a role in a number of signal transduction cascades that take place in addition to the trafficking of transmembrane receptors in the cell (Cubells et al., 2007).

Another two differentially methylated genes shared between PND7 and PND90, Ras-related protein Rab-5A (Rab5a) and TBC1 domain family member 10b (Tbc1d10b), are also involved in vesicles mediated protein transport and secretion. Endosomes mediates protein transport for either degradation via lysosomes or for release into the extracellular matrix via exosomes (Gauthier et al., 2017). GO terms associated with protein catabolism and proteolysis were highly enriched by differentially methylated genes at PND7. Internalization of transmembrane receptors through endocytosis is essential for neurotrophic factors signaling and neurotransmitters signaling mediating long-term potentiation (Park et al., 2004; Zhou et al., 2012). Endosomal abnormalities have been reported in Alzheimer's disease and Down syndrome and was linked to upregulated expression of Rab5a (Kim et al., 2016). Our results show that perinatal HFD was associated with hypomethylation in the promoter region of Rab5a, which could be linked to increased expression and associated with endosomal machinery abnormalities.

TBC1D10 (A-C) is a family of Rap GTPase-activating protein that regulate exosome secretion and endosome recycling, where overexpression of this family of proteins reduce exosome secretion and inhibit endosomal recycling (Chaineau et al., 2013; Hsu et al., 2010). In this study, we found hypomethylation in the promoter region of Tbc1d10b in HFD female offspring, which might be associated with higher expression Tbc1d10b and leading to abnormalities in endosomal

processing and exosomal secretion. Studies have reported an increase in exosomes in circulation of obese patients along with differential content of exomes in rodent models of obesity and in obese humans (Huang-Doran et al., 2017). Exosomes obtained from adipocytes of obese mice when injected into control animals induced macrophage differentiation in monocytes, which led to an increase in pro-inflammatory cytokine levels, and insulin resistance (Deng et al., 2009). Exosomes are gaining a lot of interest as novel mediators of across systems communication, which might be essential in metabolic disorders that affect several bodily systems including the nervous system.

Protein phosphorylation regulation GO terms have been enriched with differentially methylated genes in HFD offspring in both developmental stages. I found 4 core signaling pathways, MAPK, cGMP-PKG, cAMP, and calcium signaling pathways that were enriched with differentially methylated genes from both time points from the KEGG pathways enrichment analysis. These pathways are regulated through a cascades of protein phosphorylation regulation and play essential roles in several other pathways that were found from the enrichment analysis, such as axon guidance, oxytocin signaling, neurotrophin signaling, and long-term potentiation and depression.

Methyl CpG binding protein 2 (Mecp2) was one of the genes that was consistently differentially methylated across the time point examined. Its regulation through phosphorylation has been shown to modulate its activity and downstream expression of its target genes. MeCP2 phosphorylation via Ca2+/calmodulin-dependent protein kinase II (CaMKII) through neuronal activity reduce MeCP2 binding to the DNA and affect brain development (Cohen et al., 2011; Murgatroyd et al., 2009). In fact, in a model of early postnatal stress, CaMKKII increased activity in the PVN mediated a reduction of MeCP2 binding to arginine vasopressin (AVP) promoter region, consequently reducing methylation, increasing its expression, and mediating the effects of early life stress on the HPA axis of these animals in adulthood (Murgatroyd et al., 2009). Interestingly, CaMKKII was one of the genes annotated to a hypomethylated DMR at the gene body at PND7. CaMKKII is a central protein in a number of pathways that mediate neuronal development and signaling making it a key target to examine in the future to understand the effects of perinatal HFD on offspring neurodevelopment and later neuropathology.

MeCP2 is a methyl-binding protein that was described to bind to methylated CpG (mCG) to suppress gene expression (Nguyen et al., 2012). There are other reports of MeCP2 binding to non-CpG methylated DNA (mCH), which is a methylation mark that progressively increases in the brain throughout postnatal life (Chen et al., 2015; Lister et al., 2013a). Mutations in Mecp2 that affects the appropriate dosage of Mecp2 levels lead to the neurodevelopmental diseases Rett syndrome and MECP2 duplication syndrome arising from loss-of-function and gain-of function, respectively (Kinde et al., 2015). An earlier report found that global DNA hypomethylation in a number of brain regions including the hypothalamus and the prefrontal cortex in adult mice offspring exposed to maternal obesity induced by HFD consumption (Vucetic et al., 2010). Mecp2 was hypomethylated in the prompter region, which could be associated with an increase of Mecp2 transcript levels. Future work could assess the link between the levels of Mecp2 and differences in the proportion of mCG and mCH in HFD offspring.

5.4.2 PND7 specific pathways enriched with differentially methylated genes in HFD offspring

PND7 specific enriched pathways included ones that regulate neuronal development such as Wnt signaling, thyroid hormone signaling, and neurotrophin signaling pathways, which were also reflected in the number of GO terms that were involved in neuronal development. There are several reports of alterations in thyroid hormones levels and genes involved in its synthesis in offspring from obese mothers across different species. In humans, increased obesity in mothers was associated with higher thyroid hormones in mothers and their neonates (Kahr et al., 2016). Similar results were observed in weanling rats from obese dams on HFD showing an increase in their thyroid hormone levels (Tabachnik et al., 2017). In primates, fetuses from obese mothers showed a decrease in thyroid hormone levels along with a decrease in genes that mediate thyroid hormone synthesis in the hypothalamus and thyroid gland (Suter et al., 2012). Others reported an enrichment of thyroid hormone related genes in differentially expressed transcripts in the placentas of offspring in obese rat dams (Saben et al., 2014). These thyroid hormone-related transcripts showed a general downregulation in the placentas of obese animals (Saben et al., 2014).

Thyroid hormone has been documented to mediate the effects of the natural variation in maternal care behaviour in rats on HPA axis negative feedback programming. Increased tactile stimulation

through licking and grooming in rats induces an increase in thyroid hormone levels along with serotonin, both of which were associated with a recruitment of nerve-growth factor-inducible factor A (NGFI-A) to Nr3c1 promoter to induce its expression (Hellstrom et al., 2012). This recruitment was also mediated by cAMP signaling, a pathway enriched with differentially methylated genes in HFD offspring in this study (Hellstrom et al., 2012). In adulthood, animals receiving more tactile stimulation show hypomythlation in the same promoter (Weaver et al., 2004). Other studies have demonstrated that tactile stimulation is associated with a drop in pup body temperature leading to the increase in thyroid hormone (Meaney et al., 1991). Thyroid hormone is known to induce thermogenesis through BAT (Silva and Larsen, 1985). I have observed (Chapter 2&3) an increase in time spent on nest nursing in HFD dams, which could be linked with increased nest temperature. I have also discussed (Chapter 2) reported impairments in BAT thermogenic capacity in HFD offspring. Future work should examine the relationship between thyroid hormone levels, thermogenesis, maternal care behaviour in HFD animals.

In addition to thyroid hormone pathway, Wnt and neurotrophin signaling pathways were enriched with PND7 differentially methylated genes. Modification of these pathways could affect synaptogenesis and neuronal connections between the amygdala and other brain regions integrating the stress signal. For example, neuronal innervation from the amygdala to the prefrontal cortex are seen at PND7 and increases to reach adult-like patterns by PND11 (Bouwmeester et al., 2002). Therefore, impaired neuronal connections made during this early period of life could set the stage for future psychological dysfunction.

5.4.3 PND90 specific pathways enriched with differentially methylated genes in HFD offspring

On the other hand, PND90 specific KEGG pathways included Ras and Rap1 GTPase signaling pathways, which are activated by and regulate neurotransmitters signaling at glutamatergic and GABAergic synapse, another two KEGG pathways enriched specifically at PND90. This is also in line with number of GO terms that are involved in GTPase regulation enriched with PND90 differentially methylated genes. Perhaps alteration in neuronal connections at an earlier developmental stage affects the neurotransmitter signaling in mature neurons rendering them vulnerable to abnormal activation leading to psychiatric diseases. In addition, a number of disease related pathways were enriched with differentially methylated genes at PND90 including

cardiomyopathy and cancer. There are reports of programming effects of maternal obesity on myocardial functioning and development of hearth diseases (Dong et al., 2013; Fernandez-Twinn et al., 2012), however it is not clear whether differential DNA methylation in the brain would be consistent also in the heart.

Interestingly, Nr3c1 and FK506 binding protein 5 (Fkbp5), a chaperon protein that binds GR and inhibit its translocation into the nucleus and thus its genomic actions (Jääskeläinen et al., 2011), were differentially methylated at PND 90. Nr3c1 and Fkbp5 were associated with hypomethylation and hypermethylation in the gene body, respectively. Our lab has previously shown an increase in Nr3c1 expression in HFD adult female offspring in the amygdala (Sasaki et al., 2013). GR in the amygdala functions to activate the HPA axis (Joels et al., 2008). It would be interesting to validate gene expression in the same samples and draw direct correlation between gene expression and DNA methylation. GR is known to induce the expression of Fkbp5, thus indirectly regulating its own activity (Jääskeläinen et al., 2011). It is not clear whether hypermethylation in the gene body of Fkbp5 here is associated with increased gene expression and future work need to be done to demonstrate the differential expression of Fkbp5 in HFD female offspring. Increase in Fkbp5 expression is associated with single nucleotide polymorphism (SNP), which is linked to GR resistance and decreased HPA axis negative feedback efficiency (Binder, 2009). In mice hippocampus, induction of Fkbp5 expression via GR was also associated with a demethylation in intronic regions of the Fkbp5 gene (Scharf et al., 2011). These findings if explored further would shed light on a possible mechanism through which perinatal HFD programs adult female offspring phenotype described in an earlier study (Sasaki et al., 2013).

5.4.4 Conclusion

This chapter investigated for the first time a genome-wide differential methylation in response to perinatal HFD in female offspring at two different developmental time points in the amygdala. It revealed a number of conserved and age specific DMRs and differentially methylated genes across the two postnatal periods examined, which provides insights into key biological processes and pathways differentially regulated by perinatal HFD. Examining these pathways in future studies would provide us with further understanding of the molecular mechanisms underlying

perinatal HFD impacts on offspring phenotype, which can ultimately lead to the development of intervention strategies that ameliorate the effects of perinatal HFD.

Chapter 6

General discussion

6 General discussion

6.1 Thesis overview:

The main objective of this thesis was to examine the programming effects of two different environmental factors, maternal obesity and prenatal stress, and their interaction on offspring phenotype at different developmental stages, neonatal and adulthood. I utilized maternal HFD consumption prior to and throughout gestation and lactation as a model for maternal obesity and CVS during the last week of gestation as the prenatal stress paradigm. Both protocols have been previously characterized and used to program HPA-axis related behavioural, physiological, and molecular outcomes in the offspring (Boersma et al., 2013b; Mueller and Bale, 2008; Sasaki et al., 2013; Tamashiro et al., 2009). Overall, I found strong main effects of perinatal HFD on offspring HPA axis-related phenotype, including anxiety-like behaviour and Cort response to stress during the neonatal period and adulthood, while prenatal CVS showed minimal effects. However, CVS on a perinatal HFD background showed a dimorphic impact on offspring phenotype that was dependent upon the developmental period of the offspring. Specifically, CVS normalized the effects of perinatal HFD during the hyporesponsive period in neonates and in adult female offspring, while it augmented the impacts of perinatal HFD on neonatal offspring emerging from the stress hyporesponsive period. In order to better understand these outcomes in the offspring, I also characterized the maternal physiological and behavioural responses to these two environmental factors, as they might mediate their effects through alteration in the maternal phenotype. Again, HFD consumption showed strong main effects, while CVS had no major impacts on maternal phenotype. However, an interaction between HFD and CVS render the HFD mothers more sensitive to CVS exposure compared to control mothers. I also explored the link between dam and pup communication as a possible mediator of perinatal HFD by characterizing USV types and maternal retrieval.

Given the more pronounced impact perinatal HFD had on both mothers and offspring, I prioritized investigating the effects of perinatal HFD on offspring gene expression and epigenome in brain regions that regulate the HPA axis, including the PVN, ventral hippocampus and amygdala. The early activation of the HPA axis observed in HFD neonates in the stress hyporesponsive period (PND7) was associated with increased Crh gene expression in the PVN

and differential methylation of genes involved in neuronal development in the amygdala of females offspring. HFD pups emerging from the stress hyporesponsive period (PND13) exhibited a disinhibited HPA axis that was associated with decreased Nr3c1 and increased Gad1 expression in the ventral hippocampus (Abuaish et al., 2018). In adulthood, HFD females offspring showed differential DNA methylation in genes involved in protein phosphorylation, specifically GTPase related genes, in the amygdala, which are important in neurotransmitters signaling.

6.2 Mothers as mediators of environmental factors

Many studies examining maternal programing of early life environments on offspring phenotype failed to characterize the impacts of these factors on the maternal phenotype. Mothers are the primary conduits through which these factors could mediate their effects onto the offspring. Therefore, it is essential that we understand the impacts these factors have on the mothers' physiological and behavioural outcomes. One of the main aims of this thesis was to characterize maternal phenotype (Chapter 2) in response to HFD consumption during the perinatal period and the exposure to CVS during the last week of gestation.

6.2.1 Maternal physiological and metabolic phenotype

A number of reports have indicated that HFD induced obesity in rodents could be considered as a chronic stressor (Buwalda et al., 2001; Pascoe et al., 1991; Tannenbaum et al., 1997). For example, an earlier report has demonstrated an increase in anxiety-like behaviours in females exposed to HFD, which was associated with lower glucocorticoid receptors expression in the hippocampus, indicating that HFD obese females might have an already higher stress reactivity prior to pregnancy (Sivanathan et al., 2015). I have assessed HFD females' reaction to stress during pregnancy by measuring different physiological and metabolic outcomes, including their Cort response to restraint stress and their weight gain and caloric intake in response to CVS. HFD females showed increased stress sensitivity in all three measures, as they showed higher Cort levels to restraint stress, and had lower weight gain and caloric intake during gestation in response to CVS exposure. These measures, specifically weight gain and food intake, did not change in the CHD females, which was an expected feature of the CVS paradigm that has been previously reported (Mueller and Bale, 2006; Tamashiro et al., 2009). Interestingly, similar effects were observed in adult HFD obese mice exposed to CVS paradigm using social defeat

and overcrowding as stressors, where only HFD mice showed a significant decrease in body weight and a pronounced decrease in caloric intake (Finger et al., 2011). In that study, CVS exposure in adult mice was associated with a decrease in leptin levels and insulin levels in HFD animals compared to control diet levels (Finger et al., 2011). Considering these interactions between stress and obesity in modulating the maternal metabolic phenotype would help us in understanding the effects observed in the offspring.

6.2.2 Maternal behaviour and pup interaction

It is well established the effects maternal care behaviour variation have on offspring phenotype specifically HPA axis programming, where increased maternal care reduces anxiety-like behaviours, decreases Cort reactivity to stress, and increases Nr3c1 expression in the hippocampus of adult offspring (Liu et al., 1997; Suderman et al., 2012; Weaver et al., 2004). Maternal care has been reported to be influenced by both prenatal stress and HFD consumption (Bertino, 1982; Bosch et al., 2007; Champagne and Meaney, 2006; Purcell et al., 2011; Smith et al., 2004). In this thesis, I examined maternal care in two different forms: 1) daily natural maternal care provided in home cage during the first 6 postnatal days and 2) maternal pup retrieval test on PND7. Consistent across the two different cohorts examined (Chapters 2 &3), HFD dams spent more time on the nest engaging in more nursing in general compared to CHD dams, where they also showed differences in the type of posture they engaged in while nursing. Specifically, HFD dams were performing more blanket nursing, where they lay on top of their pups, and less supine or "passive" nursing, where dams lay on their side or back while pup nurse. Dams modulate their temperature and nest temperature through the time spent on the nest and the posture of nursing they use (Jans and Woodside, 1990). For example, during blanket nursing there is more heat transfer between the pups and the dam through occlusion of her ventral body, but this transfer is reduced when mothers take a passive posture (Stern and Lonstein, 1996). Interestingly, an earlier study has reported that obesity induced by HFD in rats was associated with lower body temperature in these animals (Buwalda et al., 2001). It is possible that HFD litters have an impaired thermoregulation due to dams' lower body temperature that would influence their sensitivity to the nest temperature leading to increased nursing behaviour as a byproduct. This increase in nursing behaviour observed in HFD dams has been proposed to contribute to the hyperphagic behaviour and higher weights of their pups (Purcell et al., 2011). In chapter 4, I found that HFD dams also showed an impaired pup retrieval, as they retrieved less

pups and more of them failed to retrieve all pups by the end of the retrieval test compared to CHD dams.

Maternal behaviour occurs in conjunction with pup physiological and behavioural input. In chapter 5, I investigated USVs, an essential pup behaviour that is known to modulate maternal behaviour to ensure the survival of the pup. I characterized USVs at PND7 into categories that have been proposed to have a function in maternal recruitment and reported a sex-dimorphic effect of HFD on the types of USVs produced by pups. HFD females exhibited a less optimum call type in recruiting mothers, while HFD males engaging in a more efficient USV calls. These sex differences in call type in HFD offspring might be the starting point of adult animal phenotype programming as seen in chapter 2, where HFD male offspring showed a decrease in anxiety-like behaviour, while female offspring exhibited an increase in anxiety-like behaviour.

HFD pups also showed a pronounced decrease in USVs at PND7 when isolated from their mothers, which could be linked to the lower maternal retrieval behaviour in the HFD dams as discussed in chapter 4. It is also important to consider the possible contribution of maternal care received during the first 6 postnatal days on the USV outcome at PND7. The increase in time spent nursing by HFD dams might have led to increased nest temperature. This could program the thermoregulation capacity of the HFD pups and influence their USV emissions. In fact, previous research has shown that pups raised in warm nests have a delayed thermoregulation ability and exhibit increased activity and ingest more milk (Jans and Woodside, 1990; Johanson and Hall, 1980). Interestingly, I report increased activity in HFD pups at PND7, while others report impaired thermoregulation and increased ingestive behaviour in HFD pups (Liang et al., 2016; Purcell et al., 2011). USVs are known to be in part influenced by thermoregulation ability of pups, which might explain the finding in this study (Allin and Banks, 1971; Brunelli and Hofer, 1996). In summary, physiological and metabolic changes in the dam in response to stress, including obesity, might impact their behavioural output towards their offspring influencing the pups' physiology and behaviour that in turn affect the reciprocated maternal behaviour.

6.3 Offspring HPA axis programming by perinatal HFD and its interaction with prenatal CVS

In the research presented in this thesis, I examined the programing effects on the HPA axis in offspring at the physiological, behavioural, and molecular levels. One of the highlights of this

thesis is characterizing the neonatal phenotype in chapter 3, a developmental period that is underexamined in the literature when examining programming effects of perinatal environment in offspring. The neonatal period is considered a critical period of development where the offspring are still malleable to the effects of their maternal environment. As discussed above both stressors used in this study have shown effects on maternal physiological and behavioural outcomes, which in turn could in part influence the programing of the offspring phenotype during the neonatal period.

6.3.1 Perinatal HFD effects on offspring HPA axis related phenotype

Throughout this thesis perinatal HFD showed the more prominent effect on both maternal and offspring outcomes. Neonatal HFD offspring showed altered HPA axis related behavioural, physiological and molecular phenotype during a well characterized HPA axis developmental switch. One of the strengths of this study was using ethologically relevant behavioural outcomes and stressors to examine the HPA axis phenotype in neonate. I have utilized USVs as a behavioural measure, which has been shown to be modulated by different factors of the HPA axis, including Cort and Crh levels, and is one of the main behavioural output of neonates engage in to ensure their survival (Insel and Harbaugh, 1989; Moriceau et al., 2004; Takahashi, 1995). In addition, I have used an ethologically relevant stressor to neonates by exposing them to adult male soiled bedding to examine the HPA axis reactivity (Takahashi, 1992). I observed an abnormal activation of the HPA axis in HFD neonates, with early activation observed during the stress hyporesponsive period followed by a disinhibited HPA axis during stress responsive age. I have examined gene expression alterations in two brain regions: the PVN, the main site of HPA axis activation via Crh, and the ventral hippocampus, the main region mediating the HPA axis negative feedback via Nr3c1 (Herman et al., 2016). In addition, I also evaluated the expression levels of Gad1 to shed light on the nature of the negative feedback circuit within the ventral hippocampus, which is highly regulated by GABAergic interneurons that inhibit the excitatory glutamatergic neurons output to region inhibiting the PVN (Gunn et al., 2015). Future studies discussed below need to be carried out to understand the underlying pathways that mediate these molecular differences associated with the characterized HPA axis phenotype described in this work.

In adulthood, perinatal HFD induced increased anxiety-like behaviour in the OFT for female offspring only, while HFD males showed decreased anxiety-like behaviour on the EPM. The anxiety-like phenotype was not associated with any differential Cort response to restraint stress. Despite the contradicting results in males, HFD had similar effects on female offspring in chapter 2 compared to earlier reports, where female offspring showed pronounced changes in phenotype in response to perinatal HFD (Sasaki et al., 2013). Inconsistency, however, in male offspring phenotype is one of the limitations of this study that will be discussed below.

6.3.2 Prenatal CVS effects against a perinatal HFD background in offspring

Overall, CVS alone had minimal effects on offspring in the results presented here. Earlier studies using similar prenatal CVS paradigm did not report any effects of CVS on adult offspring anxiety-like behaviour, however they showed alteration in sociality, cognition, and depressive-like behaviours (Abdul Aziz et al., 2012; Hougaard et al., 2005; Jones et al., 2013; Koenig et al., 2005; Mueller and Bale, 2008; Richardson et al., 2006). I observed a decrease in anxiety-like behaviour in adult males, while female offspring showed an increase in anxiety-like behaviour. Only one study has examined the effects of prenatal CVS on pup USVs and reported an increase in call numbers in prenatally stressed pups at PND15, indicating increased anxiety-like behaviour (Harmon et al., 2009). Others using prenatal restraint stress have also reported an increase in USV umbers at PND10 and PND14 (Laloux et al., 2012; Williams et al., 1998). Using a more severe prenatal stressor, electric shock, Takahashi et al., (1990) reported a reduction in USV counts at PND14. My results indicate a non-significant trend of an increase in USV counts at PND7. Prenatal CVS protocols implemented in the literature use different stressors that vary in their severity, with some only using mild stressors, which include could include food and water deprivation and leaving out intermediate physical stressors such as restraint and swim (Hougaard et al., 2005; Richardson et al., 2006). Others avoid using food deprivation to not induce *in utero* growth restriction, while they use restraint and swim as stressors (Abdul Aziz et al., 2012; Harmon et al., 2009; Jones et al., 2013; Koenig et al., 2005; Mueller and Bale, 2008). Therefore, the different combination of stressors used in this paradigm might make it hard to compare results across studies, especially that many of the stress paradigms reported in the literature do not provide evidence of an enhanced maternal HPA axis.

The main hypothesis of this thesis was that the combined exposure to maternal perinatal HFD and prenatal CVS would have synergistic effects on offspring HPA axis phenotype. In general, I found that prenatal CVS normalized the effects of perinatal HFD in neonates, specifically at PND7, and adult offspring. At PND7, HFD/CVS pups showed comparable Cort and ACTH levels to CHD animals. In addition, the number of USVs emitted at PND7 by HFD/CVS pups were not significantly different from CHD/Control animals (Chapter 3). In adult animals, similar normalizing effects were observed in the OFT for female offspring, where HFD/CVS spent comparable time in the centre of the open arena to CHD/Control offspring (Chapter 2). As discussed above, CVS also normalized the HFD dams' metabolic phenotype in this thesis. Although I did not investigate the levels of leptin and insulin in my animals, others have reported that chronic stress in obese animals normalizes elevated leptin and insulin levels in these animals (Finger et al., 2011). An earlier study have demonstrated elevated insulin levels during lactation due to maternal HFD consumption impaired the hypothalamic neurocircuit formation (Vogt et al., 2014). Therefore, the actions of other hormones should be considered to help understand the phenotype observed in the HFD/CVS.

At first glance, one might think that the hypothesized synergic effect of prenatal stress and HFD was observed, when HPA axis is functional, in PND13 pups, where HFD/CVS had a pronounced Cort response to stress compared to all other groups. However, alternatively, considering the total effects of HFD at different levels of the HPA axis, including both ACTH and Cort response, I observed a heightened stress response in all HFD animals in general. However, there was a dissociation between the ACTH and Cort response in the HFD/Control pups, which I propose to be due to earlier stimulation of the adrenal gland at PND7 leading to its desensitization to ACTH signal. Therefore, I argue that while the prenatal CVS might have led to temporary rescue of HFD effect on offspring through modifying maternal metabolic phenotype, as dams recover from the stress insult and continue consuming a HFD, PND13 offspring start exhibiting a heightened stress response that were spared from during the hyporesponsive period, unlike the HFD/Control animals.

6.4 The developmental epigenetic signature of perinatal HFD

Throughout this thesis HFD main effects were the most pronounced relative to the CVS effects, specifically in females, such as USV call type differences and adult anxiety-like behaviour.

Therefore, in collaboration with other lab members we examined the differential DNA methylation in response to perinatal HFD across development in neonates and adult offspring in the amygdala, a brain region that facilitate HPA axis activation and mediate emotional behaviour in response to stress, which are the two features that are heightened in HFD offspring (Herman et al., 2016). Common HFD differentially methylated genes and GO terms across the ages revealed significant enrichment in biological processes involving protein transport, secretion and phosphorylation. Three genes form the commonly differentially methylated genes (Anxa6, Rab5a, and Tbc1d10b) were involved in endosomal machinery, which is one form of transporting proteins and secreting them via a cascade of phosphorylation of different proteins (Huang-Doran et al., 2017). Endocytosis is essential for neurotrophic factors signaling and neurotransmitters signaling mediating long-term potentiation, which are KEGG pathways enriched in at both developmental stages, through internalization of transmembrane receptors (Park et al., 2004; Zhou et al., 2012). Age specific enrichment analysis indicated that alteration in neuronal connections at an earlier developmental stage suggested by the enrichment of neuronal development terms and pathways at PND7 might affect neurotransmitter signaling pathways, including glutamatergic and GABAergic synapse, which are enriched with differentially methylated genes in mature neurons at PND90, rendering them vulnerable to abnormal activation leading to psychiatric diseases.

6.5 Study limitations and future directions

There are a number of caveats one needs to keep in mind when examining the findings in this thesis, which could be addressed in future studies.

6.5.1 Maternal physiological and behavioural measures

One of the reported advantages of using a CVS paradigm was the induction of a more robust HPA axis activation (Koenig et al., 2005). However, only two studies using prenatal CVS has measured maternal Cort levels and reported an increase in pregnant females' basal serum and amniotic fluid Cort levels (Abdul Aziz et al., 2012; Tamashiro et al., 2009). In chapter 2, I was not able to observe any CVS effect on HPA axis when measuring both basal levels of Cort or its response to restraint stress. Chronic stress has been shown to facilitate a higher HPA axis response to subsequent acute novel stressors (Ferland et al., 2014). Pregnant females have been exposed to restraint multiple times during this stress paradigm and they might be showing a

habituated response to this homotypic stressor, therefore future experiment should asses their Cort response using a novel acute stress. CVS on the other hand, had pronounced effects on HFD dams weight gain and food intake. In chapter 2, HFD/CVS dams also showed a decrease in litter size and an increase in pup postpartum mortality rate, which was not observed in the second cohort presented in chapter 3. One modification I have added to the CVS paradigm was substituting the platform stressor with an EPM, because I have found that a number of females were falling off of the platform. I do not have enough data to conclude an association between the litter characteristics and animals that have fallen off of the platform, however. One observation worth mentioning in cohort 1 is that by random chance HFD/CVS seemed to be heavier than HFD/Control specifically during gestation, which might be associated with inconsistent findings across the two cohorts, specifically the litter characteristics.

I have also faced a challenge with maternal behaviour data from the first cohort with video records missing due to technical issues, with a number of animals only having 1-2 observation hours vs. 6 observation hours in animals with complete data set. There are some evidence that maternal behaviour is higher during the light period (Champagne et al., 2003). Therefore, unequal observation across day and night due to data loss might not reflect accurate results. Despite these issues, a complete set of data from the second cohort in chapter 3 have indicated consistent effects of HFD on time spent on nest nursing and differences observed in the nursing posture as discussed above. Future studies should examine the relationship between this differential nursing behaviour and thermoregulation in both dams and pups and underlying mechanisms as a possible mediator of perinatal HFD programming. I have also examined maternal emotional behaviour in dams postpartum, however I did not observe any effect of the diet or stress manipulations in the dams. Testing was done 10 days after euthanizing all pups on PND13, which lead to engorgement of mammary glands in the HFD dams and could be a confounding factor when comparing results across the diet groups. Future experiments should examine emotional behaviour in dams during their HPA hyporesponsive period, when they are more prone to develop postpartum alteration in their emotionality (Deschamps et al., 2003; Hillerer et al., 2011; Leuner et al., 2014).

6.5.2 Offspring physiological and behavioural measures

One puzzling finding in this thesis was the inconsistent results in anxiety-like behaviours compared to an earlier study published in our laboratory. Sasaki et al. (2013) reported an increased anxiety-like behaviours in HFD adult male and female offspring in the OFT and EPM, respectively. Here, I have observed decreased anxiety-like behaviour in HFD male offspring in the EPM, while females showed an increased anxiety-like behaviours in the OFT. Several reasons could explain this discrepancy. In this cohort, I switched HFD litter to CHD at PND17 to ensure that the offspring did not consume the diet themselves and the effects of the diet were mediated through the mothers only (Tozuka et al., 2009). Earlier work has indicated that withdrawal from HFD activate the HPA axis and induce anxiety-like and depressive-like behaviours (Sharma et al., 2013). While others, showed that dams exposed to HFD had an exaggerated Cort response to stress postpartum (Perani et al., 2015). I propose that the diet switch might has acted as a stressor to HFD dams, who have a reactive HPA axis during a period of a naturally blunted stress, and consequently the offspring during the postpartum period, which could be associated with the adult offspring phenotype observed in this study. In addition, this cohort did not show body weight differences throughout the pre-weaning postnatal life, a main effect of perinatal HFD that was observed by others (Bilbo and Tsang, 2010; Sasaki et al., 2013; Tamashiro et al., 2009).

The second cohort (Chapter 3-4) of animals allowed me to investigate the neonatal phenotype, including their stress reactivity and USVs. One major caveat with the USV measurement was not assessing the temperature in the pups, which could be one factor affecting their counts, especially in PND7. In a small separate cohort, I measured the temperature of the pups prior to testing but that induced higher counts of USVs relative to the second cohort in both diet groups. In addition, in this small cohort, pups at PND7 were significantly heavier than CHD pups, a finding I did not observe in the second cohort. Therefore, I could not generalize the effect of HFD on body temperature from the small cohort to the second cohort in order to explain the USV differences. As discussed above, studying thermoregulation in the future in these animals would be an interesting avenue to explore.

HFD neonates in cohort two only started showing higher weights by PND13, while earlier studies in our lab and by others have reported heavier offspring as early as PND7 (Sasaki et al.,

2013; Tamashiro et al., 2009). I was able to detect the reported higher weight at PND7 in the small cohort. This cohort was run separately from the main cohorts, which included all treatment groups HFD and stressed animals. One possible explanation could be that non-stressed animals are indirectly exposed to stress as bystander conspecifics as they were housed in the same room and on the same rack, which could explain the lack of consistency between results in this thesis earlier work publish in our laboratory. There is evidence that animals communicate stress through the release of pheromones (Mackay-Sim and Laing, 1980; Tirindelli et al., 2009). Some studies have reported programming effects in adult offspring exposed to prenatal bystander stress, when their mothers were housed with cage mates that were exposed to daily restraint stress (Mychasiuk et al., 2012, 2011b, 2011a).

Examining pups at PND13 could also be criticized as a limitation of this study because most studies examining the ontogeny of HPA axis responsiveness argue that the stress hyporeponsive period ends by PND14. However, a gradual HPA response has been observed as early as PND10 (Vázquez and Akil, 1993). In addition, I have chosen an earlier time point to capture any differences in animal maturation. Rat pups start opening their eyes by PND14, a milestone of rodent development (Bjerknes et al., 2015). This study suggests an early maturation of HFD neonates measured by the higher proportion of litters with pups with open eyes compared to the CHD litters at PND13. Since I did not want to handle the animals too frequently during the early postnatal period, I checked eye opening in pups solely on the days of testing. By testing animals at PND13, I was able to detect a more pronounced difference in eye opening proportions between diets, which would have been missed if testing was performed on animals at PND14, when more CHD animals would have had open eyes.

6.5.3 Offspring epigenetic measures

One of the limitations of this study is that we have focused on examining differential methylation in only the amygdala of female offspring. However, there is evidence, presented above, indicating female specific behavioural susceptibility to perinatal HFD, encouraging us to examine the effects on the epigenome in female amygdala in order to expand earlier findings done by our lab associated with HPA axis programming in these offspring. In addition, this preliminary study could be used as a proof of concept for using RRBS in analyzing DNA methylation in rats, where to our knowledge has been only used once before (Luo et al., 2017).

Future experiments could examine the differential DNA methylation in other limbic regions that regulate the HPA axis, including the hippocampus and the prefrontal cortex, to isolate pathways and biological processes impacted by perinatal HFD.

In this thesis, we have examined DNA methylation in the total amygdala, which could be one of the caveats of this study, since there are three nuclei in the amygdala, which display different properties mediating behavioural and physiological responses to stress and differential gene expression. For example, a microarray study examining differential gene expression signature of different brain regions, including the amygdala, reported that only 2.4% of differentially expressed genes were enriched in one brain region, with 33 genes enriched in total amygdala compared to other brain regions. Interestingly, 75% of the amygdala specific genes have shown restricted expression to the boundaries of the different nuclei of the amygdala (Zirlinger et al., 2001). Another study has demonstrated that different amygdala nuclei exhibit differential gene expression measured by RNA microarray in 129 genes, indicating differential molecular signature of these nuclei, with basal amygdala showing amore divergent signature compared to the lateral and central amygdala nuclei (Partin et al., 2013).

In addition to the different nuclei, our samples are also composed of different cell populations and not purely neuronal, whereas epigenetic signatures are to some extent cell-type specific (Lister et al., 2013b). Recent work has illustrated *in silico* that cell proportion could change in a mouse model of Gulf war illness, which contributed to epigenetic differences observed (Ashbrook et al., 2018). It is not known if perinatal HFD could influence the proportion of different cell populations in the brain, however we could not rule out that possibility and its contribution to differences in DNA methylation between diet groups. Yet, despite the mixed cell population, which could possibly dilute differences in DNA methylation, we were able to significantly detect pronounced DNA methylation differences between the two diet groups in this investigation. In order to distinguish differential DNA methylation due to perinatal HFD between neuronal and non-neuronal cells, future studies could implement Fluorescence-Activated Cell Sorting (FACS) to isolated the different cell populations and assess their epigenetic signature. Lastly, future experiments combining differential transcriptomics using RNA sequencing data to the epigenetic data will enrich our understanding of the translation of these epigenetic differences observed into more functional differences at the transcript level.

6.6 Conclusion

Despite some of the limitations of this work, this thesis has provided a number of novel findings and proposes future avenue of investigation that will aid in understanding the impact perinatal HFD has on the development and programming of offspring phenotype at different biological levels: behavioural, physiological, transcriptomic, and epigenetic outcomes. By characterizing maternal and neonatal phenotype, which is largely ignored in many studies examining maternal programing, I explored possible interaction of maternal measures (maternal care behaviour) and offspring measures (USVs) in mediating the effects of the perinatal maternal HFD consumption. In addition, this thesis also provides a unique look at the signature of perinatal HFD has on the epigenome differences across developmental stages, highlighting common genes and pathways differentially methylated across development and age-specific methylation differences. These findings could help future intervention studies by targeting specific dam-pup behavioural interaction, modulate maternal metabolic phenotype, or molecular pathways that has been differentially affected by perinatal HFD. Targeting this early period of life, when offspring brain is still under development and plastic to environmental manipulations, would yield more effective interventions in rescuing the effects observed in offspring in response to perinatal HFD and prenatal CVS.

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