Male Experience, Female Investment and Offspring Anxiety Behavior: Where Father's Nature Meets Mother's Nurture

by

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This thesis is dedicated to my wife, Jill King. You have been there for every part of this, and I appreciate all of your love, support, insightful conversations, and lunches.

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ABSTRACT

Mammals, like most species, change in physiology and behavior over the course of their lifespan. Lifetime experiences and environmental exposures can potentially alter the developmental trajectory of their offspring, not only by inherited genetic variation in the germline, but also through a change in parental behavior and, in turn, an altered nurturing environment. Such developmental variation is constrained by the effects of complex genotype by environment interactions on the spatial and temporal expression and function of genes influencing specific phenotypic traits. The question then concerns the exact social and biological mechanisms underlying the transfer of one generations experiences to the next. Innovative and novel research has suggested that the germline is not the only mode of inheritance and that previous generations may pass information regulating somatic gene expression, ultimately shaping our ability to respond and interact with the environment. In this thesis, I describe the impact of paternal (male) stress and high fat diet exposure, prior to conception, on mate preference, maternal care as well as growth and the development of stress-related behaviors in the offspring. I also examine the effect of housing quality on maternal care, and how these effects interact with preconception paternal experience. Offspring behavior and stress-related gene regulation and expression were altered by both paternal experiences and the quality of maternal care, suggesting that paternal exposures and maternal rearing conditions influence maternal behavior and the development of individual differences in stress responses in offspring. Overall, the work in this dissertation provides further evidence that development begins before conception and that gene regulation and predisposition are influenced by parental experiences.

LIST OF ABBREVIATIONS USED

DNA	deoxyribonucleic acid
HPA	hypothalamic-pituitary-adrenal
LG	licking and grooming
Evo-Devo	evolutionary-developmental
PO	predator odor
HFD	high-fat diet
GC	glucocorticoid
CNS	central nervous system
PTSD	post-traumatic stress disorder
MetS	metabolic syndrome
PVN	paraventricular nucleus
CRF	corticotropin-releasing factor
AVP	arginine vasopressin
ACTH	adrenocorticotropic hormone
GPCR	guanine-protein-coupled receptor
CRF-R1	CRF-receptor type 1
POMC	proopiomelanocortin
MC	melanocortin receptor
cAMP	cyclic-adenosine-3',5'-monophoshpate
HMGCoA	3-hydroxy-3-methylglutarylcoenzyme-A
LDL	low-density lipoprotein
11-dGC	11-deoxycorticosterone
GR	glucocorticoid receptor
MR	mineralocorticoid receptor
CRF-R2	CRF-receptor type 1
Ucn	Urocortin
CRFBP	CRF-binding protein
hnRNA	heteronucleotide RNA
EH	early-handling
MS	maternal separation
mRNA	messenger ribonucleic acid
BNST	bed nucleus of the stria terminalis
ABN	arched back nursing
5-HT	serotonin
NGFI	nerve growth factor-inducible
ER	estrogen receptor
NMDA	*N*-methyl-D-aspartate
BDNF	brain-derived neurotrophic factor
EE	environmental enrichment
MPOA	medial preoptic area
OT	oxytocin
KO	knock-out
OTR	oxytocin receptor
DA	dopamine

ir	immune-reactivity
PNS	prenatal stress
MWM	Morris water maze
LTP	long-term potentiation
LTD	long-term depression
V1aR	vasopressin 1a receptor
SH	standard housed
T2D	type 2 diabetes
DIO	diet-induced obesity
AgRP	agouti-related peptide
NPY	neuropeptide Y
ARC	arcuate nucleus
GABA	gamma-Aminobutyric acid
GHSR	growth hormone secretagogue receptor
DAG	des-acyl ghrelin
GOAT	ghrelin *O*-acyl-transferase
AG	acyl ghrelin
ObRb	leptin receptor
CART	cocaine and amphetamine regulated transcript
nc	non-coding
PCR2	polycomb repressive complex 2
H3K27	histone number, lysine number
c	cytosine
g	guanine
DNMT	DNA methyltransferases
TET	ten-eleven translocation
5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
siRNA	small interfering RNA
piRNA	Piwi-interacting RNA
lncRNA	long noncoding RNA
miRNA	micro RNA
EV	extracellular vesicles
BBB	blood-brain barrier
PS	paternal stress
EPM	elevated-plus maze
OFT	open field test
MSUS	maternal separation and unpredictable maternal stress
MeCP2	methyl-CpG binding protein 2
GWAS	Genome-wide associate studies
rDNA	ribosomal DNA
Ill3ra2	interleukin 13
JAK	Janus-kinase
STAT	signal transducer and activator of transcription
PPARA	peroxisome proliferator-activated receptor alpha
CASP12	caspase 12

PFCx	prefrontal cortex
SNH	semi-naturalistic home cage
CO	control odor
PPT	Partner preference tests
GD	gestational day
PD	postnatal day
NCP	no contact with pups
SG	self-grooming
NB	nest building
F	feeding
PN	passive nursing
SP	separated pups
PR	pup retrieval
PBS	phosphate buffered saline
ChIP	chromatin immunoprecipitation
PCR	polymerase chain-reaction
Ct	cycle threshold
dF/dT	first negative derivative
ANOVA	analysis of variance
kcal	kilocalorie
IGF	insulin-like growth factor
NSFT	novelty suppressed feeding test
IVF	*in vitro* fertilization

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CHAPTER 1 INTRODUCTION

We, all, are possessors of a certain number of characteristics which marks us as species, as members of a certain race or a certain family, and as individuals. Most of our physical and mental characteristics we have inherited from our parents, from our grandparents, from our forebears in the dark past. We have acquired only a few of these characteristics in the course of our individual life.

-Paul Kammerer (The Inheritance of Acquired Characteristics, 1924)

Currently, there is a biological and philosophical battle regarding the pathways and extent of which environmental conditions influences differential gene expression and trait variation. Accumulating evidence suggests that gene-environment interactions are mediated, in part, by epigenetic modifications of the genome that occur in response to changes in the environment. However, skeptics hold fast to a more unchanging function, similar to the initial description of epigenetics, or a 'canalization' of gene expression, giving rise to specific expression patterns, and thus, phenotypes. Indeed, Conrad Waddington's initial description of epigenetics (*the causal mechanisms by which the genes of a genotype bring about a phenotype*) left ample opportunity for interpretation represented by the vast range of ideologies regarding epigenetics (Waddington, 1942). More recently, Adrian Bird's definition of epigenetics (*the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states)* definitively identifies basic mechanistic properties (Bird, 2007). The most commonly utilized definition (*the study of heritable changes in genome function that occur without*

alterations to the DNA sequence) (Russo et al., 1996) is ideal because it leaves open the possibility for a range of application, from tissue level changes within an organism to transgenerational phenotypic inheritance.

Nearly 20 years before Waddington's first description of epigenetics, Kammerer published his life work, including a model where offspring of land-dwelling toads showed a strikingly 'water-toad' phenotype, if their parents had been forced to reproduce in water. Though Kammerer's integrity as a scientist was controversial (Vargas, 2009), his ideas were revolutionary and probably more in-line with modern epigenetic theories than Lamark's. Strikingly similar to Kammerer's toads were Waddington's Drosophila, in which altered wing pattern inheritance resulted from exposure to heat shock (Waddington, 1942). More recently, the *agouti* mouse model, in which coat colour variation is related to epigenetic marks, has revolutionized our understanding of the role of epigenetics in inheritance (Morgan et al., 1999). Together, these studies suggest that in addition to simple inheritance via the germline, complex behavioral traits are also acquired with environmental exposures and life experiences, and transferred across generations (Eaton et al., 2015).

The field of epigenetics has become very broad, meaning different things to different types of researchers. At a cellular level, epigenetics represents the deoxyribonucleic acid (DNA)-protein interactions that regulate gene transcription. This is the process by which DNA is transcriptionally regulated in developing (mitotic) tissues (Probst et al., 2009); for example, methylation of the inactive X chromosome (McGraw et al., 2013) or histone

marks being passed from mother to daughter cells (Ng and Gurdon, 2008). However, epigenetics opens the door through which early experience can drive phenotype; offspring of mouse moms that performed higher levels of pup licking and grooming (LG) have a more responsive hypothalamic-pituitary-adrenal (HPA)-axis than those from low-licking and grooming (LG) dams (Weaver et al., 2004b). These mechanisms have been implicated in early-life resilience/vulnerability to disease (Meaney et al., 2007). Further, epigenetic mechanisms have the ability to potentiate transgenerational inheritance of these acquired characteristics. The results of a landmark study showed that toxic exposure altered methylation patterns of the testis, resulting in decreased male fertility, and this reproductive phenotype was passed on to offspring that had not been exposed (Anway et al., 2005).

As stated, I will use Bird's definition of epigenetics as it relates to the ability of chromosomal changes to perpetuate altered transcriptional availability, especially focusing on the potential for transgenerational (see below) alterations. This type of inheritance requires a discussion of the role of epigenetics in evolution. Though a transgenerational passage of deleterious effects (i.e., toxicity-induced infertility) is not advantageous, it does bring to mind many questions regarding evolutionary developmental biology (Evo-Devo) hypotheses. I will outline evidence stating that many of these transgenerational effectors do have a role in boosting evolutionary fitness (Dias and Ressler, 2014), but some are susceptible to the effects of early programing or reflect a lack of fit between individual and environment, the mismatch resulting in less than ideal programming of stress and/or metabolic phenotypes. Because epigenetic changes offer

the flexibility of being dynamic, compared to DNA mutations, they can be reversed or erased within a few generations (Seong et al., 2011) and 'epimutations' allow for phenotypic diversity that outpaces DNA mutations (Jablonka and Lamb, 1995; Barber et al., 2002; Rando, 2012). These epimutations allow for increased variation during duress, improving the chance that a successful phenotype will emerge (Rando and Verstrepen, 2007). Our understanding of the underlying signalling pathways that regulate the capacity for adaptive evolution remain redimentary, but many evolutionary biologists believe that epigenetics (and Waddington's 'canalization') are partially responsible (Pigliucci, 2008), further highlighting the issues with the purely genetic driven view of phenotypic and behavioral variation (Pigliucci, 2010).

To summarize, these mechanisms may regulate inheritance in a non-Mendelian fashion, similar to imprinted genes and cytoplasmic inheritance, and are critical for our understanding of early-life programming and the eventual adult phenotypes that emerge. In order to establish a model to explore the potential for paternally inherited phenotypes, we must consider environmental factors that alter phenotypes without causing DNA mutations. For this, I utilized stress exposure of males using either predator odor (PO) or high-fat diet (HFD). Further, the potential for altered behavior of the partner in response to breeding with an 'exposed' mate must be considered. The mechanisms and background for these will be discussed.

1.1 The Stress Response

1.1.1 Modelling Stress Regulation Based on Animal Studies

Perhaps the most studied topic in behavioral neuroscience, stress has a profound breadth of effects on a wide variety of organisms, from plants to humans. In rodent, and most mammalian models, stressors and the effects of them have components that can be categorized as (1) psychological, (2) physical, and (3) homeostatic. Typically, psychological stressors (e.g., novel environment, predator threat, or social isolation) and physical stressors (e.g., foot shock, immobilization, or pain) are used as initiators of a stress response, where homeostatic components may be interacting or outcomes of stress response activation (e.g., oxidative stress, metabolic stress, or exercise). Stressors can be deployed acutely or chronically, one time or in a repeated fashion. Considering the variety in type and duration of stressors, and depending on specific hypotheses and confluent manipulations, broad ranges of response to stress are possible. Acutely, HPA axis activation serves an important role in mobilizing a 'fight or flight' response to a threat. However, under chronic stress, high levels of glucocorticoids (GCs) are deleterious to the central nervous system (CNS) and peripheral organs, implicated in psychoses from anxiety disorders to post-traumatic stress disorder (PTSD), along with obesity, metabolic syndrome (MetS), and cardiovascular disease (Juster et al., 2011; Lemche et al., 2016). Further, controllable stressors are considered beneficial, as they have the ability to reduce responding to future insults (Christianson et al., 2013). This

'behavioral immunization' implies a resilience that contradicts diathesis models, which utilize multiple stressors to generate a susceptible phenotype.

1.1.2 Stress Sensory Pathways

The initial stress response is mediated by sensory pathways, which usually pass through the reticular activating system or the thalamus, relaying information to the cortex (Amiragova, 1985). This information is then conveyed to the perirhinal cortex where it either projects directly or via the hippocampus to the amygdala (Figueiredo et al., 2003; Herman et al., 2003). Distinct nuclei of the amygdala are responsible for regulation and recognition of stress perception and fear-motivated behavior (LeDoux, 2007; Ehrlich et al., 2009). The lateral amygdala functions as a 'gate-keeper', collecting sensory stimuli from the cortex, thalamus and hippocampus, (Johansen et al., 2010; Bosch and Ehrlich, 2015) while the central amygdala is responsible for potentiating a behavioral reaction (Ciocchi et al., 2010). Amygdala axons project to the hypothalamus and give rise to autonomic, CNS, behavioral and endocrine functions; all of which are dependent on the amygdalo-hypothalamic pathway (Petrovich et al., 2001).

1.1.3 The Hypothalamic-Pituitary-Adrenal Axis (HPA-axis)

The HPA axis involves a cascade of hormone release, with numerous players converging and affecting almost every aspect of physiology. The medial parvocellular division of the paraventricular nucleus (PVN) is the primary source of corticotropin-releasing factor

(CRF) (Spiess et al., 1981; Vale et al., 1981). CRF, along with arginine vasopressin (AVP), is the principal regulator of pituitary adrenocorticotropic hormone (ACTH). Both CRF and AVP are transported via the hypophysial portal veins to guanine-protein-coupled receptor (GPCR, in this case the CRF-R1) in the anterior pituitary gland. This stimulates the synthesis of the ACTH precursor peptide, proopiomelanocortin (POMC), and release of ACTH and other POMC-derived peptides, including B-endorphin, into circulation. In the adrenal glands, ACTH stimulates the release of GCs into the blood stream (Antoni, 1986; Whitnall, 1993; Herman et al., 2005; see Figure 1).

1.1.4 Endocrine System Stress Response

1.1.4.1 Glucocorticoid (GC) Synthesis and Release

The melanocortin receptor 2 (MC2) is the primary cognate receptor for ACTH in the zona fasciculata of the adrenal cortex. MC2 is a G-protein coupled receptor (GPCR) which activates the cyclic-adenosine-3',5'-monophoshpate (cAMP) pathway, dephosphorylating 3-hydroxy-3-methylglutarylcoenzyme-A (HMGCoA) reductase. HMGCoA is the rate-limiting enzyme in the synthesis of cholesterol from dietary cholesterol, transported as low-density lipoprotein (LDL) (reviewed in Kraemer (2007)). In the mitochondria, cholesterol is modified to form pregnenolone, the precursor to all steroid hormones, including 11-deoxycorticosterone (11-dGC), which is hydroxylated and converted to active GC (Simpson and Waterman, 1983; Jefcoate et al., 1986; Miller, 1988).

1.1.4.2 Negative Feedback

Ultimately, GCs bind to receptors, specifically within the HPA axis and limbic system to mount a stress response and regulate negative feedback loops responsible for effective termination of the response. The two main receptors for GCs are the glucocorticoid receptor (GR) and mineralocorticoid receptors (MR). GRs are highly expressed and have a relative low affinity for GCs (5-10 nM), typically being bound only during times of high GC secretion (Reul and de Kloet, 1985). MRs have a much higher affinity (25-100 nM) and because of this, they are expressed in a more restricted pattern (Reul and de Kloet, 1986). Negative feedback primarily acts via the hippocampus, where both GR and MR expression is high and the hypothalamus, where GR expression outpaces MR (Reul and de Kloet, 1985). This structure of the system further suggests the role of MR binding in maintaining basal HPA-axis activity, while GR binding is more critical for stress-induced activation of the feedback system. Specifically, hypothalamic GR binding acts on CRF neurons to modify the amplitude of response.

1.1.4.3 CRF as a Marker of the Magnitude of Stress Response

CRF is often used as a proxy for the magnitude of a stress response, due to its role in initiating HPA-axis activity (Owens and Nemeroff, 1991; Caldji et al., 2000; Korgan et al., 2015). Research has shown that CRF expression (and expression of its receptor system) are modified, basally, by prior experience. The CRF system is regulated by two

receptor subtypes (R1 and R2) and involves five ligands (CRF, Urocortin1 or Ucn1, Ucn2, Ucn3, and the CRF-binding protein (CRFBP)). Each receptor and ligand is distributed in diverse regions within the CNS and peripheral tissues (reviewed in (Hostetler and Ryabinin, 2013). CRF has affinity for both R1 and CRFBP, while Ucn1 binds to both R1 and R2, Ucn 2 binds primarily to R2 and Ucn3 binds exclusively to R2. CRF is primarily synthesized within the PVN and the *crf* gene produces only one intron (Thompson et al., 1987); thus, heteronucleotide RNA (hnRNA) of the transcript can be used to quantify CRF expression levels (Imaki et al., 1995; Korgan et al., 2016). Receptor distribution of R1 in the hippocampus and amygdala highlight the role of CRF in HPA-axis functioning; however, R1 receptors in the olfactory bulb, cortex, septum and cerebellum suggest additional functions (Bale and Vale, 2004).

CRF levels in adulthood are affected by events that occur during early life, allowing these early events to affect the magnitude of adult stress responding. For example, studies of early-handling (EH) experience in rats <1-week old show decreased *crf* mRNA and protein in the PVN in adulthood (Plotsky and Meaney, 1993), while early life maternal separation (MS) increases CRF in adulthood (Ladd et al., 1996). Other studies have shown that isolation stress also increases *crf* messenger ribonucleic acid(mRNA) in the PVN in adulthood (Pan et al., 2009).

1.1.4.4 CRF Regulation of Affiliative/Avoidance Behavior

The CRF system is intricately connected to social behavior in mammals, reviewed in (Hostetler and Ryabinin, 2013). Reductions in social behavior may be expected during times of stress, but a role for CRF in social behavior outside of the HPA axis is evident by CRH activity outside of the HPA axis. Social interaction in rodents is sensitive to CRF manipulations independent of stress-induced decreases in sociality (Gehlert et al., 2005); however, this presents a limitation when interpreting CRF-dependent effects. However, Ucn or CRF treatment in the amygdala and bed nucleus of the stria terminalis (BNST) are sufficient to decrease social interaction time (Sajdyk et al., 1999; Lee et al., 2008; Donner et al., 2012). Though Ucn1 administration to the amygdala also induces an anxiogenic response (Sajdyk et al., 1999), BNST administration is specific to social anxiety (Lee et al., 2008). Together, these suggest that R1 and CRF and/or Ucn1 are critical for normal social behavior.

This is further exemplified by variations in CRFR1 and R2 receptor densities in monogamous and promiscuous vole species. The monogamous prairie and pine voles have decreases in R1 receptor densities in the shell of the nucleus accumbens, olfactory bulb, and superior colliculus, compared to polygamous meadow and montane voles. R2 densities show contradicting differences in densities in the lateral septum and hippocampus between meadow increased compared to prairie voles and montane decreased compared to pine voles. However, both monogamous species had increased R2 density in the septum, compared to polygamous species (Lim et al., 2005). Others research supports these CRF-mediated with significant differences in plasma GCs in

isolated and pair-bonded, reunited (Carter et al., 1997) monogamous prairie boles. Indeed, in mice, CRF injection into the dorsal third ventricle distrupts social behavior (Bagosi et al., 2017), further highlighting the overlap between sociality and CRF.

1.2 Maternal Behavior and Circuitry

1.2.1 Natural Variations in Maternal Care

Natural variations in maternal care have revolutionized our understanding of early-life programming of stress responsivity. Liu et al., (1997) showed that naturally-occurring variations in licking and grooming/arched back nursing (LG-ABN) were associated with adult differences in HPA stress response in offspring, dependent on GR density within the hippocampus. These differences, however, did not impact the total time dams spent with the litter, indicating that specificity of the maternal behavior is the key component (Caldji et al., 1998). Indeed, it was proposed that increases in LG-ABN increased serotonin (5-HT) turnover in the hippocampus (Weaver et al., 2004a), mediating the effect on GR density, potentially by increasing expression of transcription factors. Complimentary *in vitro* results show that 5-HT leads to increases in both GR and nerve growth factor-inducible (NGFI), a known activator of GR transcription (Weaver et al., 2014a).

Maternal behaviors are stably transmitted to female offspring, such that female offspring of high LG-ABN dams perform more LG-ABN behaviors and low LG-ABN offspring perform fewer (Francis et al., 1999b), though this effect may be biased to offspring sex-

differences in the frequency of maternal LG received (Moore and Power, 1992). This effect is not genetic, but environmentally regulated as cross-fostering results in offspring behavior similar to the rearing experience (Francis et al., 1999b; McLeod et al., 2007) and is highly plastic in response to environmental conditions (Champagne and Meaney, 2007; Leonhardt et al., 2007). Thus, expression of high vs low LG-ABN drives development of the maternal brain circuit through expression of oxytocin and vasopressin receptors in the central amygdala and BNST (Francis et al., 2002) and estrogen receptor (ER)-alpha and ER-beta expression in the MPOA (Champagne et al., 2003a; McLeod et al., 2007).

Mechanistically, high LG-ABN offspring have increased *N*-methyl-D-aspartate (NMDA) and brain-derived neurotrophic factor (BDNF) expression in the hippocampus, which is associated with enhanced spatial learning and memory (Liu et al., 2000; Barha et al., 2007). Conversely, low LG-ABN dams have offspring with deficits in NMDA binding and hippocampal-dependent spatial learning, though this can be reversed with peripubertal environmental enrichment (EE)(Bredy et al., 2003). These effects appear to be epigenetically regulated; Weaver et al., (2004b; 2014b) showed decreased methylation of the GR promoter in high LG-ABN offspring with corresponding differences in histone acetylation and transcription factor (NGFI-A) binding at the GR promoter. Further, ER-alpha promoter methylation was increased in low LG-ABN offspring (compared to high LG-ABN) in the medial preoptic area (MPOA) and this effect was also reversed with cross-fostering (Champagne et al., 2006).

1.2.2 The Maternal Brain

The maternal brain has specialized circuits not present in male or virgin female brains (reviewed in (Numan and Insel, 2003; Numan, 2007). Olfactory cues from the offspring stimulate connections between the olfactory bulb and the amygdala, resulting in primiparous parturient females quickly approaching offspring that virgin females will avoid. This contrast in behavior is driven by the endocrine-induced plasticity throughout pregnancy (Pryce, 1992; Numan and Insel, 2003). In the maternal brain, offspring exposures increase estrogen levels peripherally and reach the (MPOA). Increased neural projections from the medial amygdala appear to mediate decreased fearfulness or avoidance to offspring (Numan and Insel, 2003). Increases in ER binding in the MPOA initiates genomic response elements to regulate transcriptional activity and alters basic properties of these neurons (Loven et al., 2001; Nilsson et al., 2001). Specifically, activation of MPOA neurons results in upregulation of oxytocin (OT), which has been shown to decrease the latency to maternal behavior with intracerebrovascular infusion. This upregulation is due to binding of the ER-alpha transcription factor and is further validated in ER-alpha knock-out (KO) mice not having normal oxytocin receptor (OTR) binding (Young et al., 1998). The ventral tegmental area (VTA) receives projections from the MPOA, connecting the maternal circuit with the mesolimbic dopamine (DA) system (Tobiansky et al., 2013), highlighting the importance of central dopamine in maintaining motivation for maternal care (Champagne et al., 2004).

Though the regulation of the maternal circuit is necessary for appropriate offspring care, environmental factors play a critical role in modulating this behavior and, thus the development of the offspring. This allows fine-tuning of maternal care and offspring phenotype to suit prevailing conditions. Here, I will summarize what we have learned about the plasticity of maternal behavior using several models and the subsequent effects on offspring development.

1.2.3 Early Handling and Maternal Separation

Both early handling (EH) and maternal separation (MS) models involve removal of dependent offspring from the nest during the first week of life and both have been studied extensively. Interestingly, the simple difference in the quantity of the dams' time away from her offspring results in profound differences in the outcomes of these models.

EH models are defined by repeated removal of offspring, and separation from the mother and the nest for 15-20 min before reuniting them. Levine first reported that EH resulted in handled adult offspring with a hyporesponsive HPA response (Levine, 1957) and further research has identified potential mechanisms (Weaver et al., 2004a). One such is the effect that EH has on maternal care; EH is associated with shorter, but more frequent, bouts of maternal behavior upon reuniting of dams and pups (Villescas et al., 1977; Couto-Pereira et al., 2016). EH has also been associated with increased GC binding in the hippocampus of adult offspring (McCormick et al., 1995; George et al., 2013) as well as decreased CRF-immuno-reactivity (ir) and CRF mRNA in the PVN, central amygdala,

BNST, and locus coeruleus (Plotsky et al., 2005). These changes in CRF are further associated with differences in sociality, including rough and tumble play (Aguilar, 2010) and spatial learning and memory (Plescia et al., 2013).

Contrary to EH, early MS (180 min daily from PD 2-14) increases CRF-ir and mRNA in the PVN, central amygdala, BNST, and locus coeruleus (Plotsky et al., 2005), as well as increasing anxiety-like behavior. In contrast to EH, MS results in fragmented and inconsistent patterns of maternal care (Couto-Pereira et al., 2016), which can be reversed with CRF receptor antagonist administration in adult offspring (Maciag et al., 2002; Liao et al., 2014). Interestingly, MS-induced anxiety is attenuated if offspring are placed in a novel environment during the separation period, establishing an anxiogenic phenotype that persists into adulthood, and is driven by amygdala-dependent activation (Daskalakis et al., 2014). Predictably, early programming alterations induced by MS are not specific to the HPA-axis - MS also decreases tryptophan hydroxylase 2 (TPH2) and serotonin transporter mRNA in the dorsal raphe (Own et al., 2013) and increases vulnerability to addiction via aberrant DNA methylation in the nucleus accumbens (Anier et al., 2013).

1.2.4 Impact of stress on maternal care

Numerous studies have highlighted the impact of maternal stress on offspring development and behavior (Weinstock, 2001; Weinstock, 2017), and given the preceding sections, it is obviously relevant to note the impact of maternal stress in altering maternal behavior. Often termed gestational stress or prenatal stress (PNS), these experiences

significantly decrease OTR expression throughout the maternal brain circuit (Caldji et al., 1998; Champagne and Meaney, 2006). This insult to the maternal brain circuit decreases maternal care, specifically reducing the quantity of high quality LG-ABN behaviors (Carini and Nephew, 2013). Such decreases in maternal care are associated with HPA-axis hyper-reactivity in adult offspring (Smith, 2004). Further, gestational stress affects have consequences beyond maternal behavior, altering emotional reactivity in the dam that? persist beyond the weaning period (Darnaudery et al., 2004).

Cross-fostering studies offer insight into the effects of PNS independent of altered maternal behavior. By rearing PNS offspring with dams that were not stressed, it is possible to differentiate the roles of altered gestational endocrine exposure in offspring programming compared to altered maternal behavior following her own heightened stress response. This model has demonstrated that HPA-axis dysregulation (Maccari et al., 1995) and deficits in social behavior and oxytocin signaling (Barros et al., 2006) are reversed in offspring when they are reared with non-PNS dams. However, similar effects are not seen in learning tasks. In the Morris water maze (MWM), offspring reared by non-PNS foster dams demonstrated learning deficits and reduce long-term potentiation (LTP) and depression (LTD) (Yang et al., 2006). This discrepancy of effects sheds light on the complexity of perinatal brain development and the profound roles of gestational and early-life programming with respect to specificity of behaviors and their underlying brain circuits.

1.2.5 Housing Environment as a Modulator of Offspring Phenotypes

Early life enrichment paradigms are built on an abundance of research suggesting both protective and restorative effects of adult environmental enrichment (Fox et al., 2006; Simpson and Kelly, 2011). However, significantly less research has been conducted using models of enrichment that span early periods of life. Experimental issues, such as the lack of mobility (i.e., inability to interact with the environment) and dependence on maternal care, significantly alter the overall effect that early life enrichment can propagate. However, the convergence of increased maternal care and the eventual experience of a more naturalistic (or enriched) environment have been shown to promote stress resilience in offspring. Similar to EH models, early enrichment decreases the total time that a dam spends on the nest and overall active maternal behavior is increased, although differences in maternal LG are minimal (Connors et al., 2015; Korgan et al., 2016). Behaviorally, these offspring enter open arms more frequently and spend more time in the center of a novel open field (Connors et al., 2015; Korgan et al., 2016). Offspring reared in a socially enriched environment also develop anxiolytic phenotypes and enhanced maternal care, again dependent on OTR and vasopressin 1a receptor (V1aR) densities (Curley et al., 2009). Further, offspring of immune-challenged dams show decreased anxiety-like behavior and improved HPA-axis function if reared in enriched environment relative to non-challenged or those reared in standard housed (SH) conditions (Connors et al., 2014).

1.3 Stress, Hormones and Feeding

1.3.1Corticosterone Regulation of Feeding Behavior

Obesity is quickly becoming the most destructive and expensive epidemic of our generation (Bray et al., 2004; Gotay et al., 2013; Graversen et al., 2014). The rise in obesity is concurrent with a rise in MetS; the appearance of 'at risk' ranges of plasma lipids, progressive prediabetes, increased adiposity, especially around the abdomen, and subclinical cardiovascular issues. Several in-depth reviews have thoroughly discussed the prevalence, risk-factors, and outcomes associated with obesity, MetS, and type 2 diabetes (T2D) (Flegal et al., 2002; Bray et al., 2004; Kahn et al., 2006; Berends and Ozanne, 2012; Graversen et al., 2014). Of more immediate interest here are the consequences of diet-induced obesity (DIO) and stress-related comorbidities.

The comorbidity of stress and obesity is often attributed to the role of glucocorticoids in energy balance. Under conditions of chronic stress, high levels of GCs drive cravings for calorie-rich foods (Dallman, 2010), which can downregulate GCs, ACTH, and CRF (Dallman et al., 2003). This interplay is controlled by the orexigenic pathway, which is primarily regulated by agouti-related peptide (AgRP) and neuropeptide Y (NPY)-expressing neurons in the arcuate nucleus (ARC) of the hypothalamus. Chronic stress and exogenous GCs can both activate this pathway, stimulating food intake (Lu et al., 2002; Patterson and Abizaid, 2013). Further, the gut-derived hormone ghrelin also plays a role in regulating stress response, cognition, and energy balance. Cabral et al., (2016) recently showed that ghrelin is capable of interacting with inhibitory gamma-Aminobutyric acid producing (GABA)ergic terminals of the PVN and can activate CRF neurons through a

currently undefined pathway. Further, the adipose-derived hormone, leptin, also functions in concert with stress-induced hormonal milieus (Yang et al., 2016).

Conventionally, studies of DIO focus on the status of metabolic hormones in obesity etiology; these have been reviewed in great detail (Spiegelman and Flier, 2001). Metabolism itself is an exceptionally complex process, undoubtedly a factor in the lack of decisive conclusions regarding orexic and anorexic disturbances following stress exposure (Razzoli and Bartolomucci, 2016). Effects on sperm development, motility and epigenetic content have further implicated both stress (Rodgers and Bale, 2015; Rodgers et al., 2015) and obesity (Guo et al., 2017; Kobayashi et al., 2017) as predictors of deleterious effects. Neural control of feeding behavior is often characterized by the role of the peripheral gut and adipose hormones ghrelin and leptin, respectively (Schwartz et al., 2000; Cummings, 2006; Nogueiras et al., 2008).

1.3.2 Ghrelin in Feeding and Stress

Ghrelin is an orexigenic hormone, primarily secreted from the stomach and small intestine. It is an endogenous ligand for the growth hormone secretagogue receptor (GHSR), which stimulates growth hormone release from the pituitary. Initial descriptions of ghrelin describe a “hunger hormone”, characterizing its role in initiating food intake and energy expenditure demands to the CNS (Cowley et al., 2003). Ghrelin is encoded by the preproghrelin gene, which also encodes the peptide obestatin (Zhang et al., 2005). To bind to GHSR, des-acyl ghrelin (DAG) must be acylated by ghrelin *O*-acyl-transferase

(GOAT), resulting in the active, acyl ghrelin (AG) (Gutierrez et al., 2008; Yang et al., 2008). Ghrelin is the only known circulating hormone that increases adiposity and food intake (Tschop et al., 2000). This action occurs by activating NPY/AgRP neurons (co-localized with GHSR) in the arcuate nucleus of the hypothalamus, and in turn AgRP inhibits melanocortin receptors (MC4R), thus stimulating food intake (Cowley et al., 2003). Outside of this function, ghrelin also plays important roles in learning and memory, gut motility, circadian rhythm, reward pathways, taste, and glucose metabolism, (Andrews, 2011) reviewed in (Müller et al., 2015; Collden et al., 2017; see Figure 1). DIO results in central ghrelin resistance in ARC NPY/AgRP neurons (Briggs et al., 2010). However, GHSR KO prevents the development of DIO (Zigman et al., 2005), while reducing peripheral ghrelin by binding an exogenous GHSR decoy reduces DIO and MetS phenotypes (Gagnon et al., 2015). Indeed, stress (increases) and obesity (decreases) have regulatory effects on plasma ghrelin concentrations and suggest more profound roles in the regulation of anxiety-like behavior (Zigman et al., 2015).

The role of central ghrelin outside of the arcuate nucleus, especially within the PVN, has recently drawn the interest of neuroscientists. Indirectly, AG activates CRF neurons (Cabral et al., 2012) while DAG indirectly regulates GC secretion in a stress dependent manner (Stark et al., 2016). Chronic stress can also induce ghrelin resistance in the amygdala via decreased GHSR-ir and overconsolidation of fear memory (Harmatz et al., 2016). Finally, ghrelin also plays a role in regulating pituitary gonadotrophs, thus altering reproductive fitness (Ristic et al., 2016). Together, these studies show a critical role for

ghrelin signaling in the development of obesity and implications of ghrelin resistance in the CNS.

1.2.3 Leptin in Feeding and Stress

Leptin is an anorexigenic hormone, produced by adipose tissues in proportion to fat mass. The leptin receptor (ObRb) is found on both NPY/AgRP and POMC/cocaine and amphetamine regulated transcript (CART) neurons. Opposite to ghrelin, leptin binding activates POMC/CART and inhibits NPY/AgRP neurons, resulting in decreased feeding and increased energy expenditure. Unlike ghrelin, ObRbs are found on POMC/CART neurons, which produce α-MSH, the ligand for the MC4R found on neurons throughout the hypothalamus and amygdala and critical for inhibition of feeding behaviors (Bouret and Simerly, 2004; see Figure 1; Nogueiras et al., 2008; Perello et al., 2012). Critically, leptin knockdown in ARC promotes DIO in rats (Bian et al., 2013). Further, leptin has been shown to inhibit CRF activity during normal stress responding (Heiman et al., 1997) but not if leptin signaling is deficient or resistant, as in the case for many DIO models (Mark, 2013) and chronic or early-life stress models (Iio et al., 2014; Yam et al., 2016). This provides another mechanism by which altered hypothalamic development impairs CNS regulation of feeding behavior and stress responsivity.

1.3.4 Early Life Experience and Appetite Programming

Similar to early life programming of the HPA-axis, neural control of feeding behavior is developing during the perinatal and periadolescent periods. Thus, receiving milk contributes to the perinatal hyporesponsive HPA-axis (Spencer, 2013). Nutrient intake from the dam is dependent on nursing behavior and diet (Zambrano and Nathanielsz, 2013) and maternal behavior itself is modulated by diet. For example, low-protein diets will decrease pup retrieval and nest building but increase the amount of time spent nursing (Massaro et al., 1974; Wiener et al., 1977). Interestingly, nutrient intake is also mediated by paternally imprinted genes affecting offspring behavior (i.e. vocalizations, suckling ability, and locomotor activity) (Curley et al., 2004; Plagge et al., 2004).

Perinatal programming of the ARC is also dependent on maternal nutrition (Bouret, 2012). Not surprisingly, this programing is partially dependent on perinatal exposure to ghrelin and leptin. Maternal obesity and/or diabetes increases leptin levels in offspring, promoting leptin resistance (Steculorum and Bouret, 2011). Similarly, over-nutrition during the perinatal period can induce central ghrelin resistance in offspring (Collden et al., 2015). Together, increased ghrelin during the perinatal period diminishes leptin signaling in ARC neurons (Steculorum and Bouret, 2011). Finally, offspring of obese animals can reset metabolic homeostasis with voluntary exercise (Bahari et al., 2013; Caruso et al., 2013; Rajia et al., 2013), emphasizing the plasticity of the hypothalamic feeding circuit.

1.4 Epigenetic mechanisms of transgenerational inheritance following environmental perturbations

1.4.1 Epigenetic Mechanisms

The traditional view of epigenetic mechanisms posits that methylation marks are erased during meiosis (Cantone and Fisher, 2013; von Meyenn and Reik, 2015). This has been challenged recently, in fields ranging from botany (Iglesias and Cerdán, 2016) to human psychiatry (Lim and Brunet, 2013; Nestler, 2016). Below, I will review the three basic mechanisms of epigenetics (DNA methylation, histone modifications, and non-coding (nc) RNAs; Nestler, 2016; see Figure 1) and their potential involvement in transgenerational inheritance. This review is not complete but comprehensive reviews are continuously being updated (Lim and Brunet, 2013; Bale, 2014; Braun and Champagne, 2014; Rodgers and Bale, 2015; Day et al., 2016; Gapp et al., 2016b).

1.4.1.1 Histone modifications

Histone modifications regulate the chromatin structure, allowing for increased or decreased transcription, depending on the specific marks attached. Typically, histone acetylation is associated with increased transcriptional activity (euchromatin) and histone methylation with decreased transcriptional activity (heterochromatin). Many different types of histone modifications have been identified and are expertly reviewed in Kouzarides (2007) and Loyola et al., (2007).

During mitoses, parental histone marks associate with the histone chaperone ASF1, which is partially responsible for tetramer splitting and recycling of parental dimers in daughter strands (Groth et al., 2007). Another histone modifier, polycomb repressive complex 2 (PCR2), binds to the repressive histone mark lysine 27 on histone H3 (H3K27me3), where it is responsible for setting the methylation mark, during replication (Hansen et al., 2008). This binding ensures maintenance of the methylation mark in daughter cells and identifies a model by which other histone marks are inherited. Further, the H3k27me3 mark is present at transcriptional promoter regions in mature spermatozoa and this binding is correlated with transcriptional repression in gametes and embryos (Brykczynska et al., 2010). Finally, ATF-2 chromatin assembly is disrupted by stress and transmitted to subsequent generations in a non-Mendelian manner (Seong et al., 2011).

1.4.1.2 DNA methylation

DNA methylation is likely the most intensely studied mechanism in the field of behavioral epigenetics. Methylation of DNA is specific to cytosine (C)-guanine (G) repeats, known as CpG islands (Keshet et al., 1985; Razin, 1998). These are typically found at the start site, or promoter, of a gene and methylation of CpG islands results in transcriptional silencing of the gene (Keshet et al., 1985). DNA methylation is semi-conserved during mitosis (Gruenbaum et al., 1982) and functions by 'writing' patterns of enzyme activity. These enzymes are DNA methyltransferases (DNMT), consisting of; DNMT1, usually associated with DNA maintenance during replication (Leonhardt et al., 1992) and DNMT3, usually associated with *de novo* methylation and development

(Okano et al., 1999). DNA *de*methylation can be achieved by an 'erasing' process, where ten-eleven translocation (TET1-3) enzymes convert 5-methylcytosine (5mC), the methylated cyotosine from CpG islands, to 5-hydroxymethylcytosine (5hmC) (Ito et al., 2010).

Environmental factors have been shown to alter the methylation status of genes related to stress responding, with the most heavily studied being changes to the glucocorticoid receptor following differential maternal care (Weaver et al., 2004b). Transgenerational epigenetic inheritance of DNA methylation has been altered following exposure to HFD (Ng et al., 2010) and stress (Franklin et al., 2010) and could drive transmission of imprinted genes (Feng et al., 2010). Traditionally, research has supported the theory that sperm are 'reprogrammed' via erasure of promotor methylation marks (Hajkova et al., 2002; Lee et al., 2002), but this process does conserve some methylation marks (Reik et al., 1987; Kearns et al., 2000; Reik and Walter, 2001). Passive dilution of DNA methylation during mitosis is accomplished by preventing the function of DNMT1 at the replication fork (Rougier et al., 1998) but active demethylation processes have been identified (Zhang et al., 2007; Kangaspeska et al., 2008).

1.4.1.3 Non-coding RNAs

The regulatory effects of ncRNAs as epigenetic modifiers is based on their ability to interact with methylated DNA and histone marks, reviewed in Lim and Brunet (2013) and their ability to bind to the 3' region of mRNA, resulting in degradation or

transcriptional interference. ncRNA activity itself is epigenetically regulated and reflects a feedback mechanism within the currently identified mechanisms of epigenetics. A variety of ncRNAs have emerged from recent research, including small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), long noncoding RNAs (lncRNAs) and micro RNAs (miRNAs).

Previous studies have identified the potential for ncRNA to affect gene regulation during spermatogenesis and embryogenesis. Differential expression and alternative splicing of precursors to piRNAs and piRNAs themselves during murine spermatogenesis (Gan et al., 2011) allow for altered regulation of gene expression. Following fertilization, zygotic transfer of RNA results in phenotypic change in mice offspring tail color with altered RNA transcripts expressed in both brain and testis (Rassoulzadegan et al., 2006), providing evidence that epigenetic information can be transferred during reproduction. Recent evidence has suggested that extracellular vesicles (EVs) may be responsible for transporting miRNAs from the brain to the testis (Eaton et al., 2015), suggesting a possible mechanism for transmitting paternal experience dependent epigenetic modifications to offspring. One recent study reported transfer of RNA from somatic cells to the germline via EVs; however, use of tumor cells in this experiment led to speculation that metastasis was responsible for this finding (Cossetti et al., 2014). Further, short non-coding RNA (sncRNA) is present in high quantities in the prostasomes and epididymosomes, which are critically important for spermatogenesis (Vojtech et al., 2014). Further, EVs from the CNS can escape the blood-brain barrier (BBB) and interact

with developing gametes by expressing sperm-specific surface proteins (Noerholm et al., 2012; Kim et al., 2015).

1.4.2 Models and Evidence for Transgenerational Inheritance

Paternal transmission of biological and environnmental information including age (Smith et al., 2009; Smith et al., 2013; Atsem et al., 2016; Luo et al., 2017), chemical exposure (Skinner, 2011; Crews et al., 2012; Skinner, 2014; Carvan et al., 2017), alcohol (Kim et al., 2014; Finegersh et al., 2015; Sarkar, 2015), stress (Dietz et al., 2011; Archer et al., 2012; Hoyer et al., 2013; Mychasiuk et al., 2013; Rodgers et al., 2013; Petropoulos et al., 2014; Sharma and Rando, 2014; Harker et al., 2015; Rodgers et al., 2015; Callaghan et al., 2016; Gapp et al., 2016a; Short et al., 2016), obesity (Ng et al., 2010; McCowan et al., 2011; Fullston et al., 2013; Ng et al., 2014; Ost et al., 2014; Fullston et al., 2015; Grandjean et al., 2015; Rando and Simmons, 2015; Fullston et al., 2016a; Fullston et al., 2016b; Huypens et al., 2016; An et al., 2017), and enrichment (Mashoodh et al., 2012; Mychasiuk et al., 2012b; Gapp et al., 2016b) have the ability to alter offspring phenotypes. Elegantly simple studies have shown that olfactory imprinting can be inherited across generations in both *C. elegans* (Remy, 2010) and mice (Dias and Ressler, 2014). Further, anxiety-like phenotypes are passed to female offspring of inbred mice (Alter et al., 2009). For this thesis, I am comparing the role of stress (psychological stress) and obesity (metabolic stress) on maternal investment, offspring development and potential underlying mechanisms.

1.4.2.2 Stress

Paternal stress models, though intriguing, have shown relatively subtle effects to-date. This might be due to inconsistency in the stressors utilized (see above) or result from our presently extensive knowledge gap. Regardless, continued work in this field is critical for our understanding of the role of epigenetic mechanisms in trans- and inter-generational non-DNA dependent inheritance. Though the role of epigenetic mechanisms in inheritance is debatable, on every level of biological research from *Drosophila* (Waddington, 1953; Seong et al., 2011), 2011) to yeast (Watanabe et al., 2013; Audergon et al., 2015; Rege et al., 2015) to plants (Hsieh et al., 2016; Iglesias and Cerdán, 2016) to birds (Frésard et al., 2013; Skinner et al., 2014) to mammals (Crews et al., 2014; Bale, 2015; McGowan and Roth, 2015; Skinner, 2015), Waddington's original view of phenotypic inheritance appeals to behavioral epigenetic researchers today.

To date, paternal stress (PS) models have lacked the robust assessment of stress-induced behavior seen in maternal stress models. That said, several studies have shown increased depression and anxiety-like phenotypes in the offspring (Franklin et al., 2010). Studies have identified these phenotypes with the usual battery of behavior tests; including elevated-plus maze (EPM), open field test (OFT), sucrose preference (Franklin et al., 2010), novelty exposure, forced swim test, and light/dark box. Offspring anxiety-like phenotypes are more commonly reported in paternal stress models that induce paternal stress before adulthood, most commonly maternal separation during the early postnatal period.

These models have also demonstrated a difference in learning and fear conditioning in the offspring of paternal stress males. Bohacek et al., (2014) showed that offspring of PS males had decreased response to fear conditioning and deficits in the novel object recognition task. Similarly, Callaghan et al., (2016) reversed PS-induced learning deficits by treating the offspring of PS males with a probiotic, conceivably restoring some stress induced deficit in the gut-brain axis. Epigenetic marks on sperm have the potential to mediate these effects. In a model using maternal separation and unpredictable maternal stress (MSUS), adult male offspring had increased methylation of the methyl-CpG binding protein 2 (MeCP2) gene and decreased methylation of the CRFR2 gene (Franklin et al., 2010). These marks were transmitted to offspring, prompting altered behavior phenotypes discussed above.

The molecular effects of PS are complex. Several studies have identified abnormal stress response in PS offspring by measuring GC feedback following stress exposure (Rodgers et al., 2013) and at baseline (Dietz et al., 2011), potentially driven by inherited differential expression patterns for miRNAs (Morgan and Bale, 2011; Rodgers et al., 2013; Rodgers et al., 2015). Differences in stress responding appear via CRF regulated by epigenetic mechanisms within the PVN (Franklin et al., 2010; Korgan et al., 2016). Global methylation differences in PS offspring (Mychasiuk et al., 2013) suggest that both DNA and chromatin modifications are susceptible to PS. Interestingly, subtle differences in dendritic branching, length, and spine density were observed in PS offspring (Mychasiuk et al., 2012a), potentially highlighting differential GC exposure. Genome-

wide associate studies (GWAS) and RNA-seq experiments have identified gene-groups with differential expression and regulatory patterns in offspring of PS males. Similar to other studies of acute maternal stress, many of these are related to cell growth, plasticity and survival. However, one report identified differential expression of collagen-related proteins. As these are critical in maintaining the blood-brain-barrier, deficits in the highly vascularized hypothalamus would be deleterious for any organism. Wu et al., (2016) found that restraint stress in mice contributed to altered metabolic phenotype in offspring. Specifically, F1 offspring were found to have increased hepatic gluconeogenesis. Though they did not identify T2D or any behavioral abnormalities, paternal stress is linked to offspring metabolic homeostasis and weight gain, potentially more so in female offspring (Hoyer et al., 2013).

1.4.2.2 Obesity

The effect of paternal obesity has received significantly less attention from psychology/neuroscience research and remains an area in immediate paternal obesity exerts effects on offspring that are similar to maternal obesity. This could be due to similar mechanisms regulating non-genomic inheritance or similar investment strategies utilized by females. In Drosophila, paternal sugar consumption is associated with H3K9/K27me3 reprogramming and offspring obesity (Ost et al., 2014), potentially mediated by ribosomal (r)DNA copy number variation (Aldrich and Maggert, 2015). Conversely, high protein diets are associated with improved sperm competition, suggesting that preconception paternal effects could affect post-copulatory sexual

selection (Zajitschek et al., 2017a; Zajitschek et al., 2017b). In mice, paternal obesity is associated with decreased birth weight and length (Binder et al., 2015), similar to human neonates that are small for gestational age (McCowan et al., 2011).

Various diet manipulations have been utilized, including low-protein (Carone et al., 2010; Han et al., 2012) and protein/caloric restriction (Hardikar et al., 2015), and high-fat (Ng et al., 2010; Fullston et al., 2013; Ng et al., 2014). Fullston et al., (2013) bred HFD fed rats and showed increased prevalence of obesity and insulin resistance in F1 and F2 offspring. Further, this result seemed to propagate through sperm miRNA differences, similar to paternal stress models, and decreases in global methylation of testes. Further, sperm methylation at satellite repeats is increased in male rats following HFD, but these effects are not present in F_1 spermatozoa, suggesting that reprograming exists to normalize some paternal effects (Youngson et al., 2016), though paternal obesity can diminish offspring reproductive health (McPherson et al., 2014). Interestingly, these effects are more pronounced in female offspring, including increased B-cell dysfunction, type 2 diabetes phenotypes and adipose tissue transcriptomes (Fullston et al., 2013; Ng et al., 2014; McPherson et al., 2015). Ng et al., (2010) demonstrated that this was likely driven by observed differences in the expression of interleukin 13 (*Il13ra2*), which is involved in Janus-kinase (JAK)-signal transducer and activator of transcription (STAT) signaling. Other models of paternal obesity have identified sex-specific effects in female offspring global methylation profiles and male offspring expression of metabolic (e.g., peroxisome proliferator-activated receptor alpha; *ppara*) and apoptotic (e.g., caspase 12; *casp12*) expression (Binder et al., 2015). These negative outcomes in female offspring

can also be reversed by exercise intervention, with a specific normalization of sperm miRNAs (McPherson et al., 2015). Similarly, neonatal overfeeding of male offspring is implicated in altered insulin and glucose metabolism in two consecutive generations (Pentinat et al., 2010). Interestingly, maternal HFD models see similar changes in body weight and insulin signaling (Dunn et al., 2011), with a male germline mediated mode of inheritance, further suggesting that sperm microRNAs or paternally imprinted genes may be responsible (Dunn et al., 2011). This is also supported by studies that have not identified methylation-based differences in paternal sperm following diet manipulations (Shea et al., 2015). Future research should consider that exercise intervention could be similar to preconception enrichment protocols, which have been shown to increase maternal care of F_1 offspring. Clearly, these studies suggest that epigenetic machinery is altered by paternal diet and transmitted to offspring. However, the role that paternal obesity has on female's investment in offspring remains vastly underexplored.

1.4.2.3 Enrichment

Limited research exists describing the effects of paternal enrichment on offspring development and behavior. Like stress and obesity, research on the prospect that advantageous effects of EE can be transgenerationally inherited from parent to offspring has been more focused on the female germline. Intergeneration inheritance through the female germline has been demonstrated to play a role in F_1 offspring LTP and memory formation (Arai et al., 2009; Arai and Feig, 2011). Transgenerational inheritance through the female germline, which is only in the F_3 generation or beyond (Skinner 2008), has

shown restorative effects of EE on offspring HPA-axis function and anxiety like behavior (McCreary et al., 2016). While enriched environments are typically associated with increases in maternal care (D'Andrea et al., 2010; Connors et al., 2015), others have reported no differences based on maternal preconception EE (Bechard and Lewis, 2016), though this is more likely due to differences in animal models and scoring protocols.

Preconception paternal enrichment has been shown to increase exploratory behavior, reduce brain weight, and global methylation within the hippocampus and prefrontal cortex (PFCx) of F1 offspring (Mychasiuk et al., 2012b). This is potentially mediated by increased maternal care of F_1 offspring (Curley et al., 2011; Mashoodh et al., 2012).

1.5 Objectives and Preface to Manuscripts

Recent work has established solid evidence that paternal stress exposure has profound programming effects on offspring. However, the role of maternal investment following F_0 exposures has been critically ignored. Therefore, we sought to establish a model of F_0 exposure to psychological (PO) and metabolic (HFD) stress with attention to maternal investment and care. We also manipulated maternal care by placing dams and P0 F_1 offspring in the (SNH), which had previously been shown to enhance maternal care behavior. SNH-enhanced maternal care behaviors allow for a wider range of maternal behavior and the potential for deleterious paternal stress-induced effects on offspring to be reversed by increased quality of care in the postnatal period. Further, SNH rearing allows for enhanced behavior flexibility in offspring once they become mobile. Together,

higher-quality maternal care and room for more active play will have profound effects on offspring adult phenotypes. Following weaning, we measured offspring social, anxiety-like, stress responsive behavior, and gene regulation expression in F_1 offspring sired by F_0 males exposed to psychological stress (Chapter 2) or metabolic stress (Chapter 3 and 4).

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CHAPTER 2 EFFECTS OF PATERNAL PREDATION RISK AND REARING ENVIRONMENT ON MATERNAL INVESTMENT AND DEVELOPMENT OF DEFENSIVE RESPONSES IN THE OFFSPRING

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2.1 Abstract

Detecting past experiences with predators of a potential mate informs a female about prevailing ecological threats, in addition to stress-induced phenotypes that may be disseminated to offspring. We examined whether a male rat's prior exposure to predator (cat) odor influences a female's attraction towards a male, subsequent mother-infant interactions and the development of defensive (emotional) responses in the offspring. Females displayed less interest in males that had experienced predator odor. Mothers that reared young in larger, semi-naturalistic housing provided more licking and grooming and active arched-back-nursing behavior toward their offspring compared to dams housed in standard housing, although some effects interacted with paternal experience. Paternal predation risk and maternal rearing environment revealed sex-dependent differences in offspring wean weight, juvenile social interactions and anxiety-like behavior in adolescence. Additionally, paternal predator experience and maternal housing independently affected variations in *crf* gene promoter acetylation and *crf* gene expression in response to an acute stressor in offspring. Our results show for the first time in mammals that variation among males in their predator encounters may contribute to stable behavioral variation among females in preference for mates and maternal care, even when the females are not directly exposed to predator threat. Furthermore, when offspring were exposed to the same threat experienced by the father, hypothalamic *crf* gene regulation was influenced by paternal olfactory experience and early housing. These results, together with our previous findings, suggest that paternal stress exposure and maternal rearing conditions can influence maternal behavior and development of defensive responses of offspring.

2.2 Introduction

In the rat, postnatal maternal behavior is a critically important part of the early nurturing environment with respect to neurobehavioral development of subsequent generations (reviewed in (Weaver, 2010; Weaver, 2014)). Anticipatory parental effects, for example, through detecting the predation risk experienced by a potential mate, may allow females to adjust maternal behavior in order to increase their own survival and/or to increase the survival of offspring by preparing the neonates for living in the forecasted environment where certain threats are present (Harris and Uller, 2009; Mousseau et al., 2009). Indeed, gestational predator odor exposure has been used previously to exert effects on offspring (Korgan et al., 2014; St-Cyr and McGowan, 2015) and when administered soon after parturition increases maternal behavior (McLeod et al., 2007; Mashoodh et al., 2009) and alters anxiety in adult offspring (Mashoodh et al., 2009). This is consistent with other literature showing that female rodents are capable of altering maternal behavior based on other environmental features of their mates, such as adolescent exposure to environmental enrichment (Mashoodh et al., 2012).

Observational studies have provided evidence for stable individual differences in two main forms of mother-pup interaction, licking/grooming (LG) and arched-back nursing (ABN) posture, over the first week of lactation (Stern, 1997; Champagne et al., 2003a). Maternal LG-ABN behavior during the first week of life is associated with long-term programming of individual differences in responsiveness of the hypothalamic-pituitary–adrenal (HPA)

axis, anxiety-like and cognitive performance and reproductive behavior in the rat (Weaver, 2011). As adults, the offspring of high LG-ABN mothers show decreased expression of corticotrophin releasing factor (CRF), in the paraventricular nucleus of the hypothalamus (PVN), and a lower corticosterone response to stress by comparison to adult animals reared by low LG-ABN mothers (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999a). Offspring of low LG-ABN dams have increased DNA methylation and decreased acetylation of lysine 9 on histone H3 (H3K9) of the exon 1_7 glucocorticoid receptor-alpha (GRα) promoter region, decreased NGFI-A transcription factor association, and decreased GRα expression (Weaver et al., 2004b; Weaver et al., 2007; Weaver et al., 2014a); leading to disinhibition of CRF secretion and a higher corticosterone response to stress (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999a).

In addition to maternal behavior, recent studies have demonstrated effects of paternal age (Smith et al., 2009; Smith et al., 2013), obesity (Ng et al., 2010; Fullston et al., 2013), enrichment (Mashoodh et al., 2012), and physiological/psychological stress (Franklin et al., 2010; Dietz et al., 2011; Hoyer et al., 2013; Mychasiuk et al., 2013; Rodgers et al., 2013; Gapp et al., 2014a; Wu et al., 2016) on offspring. These paternal effects could be disseminated via sperm (Dias and Ressler, 2014), facilitated by sperm miRNA (Rodgers et al., 2013; Gapp et al., 2014a; Rodgers et al., 2015); but maternal behavior may also propagate these effects (Mashoodh et al., 2012). The differential allocation hypothesis suggests that the dam can detect prior experiences of potential mates through variation in his behavior and/or chemical cues, and then vary her own reproductive investment accordingly, including offspring rearing strategies (Burley, 1988). For example, dams

mated with males that had been reared in an enriched environment show increased LG-ABN behavior toward their offspring (Mashoodh et al., 2012). Consistent with this, we have shown that early rearing in semi-naturalistic housing (SNH) has profound effects on offspring development—induced seizure severity and number of CRF-immunoreactive neurons were reduced in juvenile rats raised in SNH compared to offspring reared in standard housing (SH) (Korgan et al., 2014; Korgan et al., 2015). This raises the question of whether the effects of SNH on *crf* gene regulation and stress responsivity are propagated by variations in maternal behavior.

In the present study, we take advantage of the properties of predator cues and the ecological validity of predation threat to examine whether maternal behavior can be indirectly influenced by a mate's prior predator experience. Herein, we examined the potential interaction of paternal predation threat and maternal environment on maternal behavior and development of social and defensive responses in the offspring. Fear and anxiety-like behaviors were examined in the adolescent offspring, along with H3K9ac association and *crf* promoter activation in the PVN.

2.3 Materials & Methods

2.3.1 Animals and Breeding

Thirty-eight Long-Evans hooded rats, 20 males and 18 females (purchased from Charles River Canada, Quebec) at ~60 days old were used for F_0 testing and breeding. All rats were housed in same sex pairs and given one week to acclimate prior to the beginning of the

experiment. Rats were housed in a colony room under a 12h:12h reversed light cycle (lights off at 0930h). Temperature in the colony room was maintained at 21 °C ± 2 °C. Rats were caged in SH, which consisted of polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids, containing pine shavings for bedding (Hefler Forest Products, Inc., Sackville, N.S. Canada) and a black PVC tube (12 cm length, 9 cm diameter), unless housed in SNH (see below). Both rat chow (Purina Lab Chow) and tap water were supplied *ad libitum*. When breeding occurred, as described below (see Figure 2.1A), one male and one naïve female determined to be in estrus were housed together for 5 consecutive days. Pups remained with the dam until weaning (Day 21; see Figure 2.1B), upon which the offspring were re-housed with a same-sex littermate. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

2.3.2 Paternal Stress Exposure

The timeline of the described experimental procedures is shown in Figure 2.1A-B. Following the one-week acclimation period, the paternal odor exposure (OE) trials began. Male cage mates were randomly assigned to one of two experimental conditions: paternal stress (predator odor, PO, n=10) or control odor (CO, n=10). Trials were 30 min in duration and took place at approximately 0930h, 1230h and 1530h for seven consecutive days. Males were transported from the colony room in covered transport cages to a designated testing room, where odor exposure trials took place under red light. Trials were performed in a clean Plexiglas test cage (60 cm x 27 cm x 35.5 cm) with a white plastic floor and a

clear Plexiglas lid with ventilation holes. For PO males, the odor stimulus was a piece of cat collar, approximately 1 cm long, attached via an alligator clip to one end wall of the box, approximately 5 cm from the top. The pieces of collar for the PO condition came from a collar that had been worn for at least 2 weeks by a reproductively intact domestic female cat housed communally in the Psychology Department. For CO males, the odor stimulus was a clean piece of collar. PO and CO conditions took place in different rooms and were performed according to an existing standard operating procedure designed to avoid cross-contamination (e.g., different gloves used and discarded in separate locations). All trials were recorded using a video camera for behavioral scoring. After each trial, rats were transported back to the colony room and placed back into their home cages. Videos were scored manually (by an observer blinded to the experimental conditions) to measure avoidance and anxiety-like behaviors, including; the frequency and duration of rearing (standing on hind paws only, with or without leaning on the perimeter wall), grooming (2 sec or longer bouts that involve; licking, nibbling and combing-like actions of the fur), the duration of time spent within 10 cm of the wall possessing the odor stimulus (vicinity time), and the frequency of odor stimulus contacts.

2.3.3 Partner Preference Test

Partner preference tests (PPT) were performed either 1 or 17 days following the final odor exposure trial (Figure 2.1A). In preparation for the PPT, sexually naïve females were vaginally swabbed; only females in estrus were used. Six females were used in the PPT 1 day following odor exposure and 4 females were used in the PPT 17 days following odor

exposure. The PPT was performed using a T-maze (base: 50 x 10 x 10 cm, arms (x2): 50 x 10 x 10 cm, and a clear Plexiglas lid; Figure 2.2A) containing two male rats and one female rat. Rats were transported to the testing room in covered cages. Trials occurred under red light and were recorded using a video camera positioned directly above the maze. Before the trial, the female rat was placed in the clean maze and allowed 5 min for habituation before being removed. Each 10-min trial was started by placing one female into the base of the maze. One PO male was placed in the end of one arm and one CO male was placed in the end of the other arm. During the test, males were restricted to the arm ends and separated by a clear Plexiglass sheet with multiple holes. The female was allowed to explore the entire maze. The total duration that the female spent inside an arm and oriented toward a male, along with the number of entries into each arm were recorded. For analysis, we calculated the percentage of the total time spent with each individual male per time spent with both males. Upon completion of a trial, the maze was cleaned with ethanol. Males were paired with sexually naïve, receptive females within 12 hours following the PPT for 5 days (Figure 2.1A).

2.3.4 Semi-Naturalistic Housing (SNH)

Dams mated with CO and PO males were observed daily for pups once they reached gestational day 20 (GD20), near the beginning of the dark cycle. Once the pups arrived (postnatal day 0, PD0), the litter was sexed, counted and weighed as quickly as possible to minimize disruption to the dams. Dams and litters randomly designated for the semi-naturalistic housing (SNH) condition (n=10) were transferred to SNH cages on PD0. Dams

and pups in the standard housing (SH) condition (n=8) were placed in clean, standard home cages. All dams and pups remained in their respective environments until the pups were weaned at PD21. The SNH (Figure 2.3A inset) consisted of two sections: an upper section, (50.5 cm x 50.5 cm x 33.5 cm) containing food and water ad libitum and a lower section, (50.5 cm x 50.5 cm x 14 cm) filled with pine shavings and a PVC tube.

2.3.5 Maternal Care Observations

Dams' maternal behavior was observed and scored daily in real-time for 60 min at 0800h, 1100h, 1300h, 1500h and 2130h. During each observation period, the frequency of the following behaviors were scored every 3 min as in Champagne (2003a) and Popoola (Popoola et al., 2015): no contact with pups (NCP), which may or may not include self-grooming (SG), nest building (NB), or feeding behaviors (F), passive nursing (PN), arched-back-nursing (ABN) ranked as level 1 (low blanket posture) and 2-4 (high postures favorable for milk ejection), licking and grooming pups, separated pups (SP) and pup retrieval (PR). The 'no contact' behaviors consist of a dam making no contact with her pups and often being accompanied by self-grooming behavior (licking, nibbling and combing-like actions of the fur), nest building (changing the positioning or location of the pine shavings around the nest), and feeding behavior (nibbling at the feeder, consuming rat chow or drinking water). Passive nursing was scored when the dam was on her side to nurse her pups, or used the sides of the cage to support her while nursing. Blanket posture or ABN1 was observed when the dam was flat over the pups. ABN consisted of graded degrees of arching, levels 2-4, based on kyphosis or the bend of the knees and steepness of

back arching of the pups. Separated pups were recorded when a dam had pups away from the nest, isolated or in small groups. Pup retrieval was the transfer of pups back into the nest.

2.3.6 Offspring Groups

The following four groups of male and female offspring were studied as juveniles and in peri-adolescence: CO-SH – father exposed to control odor and mother housed in standard housing (n=12 males, 12 females); CO-SNH – father exposed to control odor and mother housed in semi-naturalistic housing (n=10 males, 10 females); PO-SH - father exposed to predator odor and mother housed in standard housing (n=8 males, 8 females); PO-SNH – father exposed to predator odor and mother housed in semi-naturalistic housing (n=8 males, 10 females). Each group contained 2-4 males and females from multiple litters: CO-SH (n=4 litters), CO-SNH (n=5 litters), PO-SH (n=4 litters), and PO-SNH (n=5 litters).

2.3.7 Monitoring of Offspring Juvenile Play

Pup play behavior observations began on PD24, and were conducted daily at approximately 1100h, 1300h, and 1500h for five consecutive days. Observation sessions lasted one hour, during which time an experimenter would record play behaviors observed in the home cage at a fixed interval of 3 min. Behaviors scored included social grooming (licking and/or chewing the fur of the conspecific, while placing forepaws on the back or the neck); “attack” behaviors, pouncing (play initiation, forepaws extended towards play partner,

typically directed at neck, paws contact first), nose-to-nape attempted approach (nose approaches play partner's neck within 1-4 cm), nose-to-nape successful approach (nose contacts play partner's neck), and pinning (positioned over play partner with forepaws on partner); “defense” behaviors, full rotation (rolling supine, on back, to face play partner, interposing face/forepaws between attacker and nape), partial rotation (rolling towards supine to face play partner, but with at least one hind paw on floor), upright defense (turn to face play partner from an upright position on hind paws), and boxing (standing upright on hind paws, forepaws extended towards play partner in pushing or swiping motions); and evasion (swerving or leaping away from play partner, fleeing), adapted from (Field et al., 2006).

2.3.8 Anxiety-Behavior Testing of Juvenile Offspring

Offspring anxiety behavior testing was performed on PD32-35. Male and females (n=8-12 per sex, per group) were tested in the open field arena and elevated plus maze. In both tests, each trial was recorded under red light, using a vertically mounted video camera, for future behavioral scoring. Pups were transported to and from the testing room in covered home cages and the arena and maze were cleaned with 30% ethanol solution between each trial:

2.3.8.1 Open Field Test (OFT).

The OFT apparatus consisted of a solid, black Plexiglas square (79.2 cm x 78.9 cm x 35.0 cm), divided into 16, equal quadrants designated by nontoxic white paint on the maze floor. At the beginning of each 5 min trial, offspring were placed into the center of the maze. The behaviors

scored for the OFT were as follows: line crosses (all four limbs crossing into a new quadrant), time in center (time spent in the four center squares, 25% of the total area), as well as freezing, grooming and rearing (as defined for the EPM).

2.3.8.2 Elevated Plus Maze (EPM).

The EPM apparatus was constructed of solid black Plexiglas, with two open arms (11.2 cm x 50.2 cm), adjacent to two closed arms (11.3 cm x 50.4 cm x 40.2 cm), elevated 40.0 cm from the floor. At the beginning of each 10 min trial, the rat was placed in the center platform of the maze (11.2 cm x 10.2 cm). Offspring behavior in the EPM trials was scored using video recordings and were as follows: line crosses (all four limbs crossing over the central platform), time in open arms (duration of time spent in open arms), time in closed arms (duration of time spent in closed arms), entries into open arms (all four limbs crossing into an open arm), entries into closed arms (all four limbs crossing into a closed arm), attempts into open arms (stretch-attend posture at the opening of an open arm, less than four limbs entering the arm), attempts into closed arms (stretch-attend posture at the opening of an closed arm, less than four limbs entering the arm), freezing frequency and duration (2 sec or greater period without movement, but not sleeping), grooming frequency and duration (2 sec or longer bouts that involve; licking, nibbling and combing-like actions of the fur), and rearing frequency and

duration (standing on hind paws only, with or without leaning on the perimeter wall).

2.3.9 Acute Stress Exposure in Peri-Adolescent Offspring

7-10 days after anxiety-behavior testing was completed, these same offspring were randomly assigned to either PO or CO exposure (n=4-7 per sex, per group), identical to the protocol used for the paternal stress exposure (see above). The only variation in this protocol is that offspring were only exposed for one 30 min trial, followed immediately by sacrifice.

2.3.10 Sacrifice and Tissue Collection

In the preparation of fixed tissue, animals were deeply anesthetized with Euthanyl (sodium pentobarbital, 60 mg/kg, i.p.) and then perfused transcardially with heparinized saline (30-60 ml), followed by paraformaldehyde (4%) in phosphate buffered saline (PBS, pH 7.4) for 15 min. After perfusion, all brains were removed and post-fixed in the same fixation solution overnight at 4°C and then transferred to PBS containing sucrose (20%) for 48h. Tissue was frozen at -80°C until further processing. Whole brains were blocked and sectioned with a microtome. PVN containing sections (coordinates with respect to bregma were -1.6 to -2.12 mm antero-posterior, 1.5 mm lateral from the midline, and -9.0 mm dorsoventral from the dura) were identified using the rat brain atlas (Paxinos and Watson, 1986), micropunched using a 20 gauge cannula (PlasticsOne) and stored at -80 °C until

used. To confirm the dissection site, serial coronal sections (20 mm thick) of the micropunched PVN tissue were cut on the microtome, thaw mounted onto positively charged microscope slides and stored at -80 °C. Slices were then stained with 0.25% DAPI (Roche Life Science, Indianapolis, IN) for 1 min, mounted in PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific, Waltham, MA). Images were acquired with a Zeiss Axio Imager Z2 fluorescent microscope (Carl Zeiss, Oberko- chen, Germany) and a high-resolution color digital camera using a 10x objective. At least four sections were examined per animal (Figure 2.6E).

2.3.11 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation assays (Crane-Robinson et al., 1999) were performed following the ChIP assay kit protocol® (Cat#06-599, Upstate Biotechnology). Chromatin was immunoprecipitated from PVN micropunch samples using a rabbit polyclonal antibody against H3K9ac or normal rabbit IgG non-immune antibody (both from Santa Cruz Biotechnology). One-tenth of the lysate was kept to quantify the amount of DNA present in different samples before immunoprecipitation (Input). Protein-DNA complexes were uncrosslinked, by adding 20 µl NaCl (5 M) to each sample (4 h, 65 °C), followed by 10 µl EDTA (0.5 M), 20 µl Tris-HCl (1 M, pH 6.5) and 2 µl PK enzyme (10 mg/ml) (1 h, 45 °C). Following phenol-chloroform (0.5 v/v) extraction, the free-DNA was ethanol (2 v/v, 95 %) precipitated with 5 µl tRNA (10 mg/ml) and re-suspended in 100 µl 1xTE. The rat *crf* promoter region (−206 to 318, containing a cAMP response element) of the uncrosslinked DNA was subjected to polymerase chain-reaction (PCR) amplification

(Forward primer: 5′-TCAGTATGTTTTCCACACTTGGAT-3'; Reverse primer: 5′-TTTATCGCCTCCTTGGTGAC-3'). For quantitative real-time PCRs, PCR mixtures (12.5 µl) containing the immunoprecipitated DNA, SsoFast™ EvaGreen® Supermix (Cat#172-5203, Bio-Rad Laboratories) and 4 µM primer were loaded onto a 96 multiwell plate and covered with a seal (Bio-Rad Laboratories). The thermocycler (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad Laboratories) protocol involved an initial HotStart enzyme activation cycle (2 min, 95 °C, with a temperature transition rate set at 4.40 °C/sec), 40 cycles of denaturation (5 sec, 95 °C, with a temperature transition rate set at 4.40 °C/sec) and annealing (30 sec, 60 °C) with a temperature transition rate set at 2.20 °C/sec). A single fluorescence reading was acquired at the end of each elongation step. Triplicate average qPCR cycle threshold (Ct) value for input (10%) samples: ~24-26, with a 4-8 fold difference between qPCR Ct values of the H3K9ac antibody IP'ed samples (qPCR Ct value: ~29-31) or negative control IgG non-immune antibody IP'ed samples (IgG, Ct value: ~34 or not detected after 40 cycles). The specificity of the amplified PCR products was assessed by performing a melting curve analysis cycle after the PCR amplification (5 sec, 95 °C, with a temperature transition rate set at 4.40 °C/sec; 1 min, 65 °C, with a temperature transition rate set at 2.20 °C/sec) that terminated with a cooling step (30 sec, 40 °C, with a temperature transition rate set at 2.20 °C/sec). The fluorescence of the SsoFast™ EvaGreen dye bound to double stranded amplified product declines sharply as the fragment is denatured. The melting temperature of this fragment was visualized by plotting the first negative derivative (dF/dT) of the melting curve on the y-axis and temperature (°C) on the x-axis. No primer-dimers were detected that interfered with the quantification of the PCR products. The Ct values of ChIP DNA fractions were normalized

to the Ct value of the input DNA fraction for the same qPCR assay (ΔCt) to account for differences in chromatin sample preparation. Relative H3K9ac enrichment was measured by the $2^{-\Delta\Delta CT}$ method, using the DNA fractions IP'ed with IgG as the negative control (Livak and Schmittgen, 2001).

2.3.12 *RT-qPCR analysis*

Total RNA was isolated from PVN micropunch samples using the Arcturus Paradise Plus RNA Extraction and Isolation Kit (Life Technologies), which permits recovery of high quality RNA from a small number of fixed cells. Precipitated RNA was dissolved in RNase-Free H_2O and quantified (~274-335 ng RNA/50ul) with a Take3™ Micro-volume Plate on an Epoch Spectrophotometer (BioTek Instruments INC). RNA integrity was confirmed using an Experion Automated Electrophoresis System and RNA StdSens chip (Bio-Rad Laboratories). The RNA quality index value (RQI) for all samples >7.9 with low degradation. cDNA was synthesized in a 20 µl reaction volume containing 100 ng of total RNA, 40 units of Moloney murine leukemia virus reverse transcriptase (MBI), 5 µM random primer (Roche Molecular Biochemicals), a 1 mM concentration of each of the four deoxynucleotide triphosphates, and 40 units of RNase inhibitor (Roche Molecular Biochemicals). The mRNA was denatured (5 min, 70 °C), the random primers were annealed (10 min, 25 °C) and mRNA was reverse transcribed (1 h, 37 °C). The reverse transcriptase was heat-inactivated (10 min, 72 °C) and the products were stored at -20 °C. Rat PVN *crf* (NM_000756.1) heteronuclear RNA (hnRNA) was subjected to qPCR amplification (forward primer: 5'-TCAATCCAATCTGCCACTCA-3'; reverse primer: 5'-

TAAGCTATTCGCCCGCTCTA-3'). To control for equal loading, the rat *ribosomal protein L13A* (*Rpl13A*, NR_073024) exon region was also subjected to PCR amplification (forward primer: 5'-ACAAGAAAAAGCGGATGGTG-3'; reverse primer: 5'-TTCCGGTAATGGATCTTTGC-3'). The *crf* hnRNA and Rpl13A amplification were performed in parallel, using a 25 µl reaction mixture containing 1.5 µl of synthesized cDNA product and the SsoFast™ EvaGreen® Supermix (Bio-Rad) (Pfaffl, 2001). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 °C), 20-30 cycles of denaturation (30 sec, 95 °C), annealing/extension (45 sec, 60 °C), followed by a final extension cycle (5 min, 72 °C) terminating at 4 °C. The specificity of the amplification reaction was assessed by melt curve analysis and agarose gel electrophoresis of the PCR products. To control for equal loading between samples, the signal of the *crf* hnRNA was divided by the signal from the Rpl13A region amplified from the same sample.

2.3.13 ***Statistical analyses***

Differences between CO and PO males during OE were analyzed using independent Student's t-test. For each group of males (1 day and 17 days from OE), differences in time spent with CO and PO males by females in the PPT were analyzed using separate mixed-design analysis of variance (ANOVA) with paternal condition (CO, PO) as the between-subject factor and time block (first 4 min, last 4 min) as the within-subject factor. Maternal behavior was analyzed using two factor ANOVA with paternal condition (CO, PO) and maternal condition (SH, SNH) as between-subject factors for each dependent variable. Offspring wean weight and behavioral test data were analyzed using linear mixed models.

Sex, paternal condition, and maternal condition were used as between-subject factors, with litter treated as a nested factor for each dependent variable. Data from molecular endpoints were analyzed in an identical fashion with the addition of offspring odor exposure (F_1CO, F_1PO) as a fourth between subject factor. Interactions were analyzed post-hoc with simple effects analyses, with a Bonferroni correction. A threshold level of $p < 0.05$ was used to test for significance. The Statistical Package for the Social Sciences (SPSS Inc., USA) software was used for all statistical analyses.

2.4 Results

2.4.1 Predator odor exposure induces anti-predator behavior in males and reduces partner preference in females.

A summary of the research design is shown in Figure 2.1A-B. To determine avoidance behavior in males in response to predator odor, we examined time spent in proximity to the collar containing the odor. We then used a modified partner preference test (PPT) as a proxy for sexual and social preferences of virgin age-matched females toward either PO or CO males (apparatus shown in Figure 2.2A). Males exposed to PO spent significantly less time in the immediate presence of the collar compared to males being exposed to CO ($t=4.321$ (10) $p<0.001$; Figure 2.1C). There was no significant difference in the number of line crosses made by females during the PPT into arms containing CO- versus PO-exposed males, either 17 days ($F=0.058$ (1,6) $p=0.818$; CO - M=17, SE=2.979; PO - M=16.5, SE=2.979) or 1 day ($F=0.007$ (1,10) $p=0.935$; CO - M=12.333, SE=1.603; PO - M=12.167, SE=1.603) after odor exposure (not shown). During the last four minutes of the test 1 day

after OE, we detected a main effect of PO treatment, with females displaying significantly less interest toward PO males compared to CO males ($F=5.131$ (1,10) $p=0.047$; Figure 2.2B). The effect of male's predator experience on female preference remained stable, lasting more than two weeks following the final predator odor exposure ($F=6.418$ (1,6) $p=0.044$; Figure 2C). These results suggest that the olfactory experience not only influences avoidance behavior in the male, but also stably increases avoidance behavior in females. Although correlations between paternal vicinity time during odor exposures and female preference were in the positive direction, indicating greater avoidance by females of males that had shown greater responsiveness during OE, these were not statistically significant (all males – $r=0.514$, $p=0.088$; PO males only - $r=0.436$, $p=0.388$).

2.4.2 Semi-naturalistic housing increases maternal care and interacts with paternal experience.

Paternal stress (Gapp et al., 2014a), maternal nurturing behavior (Weaver et al., 2004b) and the context (Connors et al., 2015) of the early rearing environment have profound influences on postnatal development in the offspring. To examine these interactions, females were mated with PO and CO males and then raised their offspring in either standard (SH) or semi-naturalistic homes (SNH) and the mother-infant interactions were monitored during the first week of postnatal life (apparatus shown in Figure 2.3A inset). We found no effects of, or interactions between, maternal and paternal condition on litter size ($F=1.746$, (1,17), $p=0.209$), sex ratio of litters ($F=1.390$, (1,17), $p=0.260$), or birth weight ($F=2.647$, (1,17), $p=0.128$). Dams housed in the SNH behaved differently toward

offspring relative to females housed in SH (Figure 2.3A). SNH dams displayed significantly lower overall frequency of contact with their offspring ($F=18.730$, $(1,17)$, $p=0.001$); including the frequency of blanket posture (ABN1) ($F=65.369$, $(1,17)$, $p<0.001$), passive nursing ($F=4.795$, $(1,17)$, $p=0.047$), passive contact ($F=24.446$, $(1,17)$, $p<0.001$), and pups separated from the litter ($F=4.815$ $(1,15)$, $p=0.05$), but showed significantly increased frequency of active arched-back-nursing (ABN3) ($F=4.707$ $(1,17)$, $p=0.049$) and significantly more feeding behaviors ($F=10.933$ $(1,17)$, $p=0.006$). Interestingly, offspring from PO fathers received significantly more ($F=8.930$, $(1,16)$ $p=0.011$) maternal LG-ABN2 when raised in SNH relative to SH (Figure 2.3B); this pattern was not observed for offspring from CO fathers. Maternal LG-ABN3 was increased in SNH conditions, regardless of paternal condition ($F=8.658$ $(1,17)$ $p=0.011$; Figure 2.3C). Finally, pup mortality was significantly decreased in SNH conditions ($F=9.023$, $(1,16)$, $p=0.011$; Figure 2.3D). These results, together with our partner preference findings, suggest that variation among males in their predator encounters may contribute to stable behavioral variation among females in courtship and maternal care, even when the females themselves are not directly exposed to a predator.

2.4.3 Paternal odor exposure and SNH affect weaning weight and social behavior in juvenile offspring.

To determine the extent of paternal stress effects and maternal nurturing behavior within the context of postnatal growth and social behavior development, we weighed the offspring at weaning and monitored play behavior in the home cage just after weaning (Figure 2.4A-

E). Males weighed more than females (F=11.421 (1,69) p=0.001) and offspring raised in SNH weighed more than those raised in SH (F=29.445 (1,69) p<0.001; Figure 2.4A). Females groomed more than males (F=11.602 (1,39) p=0.002) and offspring reared in the SNH groomed less than those reared in SH (F=5.156 (1,39) p=0.029); Figure 2.4B). Frequencies of play attacks (F= 7.711 (1,39) p=0.008; Figure 2.4C) and defensive play behaviors (F=44.194 (1,39) p<0.001; Figure 2.4D) were greater in male offspring than females. Evade behavior in response to play attacks was decreased in offspring reared in SNH (F=6.628 (1,39) p=0.014) relative to those reared in SH, but there was also an interaction between paternal stress experience and maternal rearing environment for this behavior (F=9.322 (1,39) p=0.004; Figure 2.4E). Post-hoc comparisons revealed that CO-SH reared offspring evaded more frequently than PO-SH (p=0.002) and CO-SNH (p<0.001) offspring. These findings suggest that postnatal growth and social behavior development are altered by housing environment, whereas preconception paternal predator odor exposure affects avoidance behavior in the peri-adolescent offspring.

2.4.4 SNH affects the development of fear- and anxiety-like behavior in the offspring.

To determine the effects of paternal stress and maternal rearing environment on behavioral responses to stress in developing offspring, peri-pubertal offspring were exposed to OFT and EPM tests (Figure 2.5A-C). In the OFT, offspring reared in the SNH spent more time in the center of the open field (F=8.346 (1,69) p=0.005; Figure 2.5A), regardless of paternal experience. In the EPM, females spent more total time in open arms (F=4.635, (1,69) p=0.035; Figure 2.5B) and entered open arms more frequently (F=7.509, (1,69) p=0.008;

Figure 2.5C) than males. In addition to a significant interaction between maternal and paternal conditions (F=5.986, (1,69) p=0.017), there was a significant 3-way interaction between paternal condition, maternal condition and sex for open arm entry (F=5.112, (1,69) p=0.027; Figure 2.5C). Post hoc comparisons revealed that PO-SH females made significantly more open arm entries relative to CO-SH females (p=0.043) and PO-SH males (p=0.024). Furthermore, CO-SNH females made more open arm entries than PO-SNH females (p=0.008), CO-SH females (p=0.002) and CO-SNH males (p=0.013). Our findings show lasting sex-specific effects on stress response behaviors as a function of paternal stress experience and maternal rearing conditions.

2.4.5 Effects of offspring predator odor exposure on their behavior and hypothalamic crf gene regulation.

We exposed the offspring of CO and PO fathers to the same threat experienced by the father preconception, to determine whether paternal olfactory experience predicted behavioral avoidance in the offspring (Figure 2.6A-D). Although there was no effect of OE on male offspring behavior (Figure 2.6A-B), female offspring exposed to predator odor reared for less time (F=9.311 (1,31) p=0.005; Figure 2.6C) and engaged in fewer total rears during the OE (F=8.690 (1,31) p=0.006; Figure 2.6D). Further, there was an interaction between paternal condition and maternal condition for both rear duration (F=5.132 (1,31) p=0.031) and rear frequency (F=5.355 (1,31) p=0.027), where, CO-SNH females reared longer (Figure 2.6C) and more frequently (Figure 2.6D) relative to CO-SH (p=0.006 and p=0.006, respectively) and PO-SNH (p=0.001 and p=0.002, respectively).

Given that semi-naturalistic housing affected maternal care and fear- and anxiety-like behavior in the peri-pubertal offspring, animals were sacrificed within 30 minutes of odor exposure and tissue punches (Figure 2.6E; for details see Materials and Methods section) were taken to measure hypothalamic *crf* gene expression and chromatin marks of gene regulation. Groups were collapsed across sex because no significant sex differences were observed in either the level of histone acetylation associated with the *crf* gene promoter region ($F=0.349$ (1,32), $p=0.559$) or *crf* gene promoter activity ($F=0.110$ (1,32), $p=0.743$), respectively. H3K9ac association with the *crf* gene promoter was significantly ($F=17.441$ (1,26), $p<0.000$) increased in offspring of PO males (Figure 2.6F) and decreased in offspring reared in the SNH ($F=7.898$ (1,26), $p=0.008$; Figure 2.6G). Moreover, offspring exposed to predator odor produced enhanced levels of H3K9ac association with the *crf* gene promoter ($F=58.934$ (1,26) $p<0.000$; Figure 2.6H), in addition to higher *crf* primary transcript (hnRNA) expression in the PVN by comparison to control odor-exposed animals ($F=29.024$ (1,32) $p<0.000$; Figure 2.6I). H3K9ac association and *crf* promoter activity were positively correlated ($r=0.631$, $p<0.01$) (Figure 2.6J), suggesting stable differences in *crf* gene promoter acetylation drives hypothalamic *crf* gene expression and possibly fear- and anxiety-like behavior in the peri-adolescent offspring.

2.4 Discussion

Here, we show interactive effects of paternal experience and maternal experience on anxiety-like phenotypes and associated stress-related molecular endpoints in offspring.

Further, we have added a consideration for maternal care—a facet that had been lacking in previous paternal stress literature. Specifically, preconception PO experience in males stably influenced behavioral variation among female mates in partner preference and interacted with an enhanced maternal housing environment to affect maternal care and offspring social and defensive behavior. Paternal predator experience and maternal housing independently affected variations in histone acetylation and *crf* gene activity in response to an acute stressor in offspring.

Repeated exposure of prey to predators or their cues activates the HPA axis and initiates defensive behaviors (Mashoodh et al., 2008) that are long-lasting, in part, through programming gene expression profiles supporting the neural circuitry of endocrine and behavioral responses to stress (Morrow et al., 2002; Wright et al., 2008; Roth et al., 2011; Wright et al., 2012). PO exposure in F_0 males induced an avoidance phenotype similar to past studies, in which we have noted that a similar repeated exposure paradigm results in increased baseline GCs and avoidance behavior (Mashoodh et al., 2008; Wright et al., 2008). In the present study, repeated exposure to PO resulted in an expected decreased preference of females for PO males relative to CO. The results of our PPT suggest that female's preference for non-stressed males is based on detection of a sensory or behavioral cue, rather than simply based on territory or copulative traits. In this initial investigation, we were interested mainly in comparing offspring of CO and PO males and we did not investigate mating behavior directly. Thus, we are unable to determine whether female preference behavior incited males to increase male-directed courtship or whether elevated levels of male-directed courtship induced females to show preference

behavior. While the directionality behind this pattern is unclear at this time, feedback and negotiations between males and females are important in mutual mate choice (Sheldon, 2000) and future investigations will likely reveal interesting effects from both sexes.

The relative influence of paternal versus maternal environments on maternal behavior has not been previously studied. Here, the contribution of maternal environmental effects was investigated by varying the housing conditions of females and their offspring. Past work shows that environmental enrichment of juvenile pups can reverse maternal effects of low LG-ABN and decrease stress responsiveness in the adult offspring (Bredy et al., 2003; Bredy et al., 2004). Previously, our lab has shown alterations in severity of induced seizures and CRF-positive neuron number in the hypothalamus of juvenile offspring raised in SNH, suggesting stable and critical effects of this rearing environment on development (Korgan et al., 2014; Korgan et al., 2015). Here, we continue to use juvenile and peri-pubescent animals to focus our investigations on effects during development. It is possible that our paternal or maternal conditions could have delayed pubertal development in pups, but, because behavioral testing was complete by PD35, any delay (even in females who undergo puberty earlier) would likely not have impacted the behavioral testing. However, future studies should include measures of pubertal status.

For the first time, we show that offspring reared in SNH experience a different quality of maternal care. Similar to brief maternal separation (Liu et al., 1997; Connors et al., 2015), our SNH (which increased time away from pups) induced more active bouts of maternal care. SNH housing was associated with a marked decrease in blanket posture (ABN1),

passive nursing, passive contact frequency, and pup mortality, contributing to an overall picture that SNH promotes more active, potentially higher quality maternal care behavior. Interestingly, maternal LG-ABN2 behavior was increased by SNH, but only toward offspring that had been sired by a PO-exposed father. Thus, a dam may alter her behavior based on the past experience of her mate, but her own experience is at least as important for shaping her overall maternal care, and possibly outcomes in her offspring.

Social behavior and weight at weaning were affected mainly by sex of the offspring and rearing environment. At weaning, males were predictably heavier than females and weight was increased in both SNH reared males and females. Research is mixed on the effects of early enrichment on weight gain, likely dependent on the extent that enrichment is physical and/or social (Morley-Fletcher et al., 2003; Connors et al., 2015). Sex differences in offspring social behavior were similar to other reports (Meaney, 1988; Pellis et al., 1997); females engaged in more grooming, while males were more active in play attack, defensive and evasive behaviors. SNH rearing decreased grooming in male and female offspring. Maternal deprivation studies have identified deficits in sociality in offspring (Schneider and Koch, 2005; van Leeuwen et al., 2014). However, this difference in grooming could also be a manifestation of sex differences in play behavior, as male offspring engage in more attack grooming while females engage in more social grooming (Parent and Meaney, 2008). Future studies should distinguish between these two types of grooming behaviors. Further, in both sexes, CO-SH offspring displayed the most evasive behaviors in response to play attacks. This might suggest that both paternal

odor exposure and SNH rearing are priming offspring for a more direct response to potentially threatening stimuli.

A complex picture emerged when examining effects of maternal and paternal conditions on anxiety-related behavior in offspring. In the OFT, SNH reared offspring spent more time in the center of the arena, similar to results using adolescent enrichment (Morley-Fletcher et al., 2003) and similar to offspring of high LG-ABN dams (Weaver et al., 2004b). In the EPM, females showed higher levels in time spent in open arms and number of open arm entries relative to males. Sex differences in anxiety behavior were not unexpected. The interaction between paternal experience, rearing environment, and sex in the EPM is more interesting. In female offspring only, being sired by a father that was exposed to predator odor or being raised in semi-naturalistic housing resulted in more open arm entries, indicating anxiolytic behavior in these females.

Effects of paternal stress experience on anxiety-related behavioral outcomes have revealed inconsistent findings in previous work, as some models have shown anxiolytic effects only in juvenile male offspring following paternal stress experience (Mychasiuk et al., 2013; Rodgers et al., 2013). Differences in offspring behavior could be related to the stressors utilized or offspring age at testing. Effects of maternal experience seem more straightforward, with previous research showing the effect of prenatal stress on HPA-axis function in offspring is sex-dependent (McCormick et al., 1995; Brunton and Russell, 2010) and variations of epigenetic marks, mediated by maternal care, are more pronounced in female offspring (Champagne et al., 2003b). Postnatal enrichment has also

been shown to be more effective in female offspring (Welberg et al., 2006), consistent with our findings.

Exposing peri-adolescent female offspring to the PO paradigm resulted in the expected decrease in exploratory behavior, but male offspring exposed to PO did not show a reduction in activity relative to those exposed to CO, indicating a blunted behavioral response in males. The reason for this is unclear, but we have noted in other studies that patterns of behavioral responding to PO are sex-dependent (Mashoodh et al., 2012) and are not necessarily indicative of patterns associated with physiological responses (Mashoodh et al., 2008). Interestingly, females sired by a CO-exposed father and raised in SNH showed significantly higher levels of rearing behavior in response to CO *and* PO exposure relative to all other groups, suggestive of hyperactivity. We have observed previously that rearing behavior in female offspring is increased by manipulations that increased the levels of maternal care to which they were exposed (Mashoodh et al., 2009), suggesting a strong programming effect of maternal care on this particular behavior in female offspring.

Despite the sex differences noted in behavioral responses to odor exposure, PO exposure resulted in increases in H3K9ac association with the *crf* gene promoter in PVN as well as PVN *crf* hnRNA expression across both sexes, regardless of paternal or maternal condition. Interestingly, H3K9ac association with the *crf* gene promoter was increased in offspring sired by PO-exposed fathers but reduced in those reared in SNH. Importantly, these modest changes in acetylation did not translate into significant changes in transcript

levels—neither F_0PO nor SNH rearing significantly altered *crf* hnRNA expression—whereas the dramatic increase of histone acetylation triggered by predator odor exposure in the offspring was associated with enhanced *crf* hnRNA transcription. These results suggest that while the presence of H3K9ac chromatin marks favor a transcriptionally permissive state, they are not sufficient to influence the transcriptional rate of the *crf* gene (i.e., the actual amount of target hnRNA or mRNA) (Wang et al., 2009). The chromatin markings were also independent of sex, further suggesting additional mechanisms are involved (Elliott et al., 2010). Beyond gene expression regulation, CRF function is mediated by CRF receptors (CRF_1 and CRF_2), which can be expressed in a sex-dependent manner (Howerton et al., 2014), and exert both additive and opposing influences on fear and anxiety behavior (Risbrough et al., 2004). Although further work is obviously required to fully explore the extent of these effects and to isolate the primary mechanism, our experiments do indicate effects of both paternal and maternal history on key parameters of offspring stress responding.

Our findings provide the first evidence that variation in male predator experience influences partner preference by the female to produce stable alterations of chromatin structure and gene expression in the brain and behavioral responses to stress in the progeny. We showed an interaction between maternal condition and paternal condition on one measure of maternal care, but overall, maternal behavior was more affected by maternal condition and as such, future studies are needed to explore the mechanism(s) by which paternal behavior alters partner preference, exerts subtle effects on anxiety behavior and impacts the epigenetic status of the hypothalamic *crf* promoter in the

offspring. Clearly the gene-environment interaction is complex, especially as it pertains to early life programming and transmission of stress-induced traits. Nevertheless, our findings provide a mechanism for the inter-generational transfer of stressful paternal experience to shape adaptive responses in the offspring, through differential allocation among females in both partner preference and maternal care. These findings may help explain the immediate consequences of mothering style on pups, but the consequences are not necessarily self-perpetuating—such maternal effects appear to be dependent on the rearing environment early in juvenile development, resulting in stable alterations in phenotype of both the mother and her offspring.

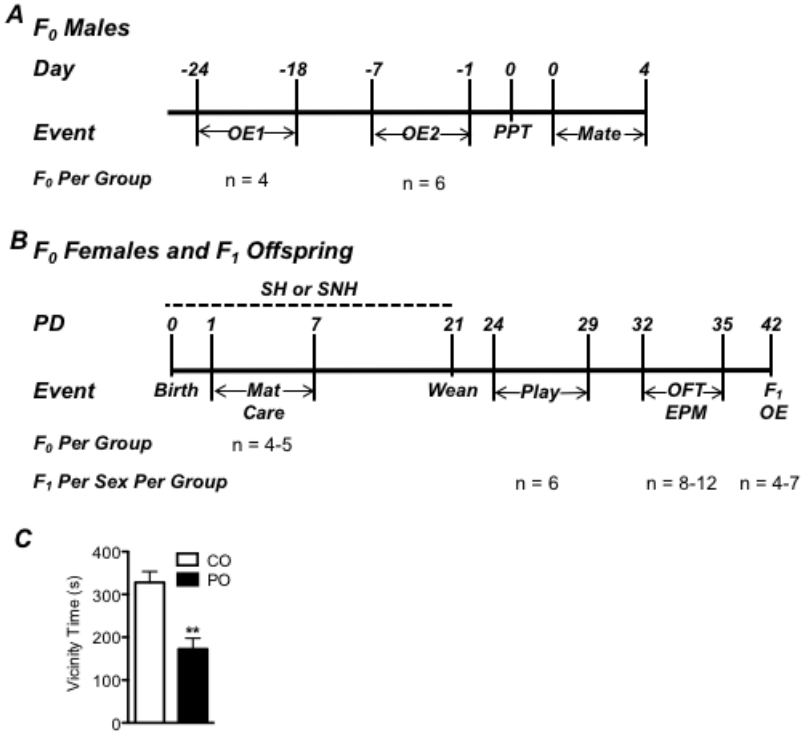

Figure 1

Figure 2.1. Experimental timelines and avoidance behavior in males during odor exposure. (A) Timeline of treatment procedures involving F0 males. During odor exposure (OE)1, males were exposed to either predator odor (PO) or control odor (CO) for 30 min, 3 times per day for 7 days beginning 24 days prior to mating. OE2 was conducted identically except that the CO and PO exposures began 7 days prior to mating. Males from OE1 and OE2 were subjected to a partner preference test (PPT) using sexually receptive virgin, naïve females. Within 12 hours of the PPT, males were bred with *different* receptive naïve virgin females. Following confirmed mating, males were removed and females were left undisturbed until offspring were born. **(B)** Timeline of treatment procedures for F0 females and the F1 offspring. Birth was considered postnatal day (PD) 0, and offspring were counted, sexed, and weighed before being transferred to either fresh standard housing

(SH) or semi-naturalistic housing (SNH), with biological mothers, until weaning. Maternal behavior (Mat Care) was scored for 1 hr, 5 times per day for 7 days. At PD 21, all offspring were weighed, weaned and placed in SH with a same-sex littermate. Play behavior was recorded in the home cage from PD 24-29, followed by exposure to the open-field test (OFT) and the elevated plus maze (EPM) on PD 32-35. F1 OE took place on PD 42 with male and female offspring being exposed to either PO or CO for 30 min and then sacrificed. Sample sizes are provided for both F_0 and F_1 groups. **(C)** Avoidance behavior in F_0 male rats was significantly increased in those exposed to PO relative to those exposed to control odor CO during OE. Data expressed as mean ± SEM; **PO different from CO, $p \leq 0.005$.

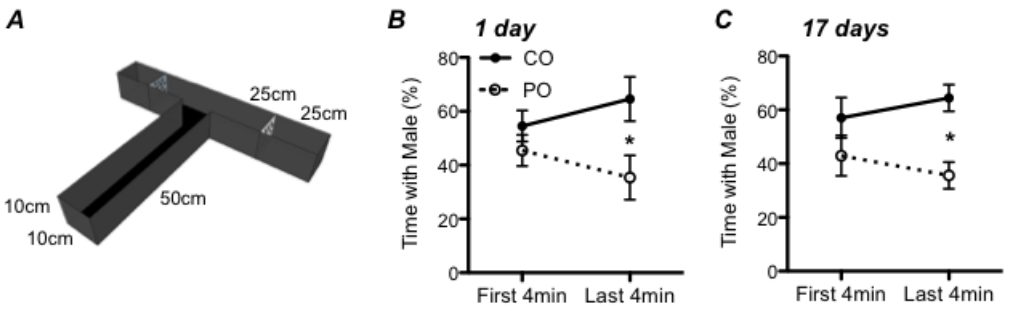

Figure 2

Figure 2.2: Use of a partner preference test (PPT) to ascertain female preference for males previously exposed to predator odor (PO) relative to control (CO). (A) Schematic representation of the T-maze used for the PPT. Males were placed in the ends of arms confined by Plexiglas shields containing many holes **(B-C)** Female rats spent less time in the vicinity of PO-exposed males relative to CO-exposed males during the last 4 minutes of a partner preference test, both 1 day and 17 days after the odor exposure had occurred in males. Percentage of time with males was calculated as the percentage of time spent with either a CO or PO male per total time spent with both CO and PO males. Data expressed as mean ± SEM; *PO different from CO, $p \leq 0.05$.

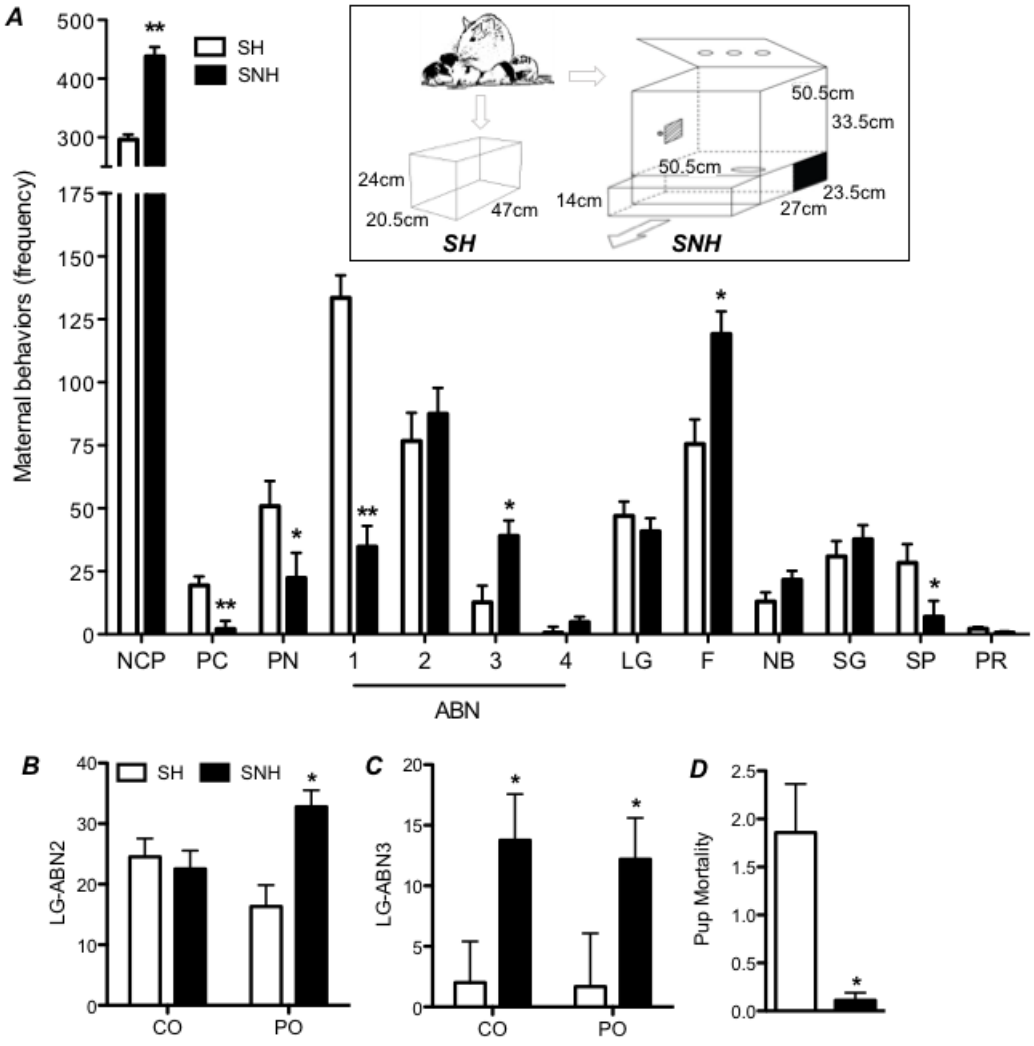

Figure 3

Figure 2.3: Maternal behaviors of females housed in semi-naturalistic housing (SNH) or standard housing (SH) raising offspring of mates that were exposed to either predator odor (PO) or control odor (CO). (A) Frequency of various maternal behaviors [NCP, no contact with pups; PC, passive contact (with pups); PN, passive nursing; ABN1, arched-back nursing 1 (blanket posture); 2, arched-back nursing 2; 3, arched-back nursing 3; 4, arched back nursing 4; LG, licking/grooming; F, feeding; NB, nest building; SG, self

groom (auto groom); SP, separated pups (from the rest of the litter); PR, pup retrieval] displayed by females housed in SH and SNH, collapsed across paternal condition. The inset shows schematics of the housing conditions; the SNH includes a lower burrow compartment (contained within a drawer that moves out to facilitate cleaning) and an upper section (containing food and water), with the two sections being connected by a hole (visible in the upper section). **(B)** The frequency of LG-ABN2 behaviors was significantly increased in dams raising offspring in SNH relative to those housed in SH, but only if offspring were from PO exposed males. **(C)** The frequency of LG-ABN3 behavior was increased in dams living in SNH relative to SH, regardless of paternal experience. **(D)** Living in SNH reduced pup mortality relative to living in SH. Data expressed as mean ± SEM; *SNH different from SH, $p \leq 0.05$; **SNH different from SH, $p \leq 0.005$.

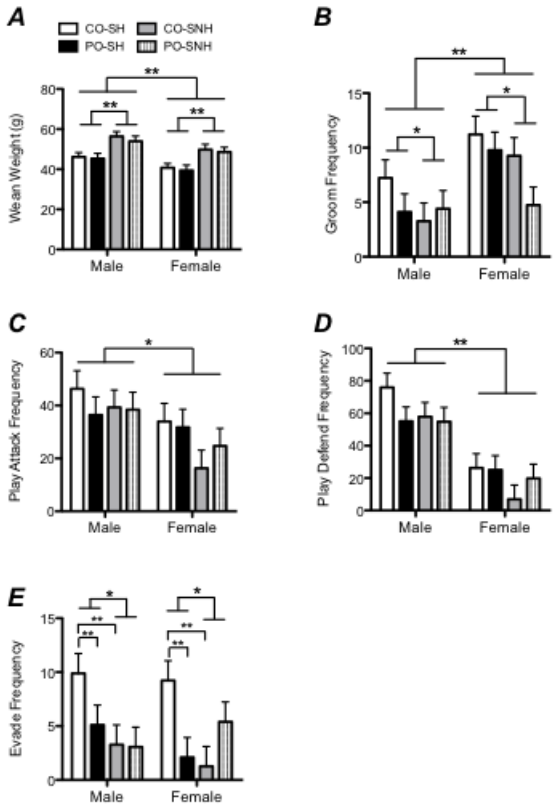

Figure 4

Figure 2.4. Weaning weight and social behavior of juvenile offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by males exposed to either predator odor (PO) or control odor (CO). (A) SNH rearing significantly increased weaning weight in both male and female offspring, regardless of paternal experience, with males weighing more overall than females. **(B)** Increased social grooming occurred in female, compared to male offspring and was higher in offspring reared in SH relative to those reared in SNH. **(C)** Males engaged in significantly more play attacks than females. **(D)** Males also engaged in significantly more play defend behaviors relative to females. **(E)** Evade behavior in response to play attacks was decreased overall in offspring reared in SNH relative to SH, but more specifically, CO-

SH reared offspring evaded more than PO-SH and CO-SNH offspring. Data expressed as mean ± SEM; Difference between indicated groups, * = $p \leq 0.05$; ** = $p \leq 0.005$.

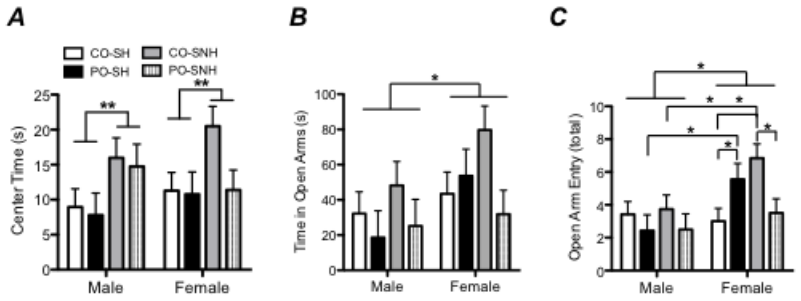

Figure 5

Figure 2.5. Development of fear- and anxiety-like behavior in the offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by males exposed to either predator odor (PO) or control odor (CO). (A) Center time in the open-field test was increased (indicative of reduced anxiety-like behavior) in offspring raised in SNH relative to those reared in SH. **(B)** In the elevated plus-maze (EPM), female offspring spent more time in the open arms relative to males. **(C)** Overall, in the EPM, female offspring displayed less anxiety (more frequent open arm entry) than males, and in particular, females of either CO-SNH or PO-SH groups displayed reduced anxiety relative to other groups. Data expressed as mean ± SEM; interaction; Difference between indicated groups, * = $p \leq 0.05$; ** = $p \leq 0.005$.

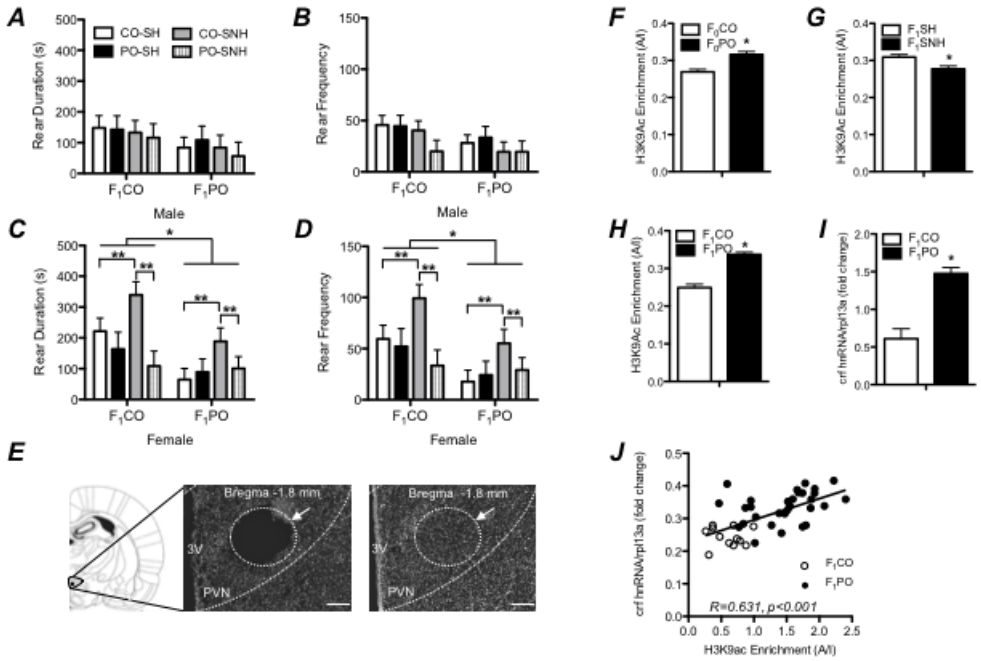

Figure 6

Figure 2.6. Effects of 30 min exposure to either control odor (F_1CO) or predator odor (F_1PO) on behavior and hypothalamic *crf* gene regulation of offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by males exposed to either predator odor (F_0PO) or control odor (F_0CO). (A) Rear duration was not significantly different in F_1PO versus F_1CO males, nor affected by paternal or maternal condition. **(B)** Likewise, rear frequency was not significantly affected by acute exposure to PO or paternal or maternal condition in males. Both rear duration **(C)** and rear frequency **(D)** were lower in F_1PO-exposed females relative to F_1CO-exposed females. Beyond the main effect, rear frequency and duration were increased in all female offspring (acutely exposed to CO or PO) of CO fathers and mothers housed in SNH. **(E)** Diagrammatic representation (left panel) and representative photomicrographs (10x

objective) of DAPI-stained coronal sections showing the dissection site (white circle/arrow) in micropunched (middle panel) and intact (right panel) paraventricular nucleus (PVN) tissue, in relation to the third ventricle (3V). H3K9ac enrichment of the *crf* promoter in PVN was **(F)** increased in F_0PO relative to F_0CO offspring, **(G)** reduced in offspring raised in SNH relative to those raised in SH, and **(H)** increased in F_1PO offspring relative to F_1CO offspring. **(I)** Levels *crf* primary transcript (hnRNA) in PVN were also increased in F_1PO offspring relative to F_1CO. **(J)** Levels of H3K9ac enrichment of the *crf* promoter and hnRNA expression were positively correlated in F_1CO and F_1PO offspring. Data expressed as mean ± SEM; Difference between indicated groups, * = $p \leq 0.05$; ** = $p \leq 0.005$; scale bar = 200 μm.

CHAPTER 3 EFFECTS OF PATERNAL HIGH-FAT DIET AND REARING ENVIRONMENT ON MATERNAL INVESTMENT AND DEVELOPMENT OF DEFENSIVE RESPONSES IN THE OFFSPRING

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In Preparation

Keywords: high-fat diet, paternal effects, affiliative behavior, maternal care, hypothalamus, *crf*, chromatin plasticity

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Preface to Manuscript

Following the establishment of a model for paternal stress induced alterations in maternal preference, maternal care, and offspring behavior, we were interested in the role of a metabolic stressor (HFD) in programming offspring social, anxiety-like, and stress responsive behavior. Again, we utilized the SNH environment during rearing to observe potential interactions with F_0 paternal HFD feeding. This was particularly pertinent considering the incidence of HFD-induced obesity driving anxiety-like phenotypes in rodent models, along with metabolic alterations seen in F_0 paternal stress models. Indeed, paternal exposure to exogenous GCs alters anxiety behavior and insulin-like growth factor *(igf)2* expression in offspring (Short et al., 2016) as well as altering growth patterns in F_1 female offspring (Hoyer et al., 2013).

3.1 Abstract

Paternal preconception risk factors correlate with metabolic dysfunction in the offspring, which is often comorbid with depressive and anxiety-like phenotypes. Detecting past dietary intake and nutritional status of a potential mate informs a female about prevailing ecological demands, in addition to adverse diet-induced metabolic phenotypes that may be disseminated to her offspring. We examined whether a male rat's prior exposure to an obesogenic high-fat diet (HFD) influences a female's attraction towards a male, subsequent mother-infant interactions and the development of defensive (emotional) responses in the offspring. Females displayed less interest in the HFD exposed males. Mothers allowed to rear young in larger, semi-naturalistic housing provided more licking and grooming and active arched-back-nursing behavior toward their offspring compared to dams housed in standard housing, although some of these effects interacted with paternal experience. Paternal HFD and maternal rearing environment revealed sex-dependent differences in offspring wean weight, juvenile social interactions and anxiety-like behavior in adolescence. Additionally, paternal HFD and maternal housing independently affected variations in *crf* gene promoter acetylation and *crf* gene expression in response to an acute stressor in offspring. Our results show for the first time in mammals that variation among males in their exposure to HFD may contribute to stable behavioral variation among females in courtship and maternal care, even when the females are not directly exposed to a HFD. Furthermore, when offspring were exposed to a predatory threat, hypothalamic *crf* gene regulation was influenced by paternal exposure to HFD and early housing. These results, together with our previous findings, suggest that paternal experience and maternal

rearing conditions can influence maternal behavior and development of defensive responses of offspring.

3.2 Introduction

Adverse intrauterine and early postnatal environments have lasting pathological consequences for hypothalamic-pituitary-adrenal (HPA) axis adaptation and metabolic programming, resulting in increased vulnerability for stress-related disorders in adolescence and adulthood (Sullivan et al., 2014; Lin et al., 2015; Weinstock, 2017). Clinical and epidemiology studies examining risk for common chronic health conditions, including obesity, hypertension, type 2 diabetes, cardiovascular disease and cancer have largely focused on maternal health before and during pregnancy, as well as perinatal factors and adult environmental exposures in the offspring (Vickers et al., 2007; Sullivan et al., 2010; Bolton and Bilbo, 2014). Maternal exposure to high-fat diets (HFD) has been implicated in programming of offspring metabolic dysfunction and obesity risk (Chang et al., 2008; Steculorum and Bouret, 2011) as well as anxiety-like behavior (Sasaki et al., 2014; Balsevich et al., 2015). Beyond the classical intergenerational transmission of cardiometabolic and anxiety traits via the germline (Rodgers et al., 2015; Fullston et al., 2016a; Rando, 2016), maternal behavior altered by maternal obesity and/or maternal HFD consumption provides a further mechanism by which programming of metabolic and stress disorders can occur (Connor et al., 2012; Bellisario et al., 2015). However, it has become clear that paternal preconception risk factors, such as advanced age (Smith et al., 2013; Atsem et al., 2016), smoking (Martinez et al., 1994; Langley et al., 2012), exposure to

stress (Franklin et al., 2010; Dietz et al., 2011; Rodgers et al., 2013; Korgan et al., 2016), drug use (Vassoler et al., 2013; Naquiah et al., 2016) and being overweight (McPherson et al., 2014; Fullston et al., 2015) also correlate with metabolic dysfunction (Carone et al., 2010; Ng et al., 2010; Rando and Simmons, 2015) and occurrence of excess weight gain (Hoyer et al., 2013; Ost et al., 2014) in the offspring, which is often comorbid with depressive and anxiety-like phenotypes (Abildgaard et al., 2011; Joseph and Golden, 2016). Therefore, it is of great interest to identify the underling mechanism(s) for the non-Mendelian inheritance of parental lifestyle and dietary risk factors that are critical for understanding the complex behavioral phenotypes in offspring, particularly with respect to metabolic programming and social-emotional development.

In the rat, we previously demonstrated that maternal environment interacts with paternal exposure to predator odor stress, altering maternal behavior (Korgan et al., 2016). Increases in licking and grooming (LG) and high quality arch-back nursing (ABN) have also been observed following paternal enrichment (Mashoodh et al., 2012), indicating that females adjust investment in offspring based on perceived mate quality (Curley et al., 2009; Pryke and Griffith, 2009). Further, we have shown that these alterations in maternal behavior result in stable differences in offspring stress responsivity and anxiety-like behavior (Korgan et al., 2016). Mechanistically, both paternal exposure and maternal care affect offspring epigenetic programming of HPA and endocrine responses to stress. The effect of maternal behavior on shaping offspring programming of glucocorticoid receptor (GR) and anxiety-like behavior is well established (Weaver et al., 2004b; Weaver et al., 2014b). We have shown that maternal rearing in semi-naturalistic housing (SNH) increases LG-ABN

behavior of the dam, decreases corticotrophin-releasing factor (CRF) immunoreactive neurons in the paraventricular nucleus of the hypothalamus (PVN), and decreases in H3K9ac at the *crf* promoter (Korgan et al., 2015; Korgan et al., 2016). Additionally, paternal predator odor stress increased H3K9ac at the *crf* promoter region, providing further evidence that paternal stress contributes to changes in offspring HPA-axis functioning.

Recent research has identified mechanisms by which paternal metabolic experience can shape offspring gene regulation, reviewed in (Rando and Simmons, 2015). In *drosophila*, paternal sugar intake alters H3K9/K27me3 regulation of metabolic genes leading to excessive weight gain in offspring (Ost et al., 2014). In rodents, paternal obesity is linked to impaired glucose tolerance in offspring (Ng et al., 2010; Fullston et al., 2013; Wei et al., 2014). Specifically, paternal exposure to HFD alters global DNA methylation in both the testes and sperm (Fullston et al., 2013), alters the sperm microRNA content (Fullston et al., 2016a), and increases offspring vulnerability to HFD feeding while decreasing reproductive success (Fullston et al., 2013). More recently, Govic et al., (2016) demonstrated that paternal exposure to caloric restriction (CR) decreased anxiety in offspring despite a corresponding decrease in maternal LG behavior, suggesting more complex variations in phenotypic alterations induced by manipulations to paternal diet.

To date, the role of paternal HFD induced obesity programming offspring anxiety-like behavior has not been observed. In the present study, we sought to identify differences in female preference for males exposed to either HFD or a control diet (CD). We then bred

these males with females and measured effects on maternal care as a result of paternal diet and/or her exposure to SH or SNH conditions. Offspring play behavior, anxiety-like behavior, stress responsivity, and *crf* expression and H3K9ac at the *crf* promotor region were then measured to identify potential consequences of paternal HFD exposure and maternal environment.

3.3 Materials & Methods

3.3.1 Animals and Breeding

Sixty-four Long-Evans hooded rats, 34 males and 30 females (purchased from Charles River Canada, Quebec) at ~60 days old were used for F_0 testing and breeding. All rats were housed in same sex pairs and given one week to acclimate prior to the beginning of the experiment. Rats were housed in a colony room maintained at 21 °C ± 2 °C under a 12h:12h reversed light cycle (lights off at 0930h). Rats were caged in SH, which consisted of polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids, containing pine shavings for bedding (Hefler Forest Products, Inc., Sackville, N.S. Canada) and a black PVC tube (12 cm length, 9 cm diameter), unless housed in SNH (see below). Both rat chow (Purina Lab Chow) or high-fat diets (see below) and tap water were supplied *ad libitum*. When breeding occurred, as described below (see Figure 3.1A), one male and one naïve female determined to be in estrus were housed together for 7 consecutive days. Pups remained with the dam until weaning (day 21; see Figure 3.1B), upon which the offspring were re-housed with a same-sex littermate. All experimental procedures were performed in

accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

3.3.2 Paternal High-fat Diet

The timeline of the described experimental procedures is shown in Figure 1A-B. Males began either high-fat (60 kcal%; Product #D12492) or protein/carbohydrate matched control diet (10 kcal%; Product #D12450J) feeding at P35 and were maintained on this diet for 60 days (Research Diets, Inc., New Brunswick, NJ). Paired male cage mates were randomly assigned to one of two experimental conditions: paternal control diet (CD, n=17) or high-fat diet (HFD, n=17).

3.3.3 Partner Preference Test

Partner preference tests (PPT) (Korgan et al., 2016)were performed either 1 or 20 days following the conclusion of the 60-day diet manipulation (Figure 1A). In preparation for the PPT, sexually naïve females were vaginally swabbed; only females in estrus were used. Fifteen females were used in the PPT 1 day following diet manipulation and six females were used in the PPT 20 days diet manipulation culmination. The PPT was performed using a T-maze (base: 50 x 10 x 10 cm, arms (x2): 50 x 10 x 10 cm, and a clear Plexiglas lid; Figure 2C) containing two male rats and one female rat. Rats were transported to the testing room in covered cages. Trials occurred under red light and were recorded using a video camera positioned directly above the maze. Before the trial, the female rat was placed in

the clean maze and allowed 5 min for habituation before being removed. Each 10 min trial was initiated by placing one female into the base of the maze. One CD male was placed in the end of one arm and one HFD male was placed in the end of the other arm; counterbalanced across runs. During the test, males were restricted to the arm ends and separated by a clear piece of Plexiglas with multiple holes and the female was allowed to explore the entire maze. The total duration that the female spent inside an arm and oriented toward a male, along with the number of entries into each arm were recorded. For analysis, we calculated the percentage of the total time spent with each individual male per time spent with both males. Upon completion of a trial, the maze was cleaned with ethanol. Males were paired with sexually naïve, receptive females within 12 hours following the initial PPT for 7 days (Figure 3.1A).

3.3.4 Semi-Naturalistic Housing (SNH)

Dams mated with CD and HFD males were observed daily for pups once they reached gestational day 20 (GD20), near the beginning of the dark cycle. Once the pups arrived (postnatal day 0, P0), the litter was sexed, counted and weighed as quickly as possible to minimize disruption to the dams. Dams and litters randomly designated for the semi-naturalistic housing (SNH) condition (n=14) were transferred to SNH cages on PD0. Dams and pups in the standard housing (SH) condition (n=15) were placed in clean, standard home cages. All dams and pups remained in their respective environments until the pups were weaned at PD21. The SNH (Figure 3.3A inset) consisted of two sections: an upper section, (50.5 cm x 50.5 cm x 33.5 cm) containing food and water ad libitum and a lower

section, (50.5 cm x 50.5 cm x 14 cm) filled with pine shavings and a PVC tube. One female did not produce a litter.

3.3.4 Maternal Care Observations

Dams' maternal behavior was observed and scored daily in real-time for 72 min at 0800h, 1100h, 1300h, 1500h and 2130h from P1-7. During each observation period, the frequency of the following behaviors were scored every 3 min (Korgan et al., 2016): no contact with pups (NCP), which may or may not include self-grooming (SG), nest building (NB), or feeding behaviors (F), passive nursing (PN), arched-back-nursing (ABN) ranked as level 1 (low blanket posture) and 2-4 (high postures favorable for milk ejection), licking and grooming pups, separated pups (SP) and pup retrieval (PR). The 'no contact' behaviors consist of a dam making no contact with her pups and often being accompanied by self-grooming behavior (licking, nibbling and combing-like actions of the fur), nest building (changing the positioning or location of the pine shavings around the nest), and feeding behavior (nibbling at the feeder, consuming rat chow or drinking water). Passive nursing was scored when the dam was on her side to nurse her pups, or used the sides of the cage to support her while nursing. Blanket posture or ABN1 was observed when the dam was flat over the pups. ABN consisted of graded degrees of arching, levels 2-4, based on kyphosis or the bend of the knees and steepness of back arching of the pups. Separated pups were recorded when a dam had pups away from the nest, isolated or in small groups. Pup retrieval was the transfer of pups back into the nest. Cumulative scores of each

behavior were utilized in analysis along with an aggregate total for ABN2/3 behaviors co-occurring with LG similar to Korgan et al.,(2016).

3.3.6 Anxiety-Behavior Testing of F_0 Males

F_0 male anxiety behavior testing was performed approximately one week following the second PPT. Males (n=12 per group) were tested in the open field arena and elevated plus maze. In both tests, each trial was recorded under red light, using a vertically mounted video camera, for future behavioral scoring. F_0 males were transported to and from the testing room in covered home cages and the arena and maze were cleaned with 30% ethanol solution between each trial:

3.3.6.1 Open Field Test (OFT)

The OFT apparatus consisted of a solid, black Plexiglas square (79.2 cm x 78.9 cm x 35.0 cm), divided into 16, equal quadrants designated by nontoxic white paint on the maze floor. At the beginning of each 10 min trial, offspring were placed into the center of the maze. The behaviors scored for the OFT were as follows: line crosses (all four limbs crossing into a new quadrant), time in center (time spent in the four center squares, 25% of the total area), as well as freezing, grooming and rearing (as defined for the EPM).

3.3.6.2 Elevated Plus Maze (EPM)

The EPM apparatus was constructed of solid black Plexiglas, with two open arms (11.2 cm x 50.2 cm), adjacent to two closed arms (11.3 cm x 50.4 cm x 40.2 cm), elevated 40.0 cm from the floor. At the beginning of each 10 min trial, the rat was placed in the center platform of the maze (11.2 cm x 10.2 cm). Behavior in the EPM trials was scored using video recordings and were as follows: line crosses (all four limbs crossing over the central platform), time in open arms (duration of time spent in open arms), time in closed arms (duration of time spent in closed arms), entries into open arms (all four limbs crossing into an open arm), entries into closed arms (all four limbs crossing into a closed arm), attempts into open arms (stretch-attend posture at the opening of an open arm, less than four limbs entering the arm), attempts into closed arms (stretch-attend posture at the opening of an closed arm, less than four limbs entering the arm), freezing frequency and duration (2 sec or greater period without movement, but not sleeping), grooming frequency and duration (2 sec or longer bouts that involve; licking, nibbling and combing-like actions of the fur), and rearing frequency and duration (standing on hind paws only, with or without leaning on the perimeter wall).

3.3.7 Offspring Groups

The following four groups of F_1 male and female offspring were studied as juveniles and in peri-adolescence: CD-SH – father exposed to control diet and mother housed in standard housing (n=14 males, 11 females); CD-SNH – father exposed to control diet and mother housed in semi-naturalistic housing (n=8 males, 10 females); HFD-SH - father exposed to high-fat diet and mother housed in standard housing (n=6 males, 7 females); HFD-SNH – father exposed to high-fat diet and mother housed in semi-naturalistic housing (n=10 males, 12 females). Each group contained 2-4 males and females from multiple litters: CD-SH (n=6 litters), CD-SNH (n=4 litters), HFD-SH (n=4 litters), and HFD-SNH (n=5 litters).

3.3.7 Monitoring of Offspring Juvenile Play

F_1 offspring play behavior observations began on PD24, and were conducted daily at approximately 1100h, 1300h, and 1500h for five consecutive days. Observation sessions lasted one hour, during which time an experimenter would record play behaviors observed in the home cage at a fixed interval of 3 min. Behaviors scored included social grooming (licking and/or chewing the fur of the conspecific, while placing forepaws on the back or the neck); “attack” behaviors, pouncing (play initiation, forepaws extended towards play partner, typically directed at neck, paws contact first), nose-to-nape attempted approach (nose approaches play partner's neck within 1-4 cm), nose-to-nape successful approach (nose contacts play partner's neck), and pinning (positioned over play partner with forepaws on partner); “defense” behaviors, full rotation (rolling supine, on back, to face

play partner, interposing face/forepaws between attacker and nape), partial rotation (rolling towards supine to face play partner, but with at least one hind paw on floor), upright defense (turn to face play partner from an upright position on hind paws), and boxing (standing upright on hind paws, forepaws extended towards play partner in pushing or swiping motions); and evasion (swerving or leaping away from play partner, fleeing), as in Korgan et al., (2016) and adapted from (Field et al., 2006).

3.3.9 Anxiety-Behavior Testing of Juvenile Offspring

F_1 Offspring anxiety behavior was tested on PD32-35. Male and females (n=6-13 per sex, per group) were tested in the open field arena and elevated plus maze. In both tests, each trial was recorded under red light, using a vertically mounted video camera, for future behavioral scoring. Pups were transported to and from the testing room in covered home cages and the arena and maze were cleaned with 30% ethanol solution between each trial:

3.3.10 Acute Stress Exposure in Peri-Adolescent Offspring

7-10 days after anxiety-behavior testing was completed, these same offspring were randomly assigned to either predator (PO) or control odor (CO) exposure (n = 3-7 per sex, per group). Offspring were exposed for one 30 min trial, followed immediately by sacrifice.

3.3.11 Sacrifice and Tissue Collection

In the preparation of fresh tissue, F_1 offspring were deeply anesthetized with Euthanyl (sodium pentobarbital, 60 mg/kg, i.p.) and rapidly decapitated. All brains were removed and flash frozen in dry ice then stored at -80°C until further processing. Whole brains were blocked and sectioned with a microtome. PVN containing sections (coordinates with respect to bregma were -1.6 to -2.12 mm anteroposterior, 1.5 mm lateral from the midline, and -9.0 mm dorsoventral from the dura) were identified using the rat brain atlas (Paxinos and Watson, 1986), micropunched using a 20 gauge cannula (PlasticsOne) and stored at -80 °C until used. To confirm the dissection site, serial coronal sections (20 um thick) of the micropunched PVN tissue were cut on the microtome, thaw mounted onto positively charged microscope slides and stored at -80 °C. Slices were then stained with 0.25% DAPI (Roche Life Science, Indianapolis, IN) for 1 min, mounted in PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific, Waltham, MA). Images were acquired with a Zeiss Axio Imager Z2 fluorescent microscope (Carl Zeiss, Oberkochen, Germany) and a high-resolution color digital camera using a 10x objective. At least four sections were examined per animal.

3.3.12 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation assays (Crane-Robinson et al., 1999) were performed following the ChIP assay kit protocol® (Cat#06-599, Upstate Biotechnology), as previously described (Korgan et al., 2016). Chromatin was immunoprecipitated from PVN

micropunch samples using a rabbit polyclonal antibody against H3K9ac or normal rabbit IgG non-immune antibody (both from Santa Cruz Biotechnology). One-tenth of the lysate was kept to quantify the amount of DNA present in different samples before immunoprecipitation (Input). Protein-DNA complexes were uncrosslinked, by adding 20 µl NaCl (5 M) to each sample (4 h, 65 °C), followed by 10 µl EDTA (0.5 M), 20 µl Tris-HCl (1 M, pH 6.5) and 2 µl PK enzyme (10 mg/ml) (1 h, 45 °C). Following phenol-chloroform (0.5 v/v) extraction, the free-DNA was ethanol (2 v/v, 95 %) precipitated with 5 µl tRNA (10 mg/ml) and re-suspended in 100 µl 1xTE. The rat *crf* promoter region (−206 to 318, containing a cAMP response element) of the uncrosslinked DNA was subjected to PCR amplification (Forward primer: 5′-TCAGTATGTTTTCCACACTTGGAT-3'; Reverse primer: 5′-TTTATCGCCTCCTTGGTGAC-3'). For quantitative real-time PCRs, PCR mixtures (12.5 µl) containing the immunoprecipitated DNA, SsoFast™ EvaGreen® Supermix (Cat#172-5203, Bio-Rad Laboratories) and 4 µM primer were loaded onto a 96 multiwell plate and covered with a seal (Bio-Rad Laboratories). The thermocycler (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad Laboratories) protocol involved an initial HotStart enzyme activation cycle (2 min, 95 °C, with a temperature transition rate set at 4.40 °C/sec), 40 cycles of denaturation (5 sec, 95 °C, with a temperature transition rate set at 4.40 °C/sec) and annealing (30 sec, 60 °C) with a temperature transition rate set at 2.20 °C/sec). A single fluorescence reading was acquired at the end of each elongation step. Triplicate average qPCR cycle threshold (Ct) value for input (10%) samples: ~24-26, with a 4-8 fold difference between qPCR Ct values of the H3K9ac antibody IP'ed samples (qPCR Ct value: ~29-31) or negative control IgG non-immune antibody IP'ed samples (IgG, Ct value: ~34 or not detected after 40 cycles). The specificity of the amplified PCR

products was assessed by performing a melting curve analysis cycle after the PCR amplification (5 sec, 95 °C, with a temperature transition rate set at 4.40 °C/sec; 1 min, 65 °C, with a temperature transition rate set at 2.20 °C/sec) that terminated with a cooling step (30 sec, 40 °C, with a temperature transition rate set at 2.20 °C/sec). The fluorescence of the SsoFast™ EvaGreen dye bound to double stranded amplified product declines sharply as the fragment is denatured. The melting temperature of this fragment was visualized by plotting the first negative derivative (dF/dT) of the melting curve on the y-axis and temperature (°C) on the x-axis. No primer-dimers were detected that interfered with the quantification of the PCR products. The Ct values of ChIP DNA fractions were normalized to the Ct value of the input DNA fraction for the same qPCR assay (ΔCt) to account for differences in chromatin sample preparation. Relative H3K9ac enrichment was measured by the $2^{-\Delta\Delta CT}$ method, using the DNA fractions IP'ed with IgG as the negative control (Livak and Schmittgen, 2001).

3.3.13 RT-qPCR analysis

Total RNA was isolated from PVN micropunch samples using the RNeasy Plus Mini Kit (Qiagen), which permits recovery of high quality RNA with effective elimination of genomic DNA. Precipitated RNA was dissolved in RNase-Free H_2O and quantified (~425-731 ng RNA/30ul) with a Take3™ Micro-volume Plate on an Epoch Spectrophotometer (BioTek Instruments INC). Rat PVN *crf* (NM_000756.1) heteronuclear RNA (hnRNA) was subjected to qPCR amplification (forward primer: 5'-TCAATCCAATCTGCCACTCA-3'; reverse primer: 5'-TAAGCTATTCGCCCGCTCTA-

3'). To control for equal loading, the rat *ribosomal protein L13A* (*Rpl13A*, NR_073024) exon region was also subjected to PCR amplification (forward primer: 5'-ACAAGAAAAAGCGGATGGTG-3'; reverse primer: 5'-TTCCGGTAATGGATCTTTGC-3'). The *crf* hnRNA and Rpl13A amplification were performed in parallel, using a 10 µl reaction mixture containing 350 ng of purified RNA and the iTaq™ universal SYBR® Green one-step kit (Bio-Rad) (Pfaffl, 2001). The thermocycler protocol involved in the reverse transcription reaction (10 min, 50 °C), polymerase activation and an initial DNA denaturation (1 min, 95 °C), 40 cycles of denaturation (10 sec, 95 °C) and annealing/extension (30 sec, 60 °C) terminating at 4 °C. The specificity of the amplification reaction was assessed by melt curve analysis. To control for equal loading between samples, the signal of the *crf* hnRNA was divided by the signal from the Rpl13A region amplified from the same sample.

3.3.14 Statistical analyses

Effects of diet (CD versus HFD) on weight gain and food intake in males were analyzed using independent Student's t-test. A repeated measures ANOVA was used to compare weight gain during the diet manipulation in F_0 CD and HFD males. For each group of males (1 day and 20 days following diet manipulation), differences in time spent with CD and HFD males by females in the PPT were analyzed using separate mixed-design analysis of variance (ANOVA) with paternal condition (CD, HFD) as the between-subject factor and time block (first 4 min, last 4 min) as the within-subject factor. Differences in litter size, sex ratio and F_1 offspring birthweight were analyzed with a three-factor ANOVA with F_0

diet, rearing environment and sex (where appropriate) as between subject factors for each dependent variable. Maternal behavior was analyzed using two-factor ANOVA with paternal condition (CD, HFD) and maternal condition (SH, SNH) as between-subject factors for each dependent variable. Offspring wean weight and behavioral test data were analyzed using a three-factor ANOVA with F_1 Sex, F_0 paternal diet, and rearing environment as between-subject factors. Data from molecular endpoints were analyzed in an identical fashion with the addition of offspring odor exposure (F_1CO, F_1PO) as a fourth between subject factor. Interactions were analyzed post-hoc with simple effects analyses, using a Bonferroni correction. A threshold level of $p < 0.05$ was used to test for significance. The Statistical Package for the Social Sciences (SPSS Inc., USA) software was used for all statistical analyses.

3.4 Results

3.4.1 Weight Gain in F_0 Males

Birth weight ($F=1.284$ (1,32) $p=0.266$) and wean weight ($F=0.005$ (1,32) $p=0.947$) were not different between the two groups of males that were subsequently randomly assigned to receive CD or HFD (data not shown). There was a significant main effect of increased in weight gain during CD and HFD feeding ($F=7.137$ (1,32) $p=0.012$; Figure 3.1C inset). Repeated measures ANOVA showed a significant difference between groups over time, with HFD males gaining relatively more weight ($F=5.015$ (2.169,69.412) $p=0.008$; Figure 3.1C). During the last five days of CD or HFD feeding food intake was measured for each cage and ANOVA revealed that CD and HFD males consumed the same kilocalories (kcal)

/gram (F=0.79 (1,9) p=0.474; Figure 3.1C inset). At sacrifice, approximately 6 weeks after HFD feeding was terminated, HFD F_0 males maintained increases in abdominal (t=2.636 (10) p=0.025) and perigonadal (t=5.902 (10) p<0.001) fat pads, increased brain weight (t=2.560 (10) p=0.028) and decreased teste weight (t=7.748 (10) p<0.001) (Figure 3.1D).

3.4.2 Anxiety-Like Behavior in F_0 Males

We tested F_0 males for anxiety-like behavior following diet manipulation and breeding (Table 3.1). In the OFT, HFD F_0 males spent less time in the center area (t=6.328 (22) p<0.001), displayed decreased rearing (t=2.856 (22) p=0.009) but did not differ in overall line-crosses (t=0.272 (22) p=0.788; data not shown). Similarly, in the EPM, HFD F_0 males spent less time and less percent of total time in the open arms (t=2.603 (22) p=0.016; t=2.675 (22) p=0.015), crossed into fewer arms (t=3.531 (22) p=0.002) and spent less time rearing (t=2.146 (22) p=0.043).

3.4.3 Partner Preference Test

Apararatus shown in Figure 2A. Immediately following the diet exposure, repeated measures ANOVA showed that, relative to HFD males, estrous females preferred CD males in the first 4 minutes of the PPT (F=7.005 (1,28) p=0.013; Figure 3.2B) and for the duration of the test (F=10.009 (1,28) p=0.004; data not shown). There were no differences in line crosses (F=0.001 (1,28) p=0.981; data not shown). Following 21 days of standard chow feeding *ad libitum*, estrous females retained a preference for CD males during the

last 4 minutes of the PPT (F=22.69 (1,9) p=0.001; Figure 3.2C) and during the entire test (F=6.728 (1,9) p=0.032; data not shown), again with no difference in line crosses (F=0.521 (1,9) p=0.491; data not shown).

3.4.4 Maternal Care

We found no effects of paternal diet on litter size (t=1.888, (27), p=0.07), litter sex ratio (t=0.135, (27), p=0.893), or birth weight t=1.16, (27), p=0.256). Dams housed in the SNH behaved differently toward offspring relative to females housed in SH (Figure 3.3A). The frequency of no contact with pups (NCP) was significantly greater in SNH dams relative to SH dams (F=8.132, (1,29), p=0.009), and this extended to a decreased frequency of blanket posture (ABN1; F=52.435, (1,29), p<0.001), passive nursing (PN; F=15.993, (1,29), p<0.001), passive contact (PC; F=85.839, (1,29), p<0.001), and self-grooming (SG; F=7.958, (1,29) p=0.009) in SNH relative to SH dams. SNH dams showed significantly increased frequency of active arched-back-nursing [(ABN3; F=26.598 (1,29), p<0.001) and (ABN4; F=12.148, (1,29) p=0.002)], and nest building (NB) behaviors (F=14.022, (1,29) p=0.001). Maternal LG-ABN2/3 was increased in SNH conditions (F=7.138 (1,29) p=0.013) and was also decreased by paternal HFD (F=4.722 (1,29) p=0.039; Figure 3.3B-C).

Interestingly, there were a number of interactions between F_0 paternal diet and maternal rearing environment. For instance, an interaction between paternal diet and rearing condition (F=4.81, (1,29) p=0.038) was observed for passive nursing, and post hoc analyses

revealed that offspring from HFD fathers raised in SH received significantly more maternal passive nursing (PN) relative to those from HFD fathers but raised in SNH (F=13.697, (1,15) p=0.003) and relative to offspring from CD fathers raised in SH (F=5.849, (1,15) p=0.031; Figure 3.4A). There were also interactions for maternal feeding (F) (F=8.383, (1,15) p=0.029; Figure 3.4B) and self-grooming (SG) (F=5.181 (1,15) p=0.032; Figure 3.4C). Dams rearing offspring in SNH environments performed fewer feeding bouts if mated with HFD males (F=7.526 (1,15) p=0.018) and dams rearing HFD offspring in SH performed less SG than those with offspring from CD males in SH (F=5.364 (1,15) p=0.038) and HFD-SNH (F=12.363 (1,15) p=0.004) dams. Finally, an interaction between paternal diet condition and rearing environment (F=6.776 (1,29) p=0.015; Figure 3.4D) revealed that while SNH increased HFD pup survival (F=7.699 (1,15) p=0.017) it was decreased in HFD pups reared in SH conditions (F=15.621 (1,15) p=0.002).

3.4.5 Play Behavior in F_1 Offspring

There was a significant interaction between paternal diet and maternal rearing environment for the total number of attack behaviors (F=4.937 (1,62) p=0.03), where offspring reared in SNH cages engaged in fewer attacks if sired by CD (F=7.343 (1,62) p=0.01), but actually engaged in more attacks if sired by HFD males (F=5.233 (1,62) p=0.029; Figure 3.5A). There was a similar interaction for total defensive behaviors (F=6.125 (1,62) p=0.016), where offspring reared in SH environments engaged in fewer defense behaviors if sired from HFD males (F=8.566 (1,62) p=0.007: Figure 3.5B). Also, males overall engaged in significantly more defensive behaviors than females (F=6.741 (1,62) p=0.012). There was

no interaction or main effect of evasive behaviors (Figure 5C; data not shown). An expected main effect of sex was observed for female grooming behavior (F=12.835 (1,62) p=0.001; Figure 3.5D; males, mean= 1.655, SE= 0.899; females, mean= 4, SE=0.767).

3.4.6 Anxiety-Like Behavior in F_1 Offspring

F_1 offspring anxiety-like behavior was tested on PD 35-42. In the OFT, there was a trend for F_1 offspring of HFD F_0 males to spend less time in the center (F=3.513 (1,69) p=0.065; Figure 3.6A). Offspring reared in SNH spent significantly more time in the center, compared to SH (F=6.834 (1,69) p=0.011; Figure 3.6B). Offspring of HFD F_0 males performed more line crosses compared to CD F_0 males (F=6.066 (1,69) p=0.016; Figure 3.6C), offspring reared in SNH performed fewer line crosses than offspring reared in SH (F=7.607 (1,69) p=0.007; Figure 3.6C) and females performed more line crosses (F=11.072 (1,69) p=0.001; data not shown). There was an interaction between paternal diet and rearing condition (F=4.516 (1,69) p=0.037), where SNH reared offspring of CD F_0 males groomed more than SH reared offspring (F=7.674 (1,41) p=0.008; Figure 3.6D) and SNH reared offspring of CD F_0 males groomed more than offspring of HFD F_0 males (F=16.197 (1,38) p<0.001; Figure 3.6D). There were no differences in rearing duration (F=0.855 (1,69) p=0.358; data not shown).

In the EPM, offspring of HFD F_0 males spent less time in open arms, compared to CD F_0 males (F=18.073 (1,67) p<0.001; Table 3.2) and female offspring spent more time in open arms than male offspring (F=8.835 (1,67) p=0.005; data not shown). This was consistent

with open arm entries (F=16.456 (1,67) p<0.001), line crosses (F=4.513 (1,67) p=0.037), and percentage of time in open arms (F=21.643 (1,67) p<0.001) for offspring of HFD F_0 males who exhibited less of these behaviors compared to offspring of CD F_0 males (see Table 2). In all cases, the behavior of F_1 females was significantly higher compared to F_1 males (F=10.157 (1,67) p=0.002, (F=4.065 (1,67) p=0.048, F=8.698 (1,67) p=0.004, respectively; data not shown). There were no differences in entries to closed arms (F=0.149 (1,67) p=0.701; data not shown). F_1 offspring of HFD F_0 males groomed less in the EPM (F=6.088 (1,67) p=0.016; Table 3.2), as did offspring reared in SNH (F=4.761 (1,67) p=0.033; Table 2), and females groomed more than males (F=8.960 (1,67) p=0.004; Table 3.2). Finally, F_1 offspring of HFD F_0 males spent more time rearing than F_1 offspring of CD F_0 males (F=8.226 (1,67) p=0.006; Table 3.2).

3.4.7 F_1 Offspring Odor Exposure Behavior

At P42, F_1 offspring were exposed to either a novel predator odor (PO) or control odor (CO) to determine stress reactivity behavior in offspring of F_0 HFD and CD males reared in either SH or SNH environments. PO exposed F_1 offspring spent less time in the vicinity of the odor stimulus (F=10.001 (1,74) p=0.002; F_1CO, mean= 588.221, SE= 47.571; F_1PO, mean= 375.469, SE=47.571) and spent less time rearing (F=4.458 (1,74) p=0.039; F_1CO, mean= 311.365, SE= 26.157; F_1PO, mean= 233.263, SE=26.157) relative to those exposed to CO, indicating an avoidance response consistent with previous observations (Korgan et al., 2016).

Interestingly, paternal diet and maternal rearing, in isolation and together, affected a number of measures having to do with F_1 offspring stress-related behavior. F_1 offspring of HFD males spent more time in the vicinity of the odor stimulus ($F=9.330$ (1, 74) $p=0.003$; Figure 3.7A) and made more stimulus contacts ($F=9.312$ (1,74) $p=0.003$; Figure 3.7B) than F_1 offspring of CD males. Further, F_1 offspring reared in the SNH contacted the odor stimulus less ($F=5.241$ (1,74) $p=0.026$; Figure 3.7C) and spent less time rearing ($F=5.930$ (1,74) $p=0.018$; Figure 3.7D) than offspring reared in SH. F_1 offspring of HFD fed males spent less time grooming ($F=7.366$ (1,74) $p=0.009$; data not shown) than those of CD males, while offspring reared in the SNH spent more time grooming ($F=10.506$ (1,74) $p=0.002$; data not shown) than those reared in SH. Post hoc analyses of an interaction between paternal diet and rearing environment ($F=5.012$ (1,74) $p=0.029$; Figure 3.7E) revealed increased grooming F_1 offspring of CD males reared in SNH compared compared to those from HFD males ($F=16.132$ (1,42) $p<0.001$) and relative to F_1 offspring of CD males reared in SH ($F=7.787$ (1,40) $p=0.008$). F_1 male offspring contacted the odor stimulus more than F_1 female offspring ($F=11.870$ (1,74) $p=0.001$; data not shown).

Finally, paternal diet and offspring sex ($F=5.775$ (1,74) $p=0.019$; Figure 3.7F) interacted and post hoc analyses revealed decreased grooming in F_1 male offspring of HFD males compared to F_1 male offspring of CD males ($F=12.871$ (1,35) $p=0.001$) and F_1 female offspring of HFD males ($F=6.506$ (1,32) $p=0.016$).

3.4.8 Body and Tissue Weights in F_1 Offspring (P42)

At P42, F_1 offspring of F_0 HFD males weighed more than F_0 CD males ($F=42.532$ (1,66) $p<0.001$; F_1CD, mean= 210.187, SE= 3.877; F_1HFD, mean= 250.506, SE=4.816); F_1 offspring reared in SNH cages weighed less than SH controls ($F=17.969$ (1,66) $p<0.001$; SH, mean= 243.450, SE= 4.748; SNH, mean= 217.243, SE=3.959); and F_1 males weighed more than F_1 females ($F=202.588$ (1,66) $p<0.001$; males, mean= 274.344, SE= 4.587; females, mean= 186.349, SE=4.145). These effects were made more complex because of interactions between paternal diet condition and rearing condition ($F=21.969$ (1,66) $p<0.001$; Figure 3.8A) where F_1 offspring of F_0 CD males had decreased body weight when reared in SNH, compared to SH ($p<0.001$) and F_1 offspring of F_0 HFD males had increased body weight, compared to F_0 CD males, if reared in SNH ($p<0.001$). There was also an interaction between F_0 diet and F_1 offspring sex. F_1 male offspring weighed more than females, from both F_0 CD ($p<0.001$) and HFD ($p<0.001$) males and F_0 HFD offspring weigh more than F_0 CD offspring in both males ($p<0.001$) and females ($p=0.009$).

At P42, F_1 offspring of F_0 CD males had heavier brains than F_0 HFD males ($F=29.573$ (1,66) $p<0.001$; F_1CD, mean= 0.934, SE= 0.014; F_1HFD, mean= 0.815, SE=0.017); F_1 offspring reared in SNH cages had heavier brains than SH controls ($F=13.103$ (1,66) $p=0.001$; SH, mean= 0.835, SE= 0.017; F_1PO, mean= 0.914, SE=0.014); and F_1 females had heavier brains than F_1 males ($F=164.585$ (1,66) $p<0.001$; male, mean= 0.734, SE= 0.016; female, mean= 1.015, SE=0.015). Further, there was an interaction between paternal diet condition and rearing condition ($F=25.275$ (1,66) $p<0.001$; Figure 3.8B) where F_1 offspring of F_0 CD

males had increased brain weight when reared in SNH, compared to SH ($p=0.001$) and F_1 offspring of F_0 HFD males, reared in SNH, had decreased brain weight, compared to F_0 CD males ($p<0.001$).

At P42, F_1 offspring of F_0 HFD males had relatively heavier abdominal fat pads than F_0 CD males ($F=43.581$ (1,66) $p<0.001$; F_1CD, mean= 0.632, SE= 0.032; F_1HFD, mean= 0.97, SE=0.04) and F_1 females had relatively heavier abdominal fat pads than F_1 males ($F=32.235$ (1,66) $p<0.001$; male, mean= 0.656, SE= 0.038; female, mean= 0.946, SE=0.034). Further, there was an interaction between paternal diet condition and rearing condition ($F=14.096$ (1,66) $p<0.001$; Figure 3.8C) where F_1 offspring of F_0 CD males had decreased abdominal fat pad weight when reared in SNH, compared to SH ($p<0.001$) and F_1 offspring of F_0 HFD males had increased abdominal fat pad weight, compared to F_0 CD males, if reared in SNH ($p<0.001$).

At P42, F_1 offspring of F_0 HFD males had relatively heavier gonadal fat pads than F_0 CD males ($F=25.138$ (1,66) $p<0.001$; F_1CD, mean= 0.148, SE= 0.017; F_1HFD, mean= 0.283, SE=0.021); F_1 offspring reared in SH cages had relatively heavier gonadal fat pads than SNH reared offspring ($F=4.469$ (1,66) $p=0.038$; SH, mean= 0.244, SE= 0.021; SNH, mean= 0.187, SE=0.017); and F_1 females had relatively heavier gonadal fat pads than F_1 males ($F=5.012$ (1,66) $p=0.029$; male, mean= 0.185, SE= 0.02; female, mean= 0.245, SE=0.012). There was an interaction between F_0 diet, rearing condition, and sex ($F=4.347$ (1,66) $p=0.041$; Figure 3.8D), and interactions between F_0 diet and rearing condition ($F=6.463$ (1,66) $p=0.013$; Figure 3.8D) and F_0 diet and offspring sex ($F=5.644$ (1,66)

p=0.020; Figure 3.8D). F_1 offspring of F_0 CD males had decreased gonadal fat pad weight when reared in SNH, compared to SH ($p<0.001$) and F_1 offspring of F_0 HFD males had increased gonadal fat pad weight, compared to F_0 CD males, if reared in SNH ($p<0.001$). Female offspring of F_0 HFD males had increased gonadal fat pad weight than both male offspring of F_0 HFD males ($p=0.022$) and female offspring of F_0 CD males ($p<0.001$). Further, female offspring of F_0 HFD males had increased gonadal fat pad weights when reared in SH, compared to F_0 CD males ($p=0.011$) and female offspring of F_0 CD males had increased gonadal fat pads than male offspring, when reared in SNH ($p=0.001$). There was no effect of F_0 paternal diet or rearing environment on F_1 male offspring teste weight (all p's>0.05).

3.4.9 F_1 Offspring Hypothalamic crf Gene Regulation -

H3K9ac of the *crf* promoter in the PVN was decreased in offspring reared in the SNH ($F=7.490$ (1,48) $p=0.010$; Figure 3.9A) compared to those reared in SH and was increased in offspring exposed to PO relative to CO ($F=10.043$ (1,48) $p=0.003$; F_1CO, mean= 0.31, SE= 0.012; F_1PO, mean= 0.295, SE=0.012). Expression of *crf* hnRNA in the PVN revealed an interaction between rearing environment and odor exposure condition ($F=7.342$ (1,48) $p=0.011$; Figure 3.9B), where offspring reared in SH and exposed to PO had increased expression ($F=13.190$ (1,48) $p=0.001$; Figure 3.9C) compared to those reared in SNH and PO exposed offspring reared in SH had increased expression ($F=26.083$ (1,48) $p<0.001$; Figure 3.9B). Expression of *crf* hnRNA was also also regulated by maternal condition, F_1 offspring sex differences and odor exposure condition. SNH increased expression

(F=10.291 (1,48) p=0.003) compared to SH. F_1 males had increased expression (F=7.510 (1,48) p=0.010) compared to F_1 females (data not shown). Finally, PO exposed F_1 offspring had increased expression (F=22.971 (1,48) p<0.001) compared to CO exposed F_1 offspring. As predicted, there was a significant correlation for all offspring between H3K9ac of the *crf* promoter and *crf* hnRNA expression in the PVN (R=0.398, p=0.005; Figure 3.9c).

3.5 Discussion

This study provides novel evidence for paternal obesity induced alterations in female preference, maternal care, and offspring behavior. Consistent with previous observations, we found paternal diet manipulation affects on maternal care and offspring behavior (Govic et al., 2016) and an interaction with maternal rearing environment. Here we found that a high-quality SNH rearing environment was shown to ameliorate deficits in maternal care and partially conserve paternal HFD exposure-induced behavioral alterations.

Exposure to an obesogenic diet in F_0 males resulted in weight gain, fat accumulation and behavioral modifications. Consistent with previous studies on obesogenic diets, male rats showed increases in total body weight, despite similar kcal intake during the conclusion of HFD exposure (Briggs et al., 2010), and accumulation of adipose tissue in perigonadal and abdominal fat pads (Klockener et al., 2011). Interestingly, the relative weight of testes was decreased in F_0 males exposed to HFD, potentially linked to type 2 diabetes induced secondary hypogonadism (Saboor Aftab et al., 2013), though there was no difference in reproductive success. Relative brain weight was also reduced in HFD expose F_0 males,

potentially due to altered expression of neuronal and glial proteins similar to that seen in obese leptin knockout mice (Bouret and Simerly, 2004) or dysregulated maintenance of apoptotic factors (Reddy et al., 2014). Finally, we were able to demonstrate that HFD induced obesity resulted in an anxiogenic phenotype with coinciding with decreased activity. These effects have been demonstrated before and are likely mediated by alterations in cholesterol and insulin levels (de Sousa Rodrigues et al., 2016) as well as altered baseline and stress induced plasma glucocorticoid levels (Sharma and Fulton, 2013) and PPARγ and adiponectin dysregulation (Guo et al., 2016). Together, these factors likely altered physical, auditory, or major histocompatibility complex (MHC) based attractiveness of male rats, resulting in increased preference for CD exposed F_0 males by reproductively active females. Indeed, previous research has shown female preference based on male stress exposure (Korgan et al., 2016), enrichment (Mashoodh et al., 2012) and caloric restriction (Govic et al., 2016).

Females' preference for CD exposed F_0 males was further demonstrated by alterations in maternal care behavior and mediated by rearing environment. A decrease in the active LG & ABN2/3 behavior in females mated with F_0 HFD exposed males suggests that females' preference resulted in altered behavioral investment in offspring. Further, maternal environment interacted with F_0 male diet; females mated with F_0 HFD exposed males performed more low quality passive nursing behavior but this was reversed if rearing offspring in the SNH. Similar to previous findings(Korgan et al., 2016), SNH rearing increased the frequency of no-contact behaviors while decreasing passive contact, passive nursing, and blanket posture or ABN1 behaviors and increasing the frequency of active

maternal behaviors; ABN3 & 4, nest building LG & ABN2/3. Interestingly, there was an interaction between F_0 diet and rearing environment regulating feeding behavior suggesting that females rearing offspring of F_0 HFD exposed males in the SNH had greater nutritional demands than other dams. Conversely, in the SH spent more time self-grooming, implicating that more self-care was necessary in the low quality environment, especially when bred F_0 HFD exposed males. Overall, maternal care variations in SNH environment was similar to that seen in brief maternal separation models (Liu et al., 1997; Connors et al., 2015) and were further impacted by the diet exposure of her mate, indicating that females were able to identify differences in mate quality and adjust behavioral investment in offspring (Pryke and Griffith, 2009; Curley et al., 2011).

F_1 social behavior was also influenced by both F_0 diet and rearing environment. Surprisingly, there were no observed sex differences in play attack behavior as seen previously (Meaney, 1988; Korgan et al., 2016); however, male offspring did engage in more defensive play behaviors while females engaged in more grooming. F_0 CD exposed males' offspring reared in the SNH performed the fewest play attacks while those reared in SH performed the most defense behaviors; together suggesting a complex programming difference in social behavior based on F_0 male diet exposure, rearing environment and maternal care behavior.

This complex relationship between F_0 diet exposure and rearing environment is exemplified by offspring behavior in the OFT and EPM. As shown previously, SNH decreases anxiety behavior in the OFT shown by increased time in the center squares, likely

a response driven by the similarity of the OFT environment to that of the SNH (Korgan et al., 2016). Interestingly, we show here that F_1 offspring of CD males and those reared in the SNH show decreases in line-crosses. This decreased locomotor activity can be interpreted as an increase in habituation, as higher anxiety phenotype rodents will show more locomotor activity prior to habituation (Brenes et al., 2009). Similarly, F_1 male and female offspring of F_0 CD males reared in the SNH displayed increased grooming in the novel OFT environment. Self-grooming requires a decrease in exploratory and vigilant behavior providing further evidence of an anxiolytic phenotype (Brenes et al., 2009). In the EPM, F_1 offspring of HFD exposed F_0 males display an anxiogenic phenotype; spending less time in open arms and entering fewer open arms. This behavioral profile is opposite of that seen in F_1 offspring of males exposed to caloric restriction (Govic et al., 2016) but is similar to that seen in F_1 offspring of males exposed to social defeat stress (Dietz et al., 2011). Further, studies have suggested diet induced programming effects on sperm by microRNA (Ng et al., 2010; Fullston et al., 2013; Grandjean et al., 2015; Fullston et al., 2016a), tsRNAs (Chen et al., 2016b) and DNA methylation (Youngson et al., 2016) (reviewed in (Chen et al., 2016a)). Recent studies have supported this by identifying substantial alterations in sperm development following HFD-induced obesity in both rodents (de Castro Barbosa et al., 2016; Fullston et al., 2016a) and humans (Soubry et al., 2016; Guo et al., 2017).

Adolescent stress responsivity behavior was tested by exposing offspring to control or predator (cat) odor. As previously shown, offspring exposed to the predator odor show a strong avoidance response and decreased rearing behavior, indicative of stress-induced

behavioral inhibition (Korgan et al., 2016). Grooming behavior is consistent with previously discussed findings in the OFT, where F_1 offspring reared in the SNH show a decrease in vigilance, though this is ameliorated in F_1 offspring of HFD exposed males. Surprisingly, F_1 offspring of HFD exposed males spent more time in the vicinity of the stimulus and contacted it more often; similar to anxiolytic phenotypes seen in F_1 offspring of calorie restricted males (Govic et al., 2016), this may be driven by an underlying motivation to explore and/or locate food (Zigman et al., 2015).

Body weight at p42, following the odor exposure, supports the idea that F_0 diet altered metabolic demands of offspring. As expected, sex differences in offspring revealed increased body weight in males, despite increased abdominal and perigonadal fat pad weight and brain weight in females. F_0 HFD exposure increased body weight, perigonadal and abdominal fat pad weight but decreased brain weight in both male and female offspring, providing more evidence for altered metabolic function in F_1 offspring, as previously described (Carone et al., 2010; Ng et al., 2010), though more thorough study of gene expression profiles in adipose tissue is required (Guo et al., 2016; Morita et al., 2016). SNH rearing has previously been associated with increased body weight (Korgan et al., 2014; Korgan et al., 2016), we have shown here that SNH rearing decreases body weight and perigonadal weight while increasing brain weight, similar to traditional environmental enrichment models (Kempermann et al., 1997; Zeeni et al., 2015). Inconsistent findings with weight differences in offspring reared in SNH (Korgan et al., 2014; Korgan et al., 2016) could be the consequence of altered maternal investment based on paternal diets and

highlights the importance comparing metabolic function and growth differences between the CD and standard laboratory chows.

Mechanistically, histone 3, lysine 9 acetylation (H3K9ac) at the *crf* promoter in the PVN were regulated by offspring rearing environment and odor exposure condition. As shown previously, F_1 offspring exposed to the PO had increased H3K9ac, with a strong reversal by SNH rearing (Korgan et al., 2016). Paternal diet induced effects on offspring central *crf* regulation have not been described, but alterations to histone regulation at leptin and adiponectin promoters have been described in F_1 adipose tissue (Masuyama et al., 2015) and global changes in H3K9me3 have been identified in fat cells from F_1 *drosophila* offspring of HFD males (Ost et al., 2014). PVN *crf* hnRNA expression was highly correlated with promoter acetylation, with SH reared offspring showing the highest expression following PO, as shown previously (Korgan et al., 2016). Though repressive and active histone methylation marks have been shown to be active in human and mouse spermatozoa (Brykczynska et al., 2010), future research is necessary to identify a role for histone acetylation in sperm.

Though recent studies have suggested that *in vitro* fertilization of F_0HFD (Huypens et al., 2016) or stress (Rodgers and Bale, 2015; Rodgers et al., 2015) are recapitulated in offspring, current work demonstrates a critical role of maternal care and rearing environment in regulating offspring anxiety and responses to PO. Future work is required to further elucidate the complex mechanisms mediating paternal germline modifications and female differential allocation in response to altered mate phenotypes.

Figures

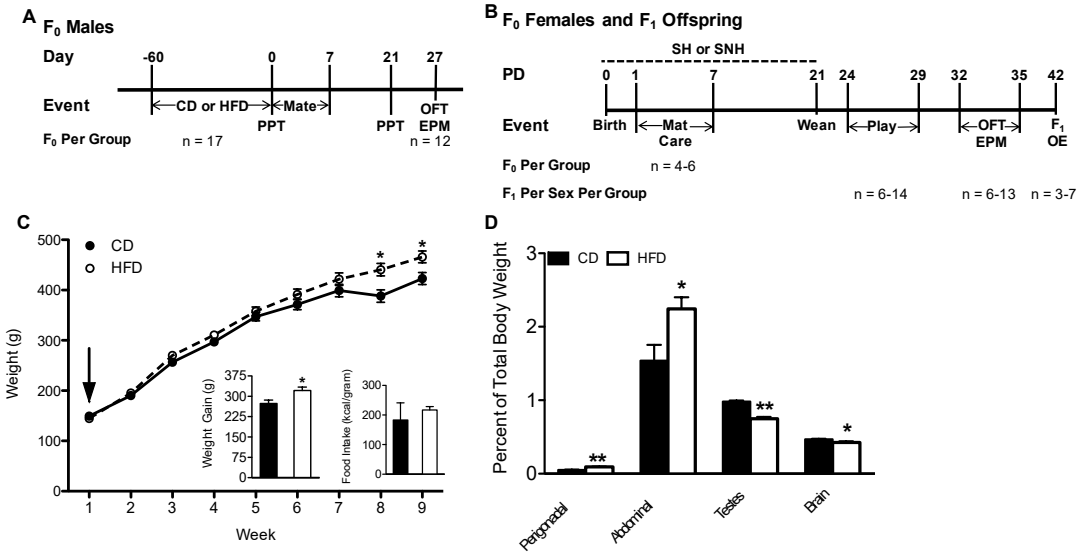

Figure 3.1. Experimental timelines, weight gain, food intake, and body weights in F_0 males following diet manipulation. (A) Timeline of treatment procedures involving F_0 males. Males were fed either control diet (CD) or high fat diet (HFD) for 60 days prior to to a partner preference test (PPT) using sexually receptive virgin, naïve females. Within 12 hours of the PPT, males were bred with *different* receptive naïve virgin females. Following confirmed mating, males were removed and females were left undisturbed until offspring were born. After mating, F_0 males were maintained on standard chow for 21 days before to a second PPT. One week after the second PPT, F_0 male anxiety behavior was assessed in the EPM and OFT. **(B)** Timeline of treatment procedures for F_0 females and the F_1 offspring. Birth was considered postnatal day (PD) 0, and offspring were counted, sexed, and weighed before being transferred to either fresh standard housing (SH) or semi-naturalistic housing (SNH), with biological mothers, until weaning. Maternal behavior (Mat Care) was scored for 72 min, 5 times per day for 7 days. At PD 21, all offspring were

weighed, weaned and placed in SH with a same-sex littermate. Play behavior was recorded in the home cage from PD 24-29, followed by exposure to the open-field test (OFT) and the elevated plus maze (EPM) on PD 32-35. F_1 OE took place on PD 42 with male and female offspring being exposed to either a control odor (CO) or predator odor (PO) for 30 min and then sacrificed. Sample sizes are provided for both F_0 and F_1 groups. **(C)** Weight gain F_0 male rats was significantly increased following HFD relative to CD, despite a decrease in food intake during the final 5 days of diet manipulation **(D)** F_0 male rats exposed to HFD showed increases in perigonadal and abdominal fat pad mass and decreases in teste and brain weights 27 days after diet manipulation. Data expressed as mean ± SEM; *****HFD different from CD, $p \leq 0.05$, ******HFD different from CD, $p \leq 0.005$.

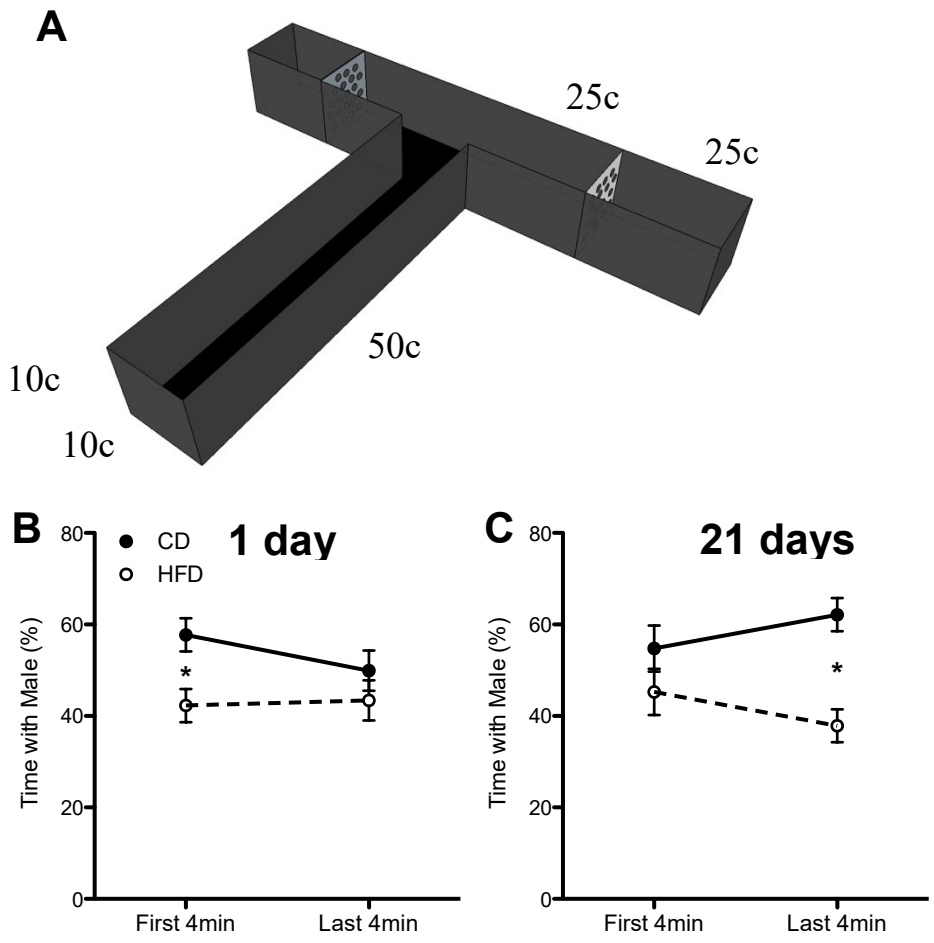

Figure 3.2: Use of a partner preference test (PPT) to ascertain female preference for males previously exposed to high fat diet (HFD) relative to control diet (CD).

(A) Schematic representation of the T-maze used for the PPT. F_0 Males were placed in the ends of arms confined by Plexiglas shields containing many holes. **(B-C)** Female rats spent less time in the vicinity of HFD fed F_0 males relative to CD fed males during PPT, both 1 day and 21 days after the odor exposure had occurred in males. Percentage of time with males was calculated as the percentage of time spent with either a CD or HFD F_0 male per total time spent with both CD and HFD F_0 males. Data expressed as mean ± SEM; *PO different from CO, $p \leq 0.05$.

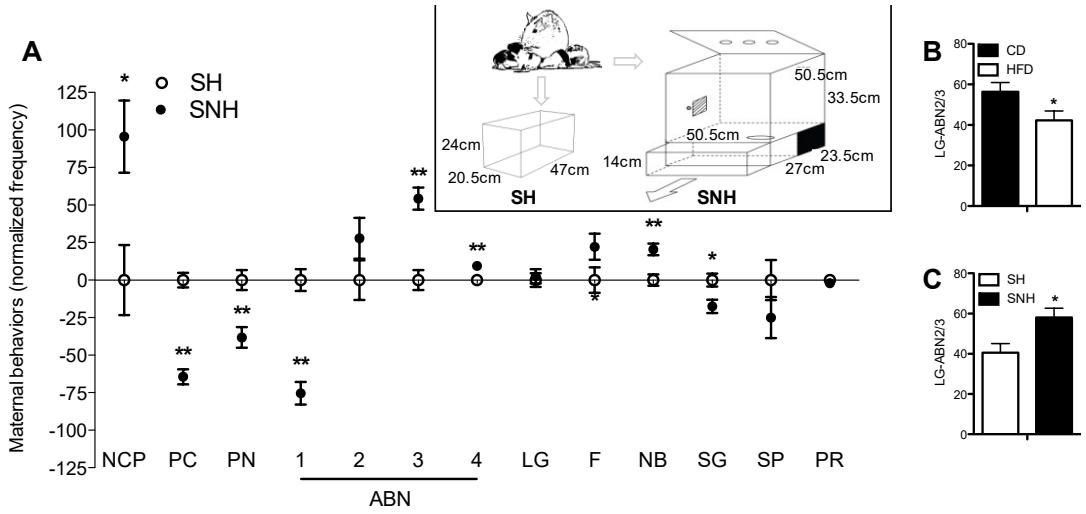

Figure 3.3: Maternal behaviors of females housed in semi-naturalistic housing (SNH) or standard housing (SH) raising offspring of F_0 males that were fed either high fat diet (HFD) or control diet (CD). (A) Frequency of various maternal behaviors [NCP, no contact with pups; PC, passive contact (with pups); PN, passive nursing; ABN1, arched-back nursing 1 (blanket posture); 2, arched-back nursing 2; 3, arched-back nursing 3; 4, arched back nursing 4; LG, licking/grooming; F, feeding; NB, nest building; SG, self groom (auto groom); SP, separated pups (from the rest of the litter); PR, pup retrieval] displayed by females housed in SH and SNH, collapsed across paternal condition. The inset shows schematics of the housing conditions; the SNH includes a lower burrow compartment (contained within a drawer that moves out to facilitate cleaning) and an upper section (containing food and water), with the two sections being connected by a hole (visible in the upper section). **(B-C)** The frequency of LG-ABN2/3 behaviors were significantly decreased in dams raising offspring of F_0 HFD males compared to those raising offspring of F_0 CD males and increased in dams living in SNH relative to SH. Data expressed as mean ± SEM; Difference between indicated groups * = $p \leq 0.05$; ** = $p \leq 0.005$.

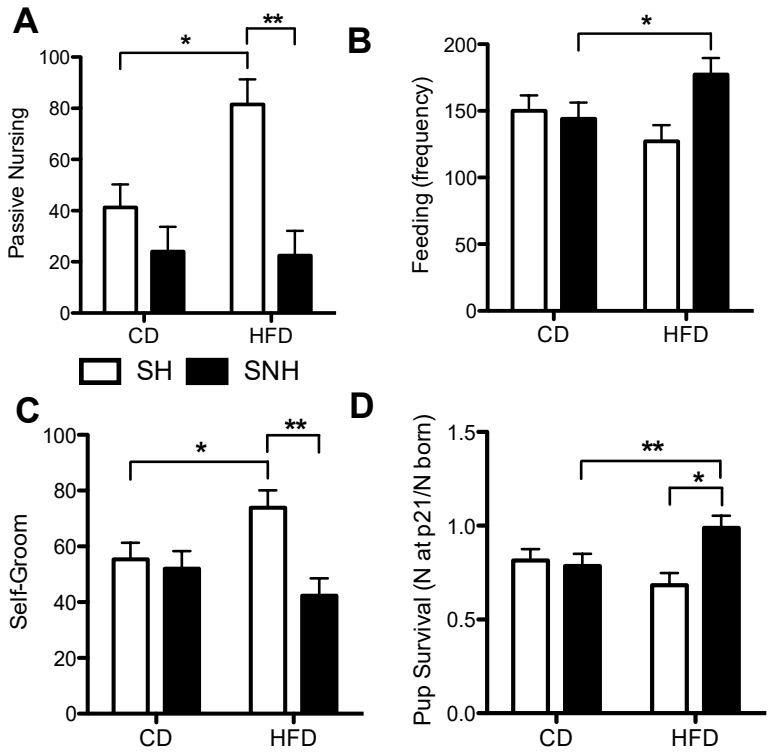

Figure 3.4: Interactions in maternal behaviors of females housed in semi-naturalistic housing (SNH) or standard housing (SH) raising offspring of F_0 males that were fed either high fat diet (HFD) or control diet (CD). (A) Females spent more time passive nursing if rearing offspring of F_0 HFD males in the SH compared to offspring of F_0 HFD males in the SNH and offspring of F_0 CD males in SH. **(B)** Females spent more time feeding if rearing offspring of F_0 HFD males in the SNH compared to offspring of F_0 HFD males in the SH. **(C)** Females spent more time self-grooming if rearing offspring of F_0 HFD males in the SH compared to offspring of F_0 HFD males in the SNH and offspring of F_0 CD males in SH. **(D)** F_1 offspring survival at P21 was increased in offspring of F_0 HFD males in the SNH compared to offspring of F_0 HFD males in the SH and offspring of F_0 CD males in

SNH. Data expressed as mean ± SEM; Difference between indicated groups * = $p \leq 0.05$; ** = $p \leq 0.005$.

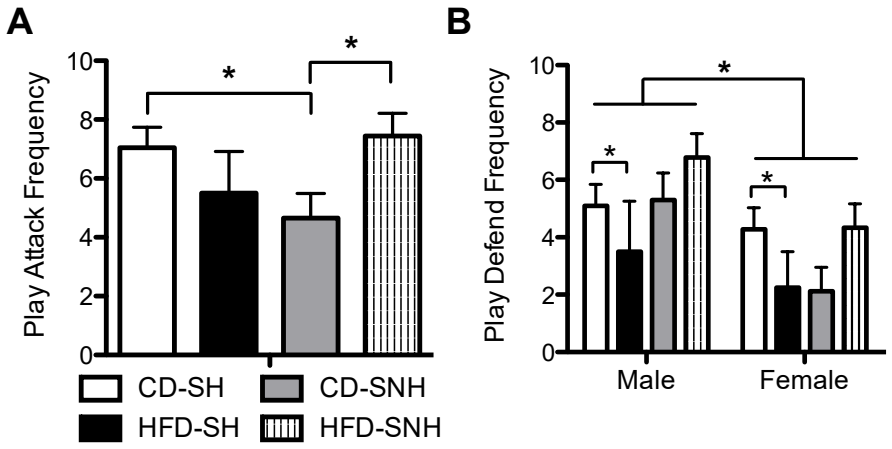

Figure 3.5. Social behavior of juvenile F_1 offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by males fed either high fat diet (HFD) or control diet (CD). (A) F_1 offspring of F_0 CD males reared in the SNH engaged in significantly fewer play attacks than F_1 offspring of F_0 CD males reared in the SH and of F_0 HFD males reared in the SNH. **(B)** F_1 males engaged in significantly more play defend behaviors relative to females. F_1 male and female offspring reared in SH engaged in fewer play behaviors if sired by F_0 HFD males compared to those from F_0 CD males. Data expressed as mean ± SEM; Difference between indicated groups, * = $p \leq 0.05$.

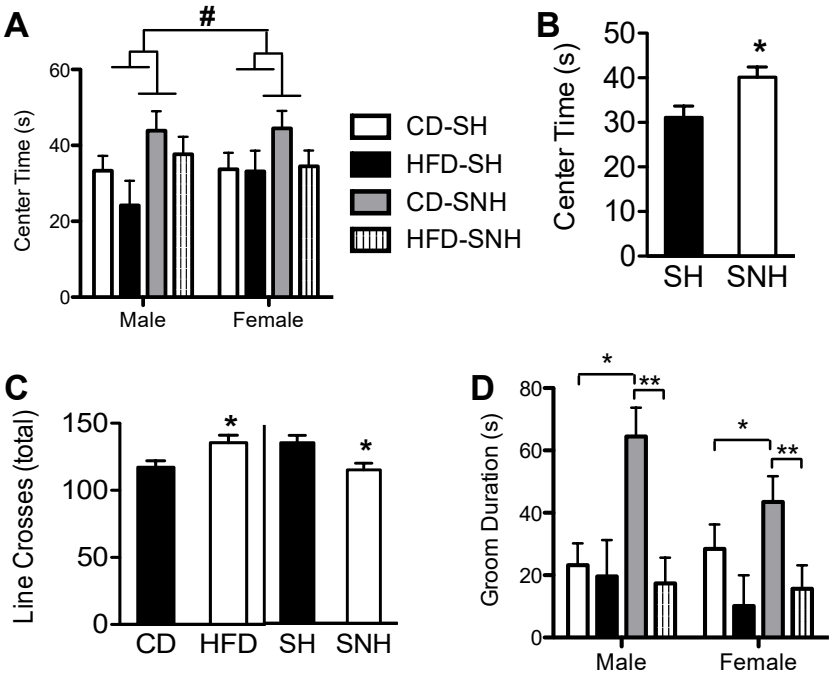

Figure 3.6. Development of fear- and anxiety-like behavior in the F_1 offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by males fed either high fat diet (HFD) or control diet (CD). (A) A trend for decreased center time in the open-field test (OFT) was observed in F_1 offspring sired by F_0 HFD males compared to those from F_0 CD males. **(B)** Center time in the OFT was increased in F_0 offspring raised in SNH relative to those reared in SH. **(C)** The number of line crosses in the OFT was increased in F_1 offspring sired by F_0 HFD males compared to those from F_0 CD males but decreased in F_1 offspring reared in SNH compared to those reared in SH. **(D)** Total time spent grooming in the OFT was increased in F_1 offspring sired by F_0 CD males reared in SNH compared to those from F_0 CD males reared in SH and F_0 HFD males reared in SNH. Data expressed as mean ± SEM; interaction; Difference between indicated groups, # = $p= 0.065$; * = $p\leq0.05$; ** = $p\leq0.005$.

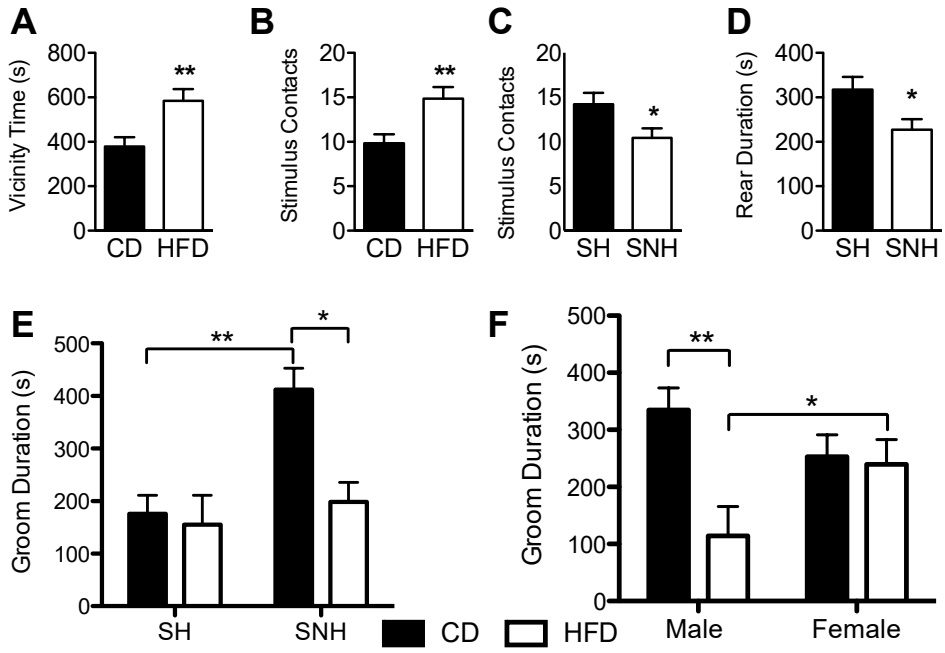

Figure 3.7. Effects of 30 min exposure to either control odor (F_1CO) or predator odor (F_1PO) on behavior of F_0 offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by F_0 males fed either high fat diet (HFD) or control diet (CD). (A-B) Vicinity time and odor stimulus contacts were significantly increased in F_1 offspring sired by F_0 HFD males compared to F_1 offspring sired by F_0 HFD males. **(C-D)** Stimulus contacts and rear duration was significantly decreased in F_1 offspring reared in the SNH compared to F_1 offspring reared in SH. **(E)** Self-grooming duration was significantly increased in F_1 offspring sired F_0 CD males reared in the SNH compared to F_1 offspring sired by F_0 CD males reared in the SH and F_1 offspring sired by F_0 HFD males reared in the SNH. **(F)** Self-grooming duration was significantly decreased in F_1 male offspring sired by F_0 HFD males compared to F_1 male offspring sired by F_0 CD males and F_1 female offspring sired by F_0 HFD.

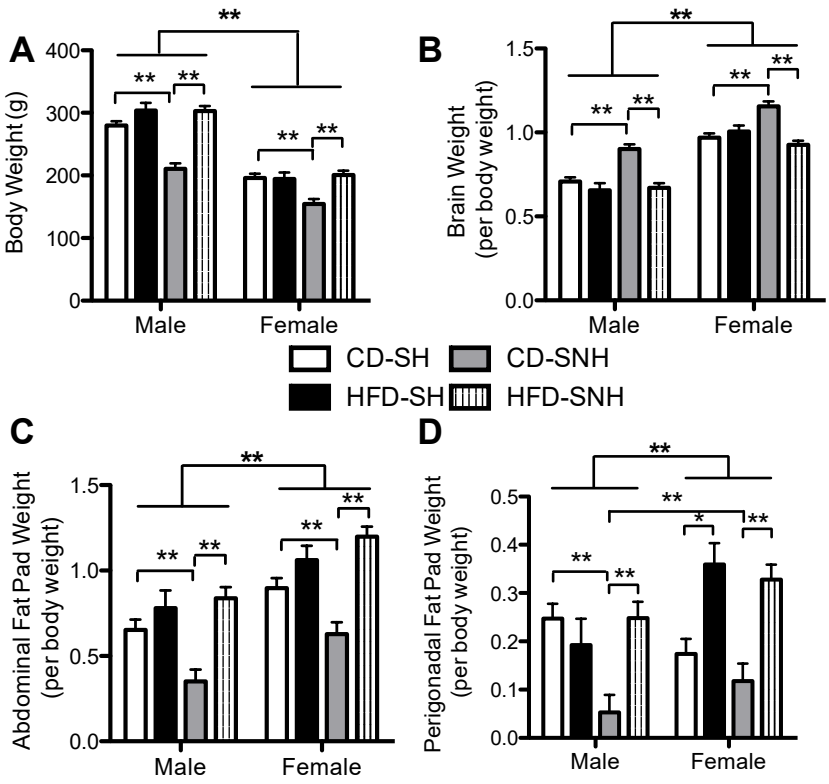

Figure 3.8. Body and tissue weights of F_0 offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by F_0 males fed either high fat diet (HFD) or control diet (CD). (A) At P42, F_1 male offspring weighed more than female offspring. Body weight of both F_1 male and female offspring was significantly decreased in F_1 offspring sired by F_0 CD males and reared in SNH compared to F_1 offspring sired by F_0 CD males and reared in the SH and F_1 offspring sired by F_0 HFD males and reared in the SNH. **(B)** At P42, F_1 male offspring had relatively lighter brains than female offspring. Relative brain weight of both F_1 male and female offspring was significantly increased in F_1 offspring sired by F_0 CD males and reared in SNH compared to F_1 offspring sired by F_0 CD males and reared in the SH and F_1 offspring sired by F_0 HFD males and

reared in the SNH. **(C)** At P42, F_1 male offspring had relatively lighter abdominal and perigonadal fat pads than female offspring. Abdominal and perigonadal fat pad weight of both F_1 male and female offspring was significantly decreased in F_1 offspring sired by F_0 CD males and reared in SNH compared to F_1 offspring sired by F_0 CD males and reared in the SH and F_1 offspring sired by F_0 HFD males and reared in the SNH. F_1 female offspring sired by F_0 CD males and reared in SNH had relatively heavier perigonadal fat pads compared to F_1 male offspring sired by F_0 CD males and reared in SNH.

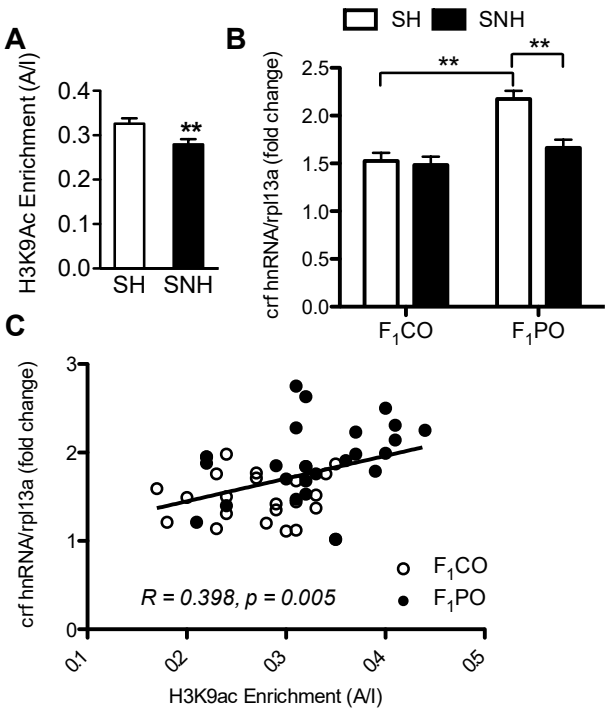

Figure 3.9. Effects of 30 min exposure to either control odor (F_1CO) or predator odor (F_1PO) on hypothalamic *crf* gene regulation of of F_0 offspring raised by females housed in semi-naturalistic housing (SNH) or standard housing (SH) and sired by F_0 males fed either high fat diet (HFD) or control diet (CD). (A) H3K9ac enrichment of the *crf* promoter in the PVN was increased in F_1 offspring reared in SNH compared to F_1 offspring reared in SH. **(B)** Levels *crf* primary transcript (hnRNA) in PVN were also increased in F_1PO offspring reared in the SH compared to F_1CO offspring reared in SH and F_1PO offspring reared in the SNH. **(C)** Levels of H3K9ac enrichment of the *crf* promoter and hnRNA expression in the PVN were positively correlated in F_1CO and F_1PO offspring. Data expressed as mean ± SEM; Difference between indicated groups, ** = $p \leq 0.005$.

Table 3.1

HFD induced anxiety behavior in F_0 Males

Behavioral Measure	Mean, SE	t, p value
Center Time (s)	CD-125.5, 10.958	6.328, <0.001
OFT	HFD-52.920, 3.385	
Rear Duration (s)	CD-156.58, 10.006	2.856, 0.009
OFT	HFD-120.08, 7.947	
Line Crosses (total)	CD-183.58, 4.209	0.272, 0.788
OFT	HFD-188.75, 8.673	
Open Arm Duration (s)	CD-214.5, 20.39	2.603, 0.016
EPM	HFD-137, 21.692	
Open Arm Time (%)	CD-35.75, 3.398	2.675, 0.015
EPM	HFD-22.833, 3.615	
Line Crosses (total)	CD-31.420, 1.474	3.531, 0.002
EPM	HFD-22.5, 2.05	
Rear Duration (s)	CD-66.750, 5.059	2.146, 0.043
EPM	HFD-50.420, 5.686	

Table 3.2

Anxiety behavior in F_1 offspring in the EPM

Behavioral Measure	Mean, SE	f, p value
Open Arm Duration (s)	CD-F_1-55.274, 5.686	18.073,
	HFD-F_1-16.683, 7.076	<0.001
Open Arm Entries (total)	CD-F_1-4.239, 0.422	16.456,
	HFD-F_1-1.506, 0.526	<0.001
Line Crosses (total)	CD-F_1-11.471,0.999	4.513,
	HFD-F_1-8.083, 1.243	0.037
Open Arm Time (%)	CD-F_1-9.212, 0.939	21.643,
	HFD-F_1-2.505, 1.094	<0.001
Groom Duration (s)	CD-F_1-15.365, 3.108	6.088,
	HFD-$F_1$27.608, 3.868	0.016
Groom Duration (s)	SH-16.074, 3.8	4.761,
	SNH-26.9, 3.191	0.033
Groom Duration (s)	M-14.061, 3.691	8.960,
	F-28.913, 3.316	0.004
Rear Duration (s)	CD-F_1-42.448, 3.142	8.226,
	HFD-F_1-56.838, 3.911	0.006

CHAPTER 4 EFFECTS OF PATERNAL HIGH-FAT DIET AND REARING ENVIRONMENT ON MATERNAL CARE AND FEEDING AND ANXIETY BEHAVIOR

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Preface to Manuscript

Following the establishment of a model for paternal stress and diet induced alterations in maternal preference, maternal care, and offspring behavior, we were interested further quantifying the role of paternal HFD in programming offspring feeding and anxiety-like behavior. For this, we utilized the novelty suppressed feeding test (NSFT) to measure anxiety behavior and feeding motivation in F_1 offspring.

4.1 Abstract

Paternal preconception risk factors correlate with metabolic dysfunction in the offspring, which is often comorbid with feeding and anxiety disorders. We examined whether a male rat's prior exposure to an obesogenic high-fat diet (HFD) influences subsequent mother-infant interactions on growth and feeding behavior as well as the development of defensive (emotional) responses in offspring reared in semi-naturalistic housing (SNH). Paternal HFD and maternal rearing environment revealed sex-dependent differences in offspring wean weight, food intake, frequency of feeding bouts as well as changes in weight and anxiety-like behaviors following food deprivation. Our results show that variation among males in their exposure to HFD may contribute to stable behavioral variation among offspring feeding and anxiety behavior in response to stress, even when the offspring themselves are not directly exposed to a HFD. Furthermore, when offspring were exposed to a food deprivation, the sex-dependent differences in weight loss and anxiety behavior were influenced by maternal care the offspring had received during the first week of postnatal life. These results, together with our previous findings, suggest that paternal experience and maternal rearing conditions can influence maternal behavior and development of defensive responses of offspring.

4.2 Introduction

Consuming high-fat diets (HFD) has the ability to alter neuroendocrine responses to stress (Dutheil et al., 2016) and feeding patterns either during or after a stressor. These

neuroendocrine responses are critical to our understanding of the developing obesity epidemic (Gotay et al., 2013; Graversen et al., 2014) and comorbid anxiety disorders (Anderson et al., 2001; Castanon et al., 2014). While the role of gestational, perinatal (Sasaki, 2013;2014; Vogt, 2014) and direct exposure to HFD (De Souza et al., 2005; Stienstra et al., 2011; Dutheil et al., 2016)) has gained recent attention, the impact of the paternal diet remains unclear.

Previous research has shown alterations in anxiety (Grissom (Sullivan et al., 2010; Sullivan et al., 2014; Grissom et al., 2015) and feeding behavior (Purcell et al., 2011) in offspring of dams exposed to HFD, likely driven by glucocorticoid mediated inhibition of ghrelin signaling (Zigman et al., 2015) and food preference (Vucetic et al., 2010). However, other studies suggest that HFD exposure shapes comfort feeding, resulting in an increased desire to feed during a mildly stressful experience (Maniam and Morris, 2010). Further, gestational programming of metabolic function by maternal HFD exposure results in increased body-weight and susceptibility to metabolic disorders (Kirk et al., 2009; Li et al., 2011; Hoeijmakers et al., 2015). Paternal exposure to HFD does induce metabolic pathologies, resulting in metabolic syndrome (MetS) and/or type 2 diabetes (t2d)-like phenotypes (Fullston et al., 2013; Ng et al., 2014; Ost et al., 2014; Fullston et al., 2016b). Further, paternal exposure to HFD induces an anxiogenic phenotype in F_1 offspring (Chapter 3), opposite to that seen in offspring sired by F_0 caloric restricted males (Govic et al., 2016).

Both the early rearing environment and maternal care further shape offspring anxiety and metabolic phenotypes. Offspring of high-LG dams display anxiolytic phenotypes in elevated plus maze and open field tests (Caldji et al., 1998; Weaver et al., 2006) and increases in maternal LG-ABN are associated with decreased latency to feed in the novelty suppressed feeding test (NSFT) and in offspring body weight. Further, high-quality maternal behavior patterns shape metabolic programming (Sauce et al., 2017), likely contributing to resilience to stress induced obesity and subsequent MetS phenotypes (Meaney et al., 2007). Early enrichment and early handling facilitate these increases in maternal care and associated decreases in offspring anxiety and body weight throughout development (Francis et al., 1999b; Connors et al., 2015; Korgan et al., 2016).

In the current experiment, we sought to explore the role of paternal HFD exposure and offspring rearing in a semi-naturalistic home cage (SNH). The novelty-suppressed feeding task is unique in animal behavior models, as animals undergo food deprivation, novelty-induced anxiety coupled with the opportunity to feed, and a subsequent feeding period in a familiar environment (Samuels and Hen, 2011). Thus, feeding and anxiety-like behavior NSFT was assessed to uncover potential interactions between paternal and early-life experiences. Further, correlations between offspring weight and behavior and maternal care behaviors suggest early programming effects of the maternal care in offspring development.

4.3 Materials & Methods

4.3.1 Animals and Breeding

Eighty Long-Evans hooded rats, 38 males and 42 females from a previously described experiment (Chapter 3) at 60±5 days old were used for F_1 testing. All rats were housed in same sex pairs in a colony room maintained at 21 °C ± 2 °C under a 12h:12h reversed light cycle (lights off at 0930h). Rats were caged in SH, which consisted of polypropylene cages (47 cm x 24 cm x 20.5 cm) with wire lids, containing pine shavings for bedding (Hefler Forest Products, Inc., Sackville, N.S. Canada) and a black PVC tube (12 cm length, 9 cm diameter. Both rat chow (Purina Lab Chow) and tap water were supplied *ad libitum*. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

4.3.2 Paternal High-fat Diet and Semi-Naturalistic Housing (SNH)

Males began either high-fat (60 kcal%) or protein/carbohydrate matched control diet (10 kcal%) feeding at P35 and were maintained on this diet for 60 days (Research Diets, Inc., New Brunswick, NJ) as described in Chapter 3. Dams and litters were randomly designated for the semi-naturalistic housing (SNH) condition and transferred to SNH cages on PD0. Dams and pups in the standard housing (SH) condition were placed in clean, standard home cages as described in Chapter 3.

4.3.3 Maternal Care Observations

Dams' maternal behavior was observed and scored daily in real-time as described in Chapters 2 and 3. Cumulative scores of each behavior were utilized in analysis along with an aggregate total for ABN2-4 behaviors and those co-occurring with LG similar to Korgan et al., (2016).

4.3.4 Novelty-suppressed Feeding Test of F_1 Offspring

F_1 offspring anxiety and feeding behavior was tested at 60±5 days of age in the novelty-suppressed feeding test (NSFT) (Bodnoff et al., 1988; Santarelli et al., 2003; Snyder et al., 2011). Each trial was recorded under white light, using a vertically mounted video camera, for future behavioral scoring. Following 24hr food deprivation, F_1 offspring were transported to and from the testing room in covered home cages and the arena was cleaned with 30% ethanol solution between each trial. The NSFT test was conducted in a Plexiglas square (79.2 cm x 78.9 cm x 35.0 cm) constructed from opaque black panels, with a floor covered by a thin layer of wood chip bedding. A small platform (2 cm) with a standard chow pellet is positioned ~20cm from the center of one side of the arena. At the beginning of each 10-min trial, offspring were placed in the center of the opposite side of the arena. The behaviors scored for the NSFT were as follows: thigmotaxic time (total time that the rat was within one body width of a wall), center crosses (the total number of passages across the center (10 cm x 10 cm) area), latency to feed (duration (seconds) prior to any feeding, carrying, or other manipulation of the food pellet), as well as the number and

duration of freezing (2 sec or greater period without movement, but not sleeping), grooming (2 sec or longer bouts that involve; licking, nibbling and combing-like actions of the fur), and rearing (standing on hind paws only, with or without leaning on the perimeter wall). Following the 10-min test, the rat was returned to its home cage and *ad libitum* feeding behavior (latency to feed, feeding bouts) is scored for 5 min to establish potential differences in feeding motivation. Both the open field and the home cage food samples were weighed before and after testing, as a measure of food intake.

4.3.5 Offspring Groups

The following four groups of F_1 male and female offspring were studied, as in previous experiments: CD-SH – father exposed to control diet and mother housed in standard housing (n=12 males, 14 females); CD-SNH – father exposed to control diet and mother housed in semi-naturalistic housing (n=8 males, 12 females); HFD-SH - father exposed to high-fat diet and mother housed in standard housing (n=6 males, 6 females); HFD-SNH – father exposed to high-fat diet and mother housed in semi-naturalistic housing (n=12 males, 10 females). Each group contained 2-4 males and females from multiple litters: CD-SH (n=7 litters), CD-SNH (n=6 litters), HFD-SH (n=3 litters), and HFD-SNH (n=6 litters).

4.3.6 Statistical analyses

Offspring P60 weight and behavioral test data were analyzed using a three-factor ANOVA with F_1 Sex, F_0 paternal diet (CD vs. HFD), and rearing environment (SH vs SNH) as

between-subject factors. Interactions were analyzed post-hoc with simple effects analyses, using a Bonferroni correction. Correlations were calculated using either Pearson's R or Spearman's ρ for linear and nonlinear data, respectively. A threshold level of $p < 0.05$ was used to test for significance. The Statistical Package for the Social Sciences (SPSS Inc., USA) software was used for all statistical analyses.

4.4 Results

4.4.1 P60 F_1 Weight and Correlations with Maternal Care

F_1 bodyweight was measured prior to 24hr food deprivation on P60. There was an interaction between F_0 paternal diet and F_1 offspring sex ($F=6.302$ (1,80) $p=0.014$; Figure 4.1A). Post-hoc analyses revealed increased body weight in male F_1 offspring sired by CD ($F=67.354$ (1,46) $p<0.001$) and HFD ($F=68.146$ (1,34) $p<0.001$) F_0 males compared to females. Further, both male ($F=9.583$ (1,38) $p=0.004$) and female ($F=5.593$ (1,42) $p=0.023$) F_1 offspring sired by F_0 HFD males weighed more than those sired by F_0 CD males. SNH rearing also decreased bodyweight at P60, independent of F_0 diet and F_1 sex ($F=28.800$ (1,80) $p<0.001$; Table 4.1).

Linear correlations between maternal care frequencies of high- and low-quality maternal care behaviors predicted F_1 male and female P60 bodyweights. Blanket posture (BP) nursing was positively correlated with F_1 weight in both males ($R=0.233$, $p=0.002$; Figure 4.1B) and females ($R=0.203$, $p=0.003$; Figure 4.1C). Maternal ABN2-4 and LG-2 frequency were negatively correlated with F_1 weight in both males ($R=-0.475$, $p<0.001$;

R=-0.712, p<0.001; Figure 1D-F) and females (R=-0.483, p<0.001; R=-0.557, p<0.001; Figure E-G), respectively.

4.4.2 NSFT in F_1 Offspring

Following the 24hr food deprivation, F_1 male offspring lost more weight than females (F=31.663 (1,80) p<0.001; Table 4.1). During the NSFT, there were no differences in latency to feed (all p's > 0.05). Post hoc analyses of an interaction between F_0 diet and rearing environment (F=5.564 (1,80) p=0.021; Figure 4.2A) for center cross frequency revealed increased center crosses in F_0 offspring sired by CD males if reared in the SNH compared to SH (F=11.332 (1,46) p=0.002) and increased center crosses in SH reared offspring of F_0 HFD males compared to CD (F=5.357 (1,38) p=0.026). Analyses of a similar interaction for thigmotaxis time (F=8.224 (1,80) p=0.005; Figure 4.2B), revealed increased thigmotaxis in offspring sired by F_0 CD males reared in the SH compared to SNH (F=9.613 (1,46) p=0.003) and increased thigmotaxis in SNH reared offspring of F_0 HFD males compared to F_0 CD (F=5.720 (1,42) p=0.022). Following the NSFT, there were no differences in latency to feed (all p's > 0.05). There was an interaction between F_0 diet, rearing environment and sex (F=4.764 (1,80) p=0.032; Figure 4.2C), and post hoc analyses revealed an increase in food intake in male offspring (F=7.043 (1,80) p=0.010; Table 4.1) and increased food intake in F_1 male offspring sired by F_0 HFD males and reared in SH compared to CD-SH (F=8.050 (1,18) p=0.008) and HFD-SNH (F=5.145 (1,18) p=0.038). Further, F_1 male offspring engaged in more feeding bouts than F_1 female offspring (F=8.431 (1,80) p=0.005; Table 4.1).

4.4.3 F_1 NSFT Behavior and Maternal Care Correlations

A nonlinear correlation between food deprivation (24hr) weight loss and maternal ABN2-4 frequency (ρ=0.394, p=0.014; Figure 4.3A) was found for F_0 male offspring. During the NSFT, rear duration was correlated with PN frequency for F_0 males (ρ=-0.470, p=0.003; Figure 4.3B). Following the NSFT, F_0 latency to feed was also correlated with PN frequency (ρ=0.365, p=0.024; Figure 4.3C). Center crosses were negatively correlated with maternal ABN2-4 (ρ=-0.406, p=0.007; Figure 4.3D) and maternal LG frequency (ρ=-0.367, p=0.017; Figure 4.3E) in F_0 female offspring, while thigmotaxis time was correlated with maternal LG-3 frequency (ρ=-0.337, p=0.033; Figure 4.3F).

4.5 Discussion

Overall, the current study identifies early paternal and early life programming events that may dictate offspring growth, feeding, and anxiety-like behavior patterns in early adulthood. These novel findings reveal significant interactions between F_0 HFD exposure and rearing in the SNH environment. These perinatal exposures program sex-specific alterations in F_0 offspring adult weight, anxiety behavior, and home cage feeding behavior.

In both F_1 male and female offspring, SNH rearing decreased and paternal F_0 HFD exposure increased P60 body weight. These effects are similar to those seen at weening

(Chapter 3). Differences in weening and adult body weights could be dictated by alterations in body composition and fat accumulation, driven by altered metabolic programming (Ng et al., 2010; Carone et al., 2014). Interestingly, in both male and female offspring active maternal care behaviors were negatively correlated with P60 weight, while passive maternal care behaviors were positively correlated. While others have shown that early enrichment alters adult growth patterns (Connors et al., 2015), these correlations also fit with our current findings indicating increased active maternal behaviors in offspring of F_0 CD males, while offspring sired by F_0 HFD males receive increased passive maternal care behavior. Further, increases in active maternal care behavior in F_1 offspring reared in SNH receive increased frequencies of active behaviors and weigh less than SH controls (Chapter 3). Interestingly, both high and low frequency of high quality maternal care behavior (ABN2-4) were correlated with F_1 male weight loss during the food deprivation, suggesting different mechanisms regulating weight loss and increased metabolic stability in F_1 offspring receiving 'medium' levels of ABN2-4. Further support for differential mechanisms is the increase in post NSFT latency to feed in offspring receiving more PN.

In the NSFT, F_1 diet and rearing environment interacted to alter anxiety-like behavior patterns in F_0 offspring. The increase in center crosses by offspring reared in SNH or those of F_1 HFD males, compared to CD-SH controls, indicates that both paternal HFD and SNH rearing have the potential program anxiolytic phenotypes. Offspring sired by F_0 males may exhibit less anxiety-like behavior due increased motivation to explore, especially when a feeding opportunity is present (Zigman et al., 2015), though we did not

observe differences in food intake or latency to feed in the NSFT. Contrarily, offspring reared in the SNH may have similar exploration motivation, but are desensitized to the novelty of the testing arena (Korgan et al., 2016). Decreased thigmotaxis time in F_1 offspring sired by F_0 CD males and reared in the SNH, compared to those reared in SH and those sired by F_0 HFD males, indicated that SNH rearing alone decreases anxiety-like behavior and raises the possibility that offspring sired by F_0 HFD males may have increased motivation to explore (especially for a food reward) but are still more anxious than offspring sired by F_0 CD males (Chapter 3).

Following the NSFT, home cage observations allow for identification of non-novelty-suppressed feeding behavior. In the home cage, male offspring sired F_0 HFD males that were reared in the SH ate more than CD-SH and HFD-SNH males. Similar to studies of gestational HFD exposure, sex-differences in metabolic programing may influence in male offspring more than females (Edlow et al., 2016). This indicates that F_0 HFD induces altered metabolic demands in F_1 offspring (Carone et al., 2010; Fullston et al., 2013; Ost et al., 2014; Rando and Simmons, 2015), particularly in males. Further, increases in active maternal behaviors and the SNH environment may be sufficient to reverse this altered metabolic programming. Indeed, manipulations to the early rearing environment alter F_1 growth (Weinberg et al., 1995) and may represent effects on metabolic programming based on differential allocation of maternal resources (Burley, 1988; Mashoodh et al., 2012).

Future studies should further characterize the development of metabolic circuitry, receptor sensitivity, and subsequent anxiety-like behavior in offspring sired by F_0 HFD males. Potential programming of insulin, ghrelin, leptin, and inflammatory pathways might influence anxiety and feeding behavior (Steculorum and Bouret, 2011; Warchoł et al., 2014; Lockie et al., 2015; Dutheil et al., 2016; Johnson et al., 2016). Further, the influence of maternal care behavior and/or the early rearing environment in mediating effects of paternal exposure, especially to HFD, remain unclear.

Figures

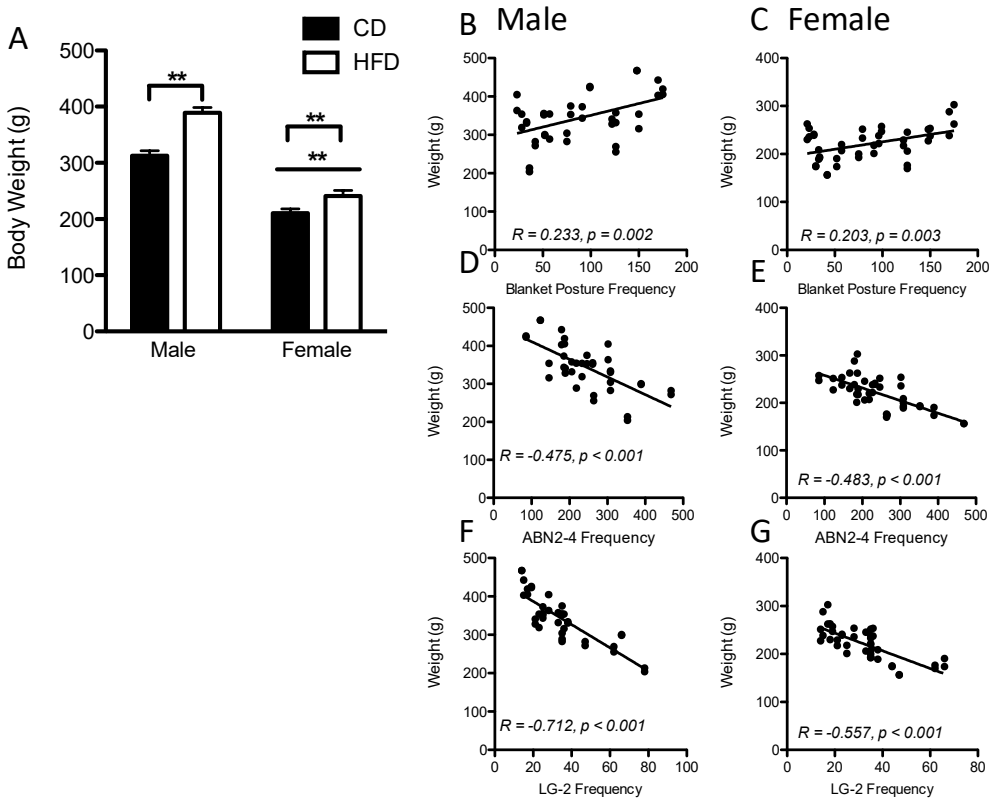

Figure 4.1. P60 Body weight and correlations with maternal care behaviors. (A) F_1 offspring sired by F_0 HFD males weigh more than those sired by F_0 CD males. F_1 male offspring weigh more than females, regardless of F_0 diet manipulation. (B-C) Maternal blanket posture frequency is positively correlated with P60 weight in F_1 male and female offspring. (D-E) Maternal ABN 2-4 frequency and LG-2 (F-G) is negatively correlated with P60 weight in F_1 male and female offspring. Data expressed as mean ± SEM; **HFD different from CD and male different from female, $p \leq 0.005$.

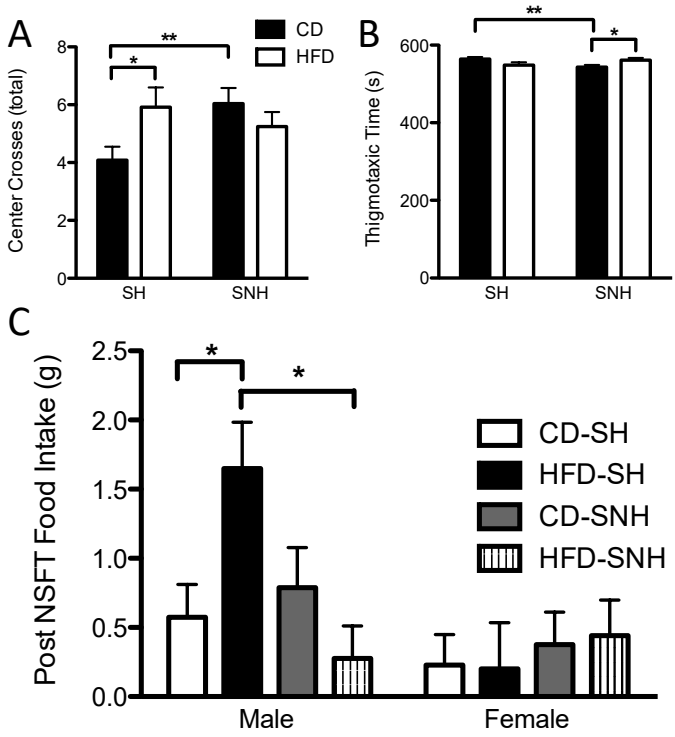

Figure 4.2: F_0 anxiety behavior the NSFT and home cage feeding behavior.

(A) F_1 CD-SH offspring performed fewer center crosses than F_1 HFD-SH and CD-HFD offspring. (B) F_1 CD-HFD offspring displayed increased thigmotaxis behavior compared to CD-SH and HFD-SNH offspring. (C) F_0 HFD-SH males consumed more food during the home cage observation CD-SH and HFD-SNH male offspring. Data expressed as mean ± SEM; Difference between indicated groups * = $p \leq 0.05$; ** = $p \leq 0.005$.

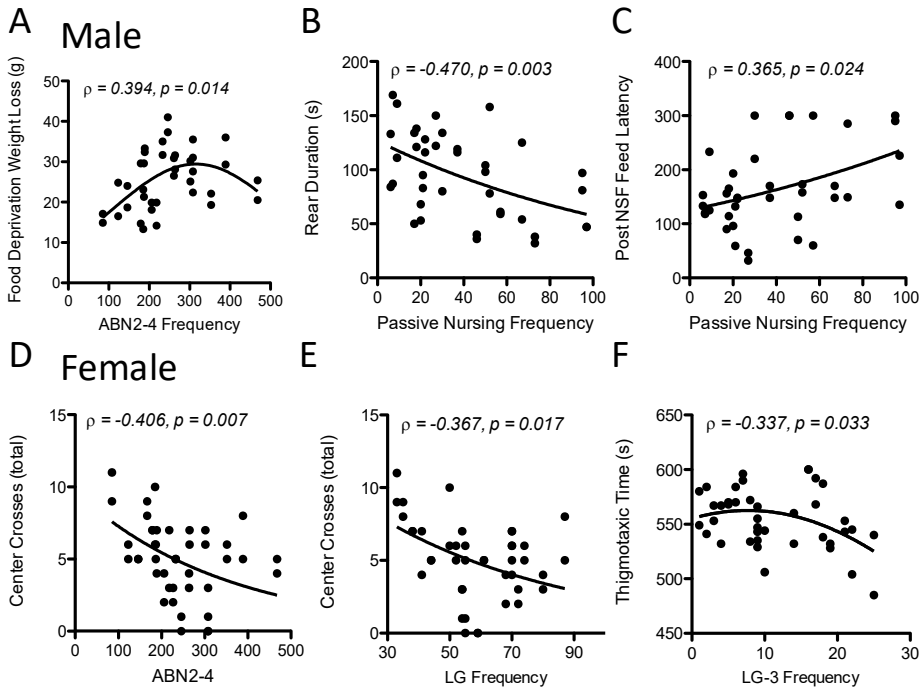

Figure 4.3: Maternal behaviors correlate with metabolic and anxiety-related offspring phenotypes. (A) Both high and low maternal ABN2-4 frequencies were associated with decreases in food deprivation weight loss in F_1 male. (B) Maternal passive nursing was negatively correlated with decreased rearing behavior in F_1 male offspring. (C) Maternal passive nursing frequency was positively correlated with F_1 male offspring latency to feed in the home cage. (D-E) Maternal ABN2-4 and LG frequencies were negatively correlated with center crosses frequency in F_1 female offspring during the NSFT. (F) Maternal LG-3 frequency was negatively correlated with F_1 female thigmotaxis time in the NSFT.

Table 4.1
F_1 Offspring Differences in Feeding and Weight Pre- and Post-NSF Testing

Behavioral Measure	Mean, SE	F, p value
Body Weight	SH-312.675 SNH-263.979	28.800, <0.001
Food Deprivation Weight Loss (g)	M-25.257, 1.071 F-16.907, 1.027	31.663, <0.001
Post NSF Food Intake (g)	M-0.822, 0.139 F-0.311, 0.133	7.043, 0.010
Post NSF Feeding Bouts (total)	M-2.406, 0.289 F-1.242, 0.278	8.431, 0.005

Chapter 5 Discussion

5.1 Summary of Findings

The above studies provide new evidence for maternal preference, investment and rearing environment affecting offspring development and behavior. Previous studies have generally not accounted for the role of maternal preference, allocation of resources, or rearing environment. However, we provide evidence that early-rearing dependent developmental alterations program offspring sociality, stress responsivity and underlying chromatin modification and gene expression patterns that are critical in our interpretation of paternal effect experiments.

In mammals, females are able to detect mate quality based, partially, on odor and changes in testosterone (Willis and Poulin, 2000) and breeding with preferred mates can increase offspring viability (Drickamer et al., 2000). This could have driven the novel findings in this thesis of reduced preference for HFD or chronically stressed males. Chronic PO exposure induced avoidance behavior in adult male rats, which was related to decreased preference in females. More specifically, there was no female preference during the first four minutes of the test. Preference was only decreased during the last four minutes of the test, suggesting that females required time to evaluate male quality and adjusted their responses based on this appraisal. Most interestingly, this effect was consistent both immediately following the PO exposure protocol and following a 17 day lag period, suggesting that PO exposure alters male attractiveness on a semi-permanent timescale.

PO exposure is an ethologically valid stressor model that replicates the potential differences in predator salience that might exist in natural settings. Similarly, males exposed to HFD also were subject to decreased female preference. Contrary to the PO experiment, CD exposed males, compared to HFD, were preferred during the first four minutes with a change to equal preference for the final four minutes when tested immediately following the last day of diet manipulation. However, when tested 17 days later, females only preferred CD males, compared to HFD, during the final four minutes of testing. This might suggest an immediate aversion to hyper-anxious HFD exposed males, followed by habituation in females during the initial testing. However, that habituation in preference is lost following 17 days of standard chow feeding. This could be driven by a changing anxiety profile as HFD males adjust to the lower fat standard chow diet.

While it could be argued that surviving a predatory experience (PO) is a benefit, we were exposing males to a chronic stressor to induce an anxious and recognizable phenotypic difference utilizing an established stressor. Other models of PO exposure allow for more impactful avoidance, allowing for a more controllable stressor, and are shorter in duration. Our model of chronic exposure for one week increases stress behavior and GC levels, though not potentiating the same anxiety-like phenotype seen in adolescent exposure models (Wright et al., 2012). In mice exposed to chronic predator (cat) odor, urinary attractiveness is increased (Zhang et al., 2008). However, this study utilized cat urine rather than cat collars containing dander. Further, females were exposed to urinary cues from males, rather than the males themselves. While pheromone-dependent cues

may have increased attraction to male odor, the reported increase in aggression might have a different effect if females were given the opportunity to select males. This potential confound highlights the need for replication utilizing other stressor protocols, such as chronic social defeat stress and early maternal separation, prior to PPT, as well as careful control of sensory cues during the PPT.

Large males are not preferred in rodent preference tests (Lovell et al., 2007; Zewail-Foote et al., 2009; Winland et al., 2012). Our finding of altered fat accumulation in HFD exposed males further denigrates any benefit of being larger. Indeed, mate preference is more refined than a positive correlation between size and attractiveness. Human (Jackson and McGill, 1996) studies have shown preferences for non-obese body types. Indeed, these mechanisms are invariably complex and differ between species (and reproductive strategies) but important determinants of mate attractiveness or health appear to be maintained throughout taxa. Similarly, both the parasitism model (Willis and Poulin, 2000), chronic stress (Apfelbach et al., 2005) and HFD feeding (Liu et al., 2012) have been shown to reduce plasma testosterone, potentially mediating given effects on female preference. This is further supported when considering that males exposed to endocrine disrupters, such as vinclozolin, receive decreased female preference, even three generations after the exposure (Crews et al., 2007). Together, these data suggest that mate preference has significant impact on evolution, further highlighting the important role that epigenetics have in these processes (Crews and McLachlan, 2006).

The SNH rearing environment showed novel and consistent effects on maternal care and offspring development. While previous studies have identified changes in offspring seizure severity (Korgan et al., 2014) and CRF-ir (Korgan et al., 2015), this is the first detailed analysis of maternal care behavior in the SNH. The alterations to F_1 anxiety-like behavior and regulation of *crf* gene expression align with our current understanding of perinatal programming by maternal behavior and the effects seen in early enrichment and brief maternal separation models. Specifically, variations in maternal care (Francis et al., 1999c) and brief maternal separation-induced increases of LG-ABN behavior (Plotsky et al., 2005; Couto-Pereira et al., 2016) result in offspring with decreased stress responsivity, measured by differences in *crf* expression and plasma GCs. Early enrichment and our SNH propagate similar changes to maternal behavior and subsequent offspring programming while maintaining ecological validity.

Differences in the interaction of paternal exposures, either PO or HFD (compared to appropriate controls), and maternal behavior in the SH and NSH suggest that female allocation of maternal care is not only altered by exposure to PO or HFD, but also by exposure to CO and CD. Increased LG-ABN2 behavior in female rats rearing offspring in the SNH who were mated with PO exposed males, compared to those rearing offspring in the SH suggest that females might be attempting to rescue some deleterious consequence of having a PO father. This effect was not seen in females rearing offspring in the SNH who were mated with HFD males. Conversely, HFD mated females in the SH actually performed more passive nursing behaviors. Overall, this might suggest that F_0 HFD transmits worse or more irreversible effects to F_1 offspring than F_0 PO exposure. Further,

females raising offspring of F_0 HFD males in the impoverished SH conditions provide the lowest quality of care or decreased allocation of maternal resources. Clearly, these interactions are equally complex and dynamic, demanding future investigations on diet and stress exposures in cross-fostering and direct comparison experiments while utilizing the novel and ethologically valid SNH manipulation.

5.2 Are the Results of Paternal Exposures Necessarily Maladaptive?

While most studies on paternal effects focus on negative outcomes preceded by increased paternal age, stress, diet, and toxic environmental exposures, the question of adaptive evolutionary traits in offspring remains. Clearly, increased paternal age and paternal toxic exposure have deleterious effects on offspring. However, the function of paternal transgenerational inheritance implies an evolved mechanism, capable of conferring some advantage to improve offspring survival. Dias et al., (2014) showed that F_0 paternal fear conditioning to a novel odor stimulant heightened F_1 and F_2 sensitivity to the odor by altered epigenetic regulation of the neuroanatomical representation of the odor binding pathway. Indeed, studies have suggested that paternal stress alters offspring stress sensitivity to improve coping in high stress situations (Gapp et al., 2014b). Similar to theories pertaining to maternal stress studies (Zalosnik et al., 2014), this altered sensitivity may be deleterious in an environment without high stress situations. In this case, having increased transcriptional availability of stress related genes (*crf*) may result in dysregulated HPA-axis function and a hyper-anxious phenotype.

Paternal diet manipulation appears to alter offspring metabolism development similar to that of maternal diet manipulation. Offspring exposure to HFD through maternal milk programs 'thrifty' metabolic pathways in offspring, resulting in increased adiposity and risk for MetS (Gluckman and Hanson, 2004; Srinivasan et al., 2006). A more complex mechanism in F_0 males exposed to HFD appears to alter spermatogenesis and miRNA, resulting in similar vulnerabilities in F_1 to F_3 offspring. In theory, a thrifty metabolic phenotype might be advantageous in environments with low food availability (Sauce et al., 2017). However, in most rodent and human populations this is not the case. Easy access to high fat or western diets (in humans) or *ad libitum* feeding in rodents instead allows for over-accumulation of adipose tissue and perpetuation of obese-phenotypes.

Another theory has recently emerged to explain the differential effects of transgenerational control of gene expression and phenotypic variation. Stochastic alterations to the paternal germline and epigenome could function to increase variability in offspring, allowing for increased flexibility in an unpredictable environment, at a frequency that outpaces DNA mutation rates (Rando and Verstrepen, 2007). Evidence for stochastic events comes from prominent bacterial models, but more recent evidence has pointed to targeted changes in gene expression. In *escherichia coli* (*E. coli*), fimbriae (*Fim*) genes are surrounded by inverted sequence repeats, which are controlled by the orientation of a promoter element. This promoter element is driven by upstream genes, driving a phase shift, which promote fimbriae development or leave the bacteria bald (Klemm, 1986; Olsen et al., 1998). This phenotypic variation is advantageous as the bacteria enters a host and is heritable in experimental paradigms. In voles, a highly

variable repeat locus upstream of the *avpr1a* gene, which influences social, pair bonding, and paternal behavior, is associated with increased mutation rate (Hammock and Young, 2005). Phenotypic differences in social behaviors might allow for a more adaptive response to the environment, though this has not been confirmed experimentally.

In the present studies, F_0 paternal exposure to PO and HFD contributed altered anxiety-like phenotypes, partially mediated by differences in regulation of the *crf* promoter, which enhanced gene transcription, with no differences in modifications that inhibit expression (e.g. H3K9me3; Appendix A). While an evolutionary advantage is possible, our PO and HFD may have conferred seemingly non-adaptive alterations to anxiety-like behavior and, for the F_1 offspring of PO exposed males, *crf* regulation. Clearly, this complicated interaction of preconception exposures affecting development, along with early life rearing environments, prompt substantial need for continued investigation.

5.3 Limitations and Future Directions

While other examinations of predator odor stress have shown profound effects on adult anxiety-like and depressive behavior (Dielenberg and McGregor, 1999; Apfelbach et al., 2005; Masini et al., 2010), our outcome measures in Chapter 2 did not include such outcomes in F_0 males. While we did observe alterations in avoidance behavior between CO and PO exposed male, potentially highlighting differences in resilience responses to the stressor (Dielenberg and McGregor, 1999), future studies should consider the role of habituation in mediating the F_0 exposure. Indeed, many studies utilizing paternal stress

models use stressors that are less conducive to habituation, especially early maternal separation (Franklin et al., 2010) and chronic social defeat (Dietz et al., 2011). Further, a novel odour stimulus paired with a negative conditioned stimulus (Dias and Ressler, 2014) may elicit the least amount of habituation. The chronicity and external and ethological validity of paternal stressors are characteristic design issues in murine stress research and present a wide array of potential for future studies.

In Chapter 3, we adjusted our protocol to allow for anxiety-like behavioral testing in F_0 males. Expected HFD-induced effects in the OFT and EPM support our paternal transmission hypothesis; that F_0 metabolic stress could produce anxiety-like behavior in F_1 offspring. While the mechanisms for this are still unclear, alterations in maternal care and adipose tissue accumulation allow the potential for both the inheritance of F_0 epigenetic modifications and/or differential allocation of maternal investment programming anxiety-like and HFD-susceptible offspring. Another potential mechanism is the alteration of F_0 gut-brain interactions induced by HFD (Abildgaard et al., 2011; Abildgaard et al., 2013; Foster and McVey Neufeld, 2013; Wong et al., 2016). Changes in microbiome diversity could potentiate anxiety-like behavior (Kang et al., 2014; Abautret-Daly et al., 2017) and be passed to offspring via seminal fluid microbiome (Chen et al., 2016b; Isganaitis et al., 2016; Javurek et al., 2016). Alternatively, alterations to the F_0 male microbiome could be shared with F_0 females during breeding (Lees et al., 2014); facilitating a gestational shift to the placenta microbiome of HFD-paired F_0 females, which has been shown to regulate the development of offspring behavior (Zijlmans et al., 2015; Neu, 2016; Jasarevic et al., 2017). Future studies could test F_0

female behavior after breeding to assess anxiety-like phenotypes, while cross fostering or *in vitro* fertilization (IVF) studies might partially occlude this confound.

Several studies have utilized IVF to prevent mating behavior or cue-induced changes to maternal allocation and investment. Significant advances in IVF technology have changed our understanding of epigenetic mechanisms of male germline inheritance (Ventura-Junca et al., 2015). Dietz et al. (2011) showed that IVF prevented paternal stress-induces changes to offspring anxiety-like behavior, promoting the role of male-female interactions during mating as a necessary component of paternal transgenerational inheritance. However, Rodgers et al. (2015) discovered stress induced changes to F_0 sperm miRNA and perpetuated an anxiety-like phenotype to F_1 offspring by supplementing miRNA from F_0 seminal fluid for the IVF procedure. Similar changes have also been detected in HFD exposed F_0 males (Fullston et al., 2016a). Future research should be directed at elucidating interactions between F_0 male-female interactions, their environment (Champagne, 2016) and epigenetic effects regulating spermatogenesis, not limited to miRNA but also methylation (Shea et al., 2015; Soubry et al., 2016) and chromatin modifications (Brykczynska et al., 2010; Ma et al., 2015). As our understanding of these transgenerational mechanisms and technologies available to study IVF-based inheritance develop, future research will be able to more clearly delineate underlying mechanisms.

5.4 Concluding Remarks

The field of transgenerational epigenetics has grown substantially in the past five years, with high-impact reviews published regularly. Overall, most studies show useful and novel additions to our current understanding. However, as more researchers build programs around a transgenerational platform, a need for consistency and control within these studies will be vital. Within the studies presented in this thesis, we have demonstrated profound influences of maternal preference, environment and care on offspring development of anxiety-like phenotypes. Clearly, significant contributions to this work will follow as we continue to characterize a previously overlooked biological process.

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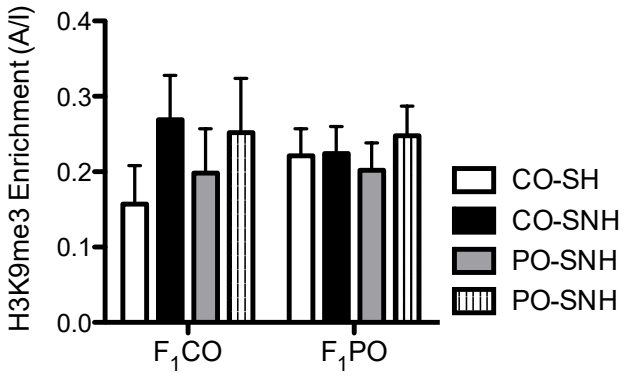
Appendix A



H3K9me3 enrichment of the *crf* promoter in PVN. There were no differences in H3K9me3 enrichment based on F_0PO relative to F_0CO offspring, offspring raised in SNH relative to those raised in SH, or F_1PO offspring relative to F_1CO offspring. Data expressed as mean ± SEM; utilizing univariate ANOVA all p's <0.05.